April 20, 2015

VIA EMAIL
(P65PUBLIC.COMMENTS@OEHHA.CA.GOV;
MONET.VELA@OEHHA.CA.GOV)

Ms. Monet Vela
Office of Environmental Health Hazard Assessment
P.O. Box 4010, MS-58D
Sacramento, California 95812-4010

Re: Bisphenol A – Female Reproductive Toxicity

Dear Ms. Vela:

On behalf of The Art and Creative Materials Institute, and in conjunction with Technology Sciences Group Inc., I am submitting comments on the Proposition 65 Bisphenol A Hazard Identification Materials for consideration by the Developmental and Reproductive Toxicant Identification Committee and the Office of Environmental Health Hazard Assessment. ACMI also supports and joins in the comments submitted by the American Chemistry Council.

The Art and Creative Materials Institute (ACMI) is a non-profit international trade association comprised of approximately 190 companies. ACMI's mission is to create and maintain a positive environment for art, craft and other creative materials usage; to promote safety in these materials; and to serve as an information and service resource on such products. Since 1936, ACMI has sponsored a certification program for children's art materials, which it expanded in 1982 to include certification of adult art material products as well. In its current form, the certification program incorporates the requirements of ASTM Standard D4236, the federal Labeling of Hazardous Art Materials Act and the acute health hazards provisions of the Federal Hazardous Substances Act.

Bisphenol A (BPA) is used to make polycarbonate components of certain art material products, as well as certain packaging used for art materials. Accordingly, ACMI has a strong interest in the consideration of BPA for listing as a reproductive toxicant under Proposition 65.

As explained further below, the studies identified in the Hazard Identification Materials do not demonstrate that BPA has been "clearly shown through scientifically valid testing according to generally accepted principles" to cause female reproductive toxicity. Further, a recent study demonstrates that there is ***no*** causal effect between BPA exposure and female reproductive toxicity. BPA thus is not ***known*** to cause reproductive toxicity within the meaning

50 California St. | Suite 1500 | San Francisco CA 94111 | T: 415-463-5186 | F: 415-358-4467
ann.grimaldi@grimaldilawoffices.com | www.grimaldilawoffices.com

of Proposition 65. Therefore, this substance should not be listed as a female reproductive toxicant under Proposition 65.

ACMI looks forward to presenting its position at the upcoming DARTIC meeting on May 7, 2015 and requests that sufficient time be reserved for it to do so.

I. EXECUTIVE SUMMARY

BPA will be considered for listing under Proposition 65 as a chemical known to the state to cause female reproductive toxicity at the meeting of the Developmental and Reproductive Toxicant Identification Committee (DARTIC or the Committee) scheduled for May 7, 2015. The proposed listing is being made pursuant to the State's Qualified Experts (SQE) mechanism. Under that mechanism, "a chemical is known to the state to cause…reproductive toxicity if in the opinion of the state's qualified experts it has been clearly shown through scientifically valid testing according to generally accepted principles to cause…reproductive toxicity…." As explained further in this submission, BPA does not meet this requirement for listing.

The U.S. Food and Drug Administration (FDA) BPA Joint Emerging Science Working Group (FDA Working Group) has reviewed the scientific literature on BPA in detail periodically since 2008 and characterized the utility of available study reports for use in hazard identification and risk assessment. The FDA, an authoritative body under Proposition 65, ***has ruled out female reproductive toxicity*** as an endpoint of potential hazard from BPA exposure, based on the weight of the evidence in the scientific literature.

OEHHA has presented a recent review article by Peretz and coauthors as the primary new document in the BPA Hazard Identification Materials (HIM). This review article concluded that BPA is both an ovarian and a uterine toxicant. The FDA Working Group reviewed a majority of the underlying studies. Applying scientifically sound acceptance criteria that are aligned with the DARTIC and statutory criteria for listing, the FDA Working Group either concluded that these studies were not suitable for consideration or established that the studies did ***not*** demonstrate a causal connection between BPA exposure and reproductive toxicity.

Technology Sciences Group Inc. (TSG) has analyzed the studies reviewed by Peretz et al. Summaries of its review appear in the tables attached as Exhibit 4-9 to this submission. In short, the studies evaluated by Peretz and her coauthors deviate substantially from the mandatory DARTIC and statutory criteria for listing and therefore cannot support the listing of BPA as a female reproductive toxicant.

Importantly, one new study of BPA exposure conducted according to scientifically sound hazard identification criteria determined that BPA does not cause female reproductive toxicity to rats following low dose exposure during gestation and early postnatal development. (Delclos, 2014.) This recent study conducted by the National Center for Toxicological Research (NCTR) exposed rats orally to BPA at very low doses or high doses during gestation and for 90-days after parturition. There were no effects of BPA on female reproductive organs, estrous cycles, or female hormone levels in rats exposed to BPA during development at doses below those that caused significant systemic toxicity. These results support the determination that BPA does ***not*** cause female reproductive toxicity.

Thus, BPA is not ***known*** to cause female reproductive toxicity within the meaning of Proposition 65, and must not be listed. Listing BPA in these circumstances will lead to Proposition 65 warnings that will not promote the public health, and will lead to enforcement actions that are unconnected to the public interest. Such consequences are directly contrary to OEHHA's larger program goals of improving Proposition 65 by reducing unnecessary warnings and lawsuits.

II. THE ART AND CREATIVE MATERIALS INSTITUTE HAS A STRONG INTEREST IN THE POTENTIAL LISTING OF BPA AS A PROPOSITION 65 REPRODUCTIVE TOXICANT

For decades ACMI has sponsored a certification program for the evaluation of art materials used by children and adults. Under ACMI's certification program, art material products undergo toxicological evaluations for the assessment of acute and chronic health hazards. These evaluations are undertaken by a toxicology consulting team at Duke University Medical Center in Durham, North Carolina. Based on the outcome of these evaluations, member companies are granted the use of the program's seals on the certified products, and the certified products are required to be labeled in accordance with federal law and the certification program's requirements.

Art materials found to contain no materials in sufficient quantities to be toxic or injurious to humans, including children, or to cause acute or chronic health problems, are designated with an "AP" seal. The "CL" seal is used for products that are certified to be properly labeled in a program of toxicological evaluation by a medical expert for any known health risks, and that bear information on their safe and proper use.

Various states, including California, restrict school districts' purchases of hazardous art materials for grades K-6. In 2014, the Office of Environmental Health Hazard Assessment (OEHHA) published an updated list of hazardous art material products that are prohibited from being purchased by California schools for use by children in grades K-6. Virtually the entire list consisted of CL seal-bearing ACMI member products, even though not all art material manufacturers are ACMI members – thereby demonstrating the extensive reliance, even by OEHHA itself, on ACMI's certification program.

Bisphenol A (BPA) is present in polycarbonate components of certain art material products as well as their packaging. The listing of BPA as a female reproductive toxicant under Proposition 65 therefore will have a direct effect on ACMI's members, many members of which have been targeted in Proposition 65 actions over the years. ACMI members will have to choose between:

- Providing Proposition 65 warnings to avoid enforcement actions, whether they are brought on meritorious grounds or not; or

- Conducting and relying on exposure assessments as a defense to enforcement actions. ACMI members know from past experience that sharing exposure assessments with Proposition 65 enforcers in pre-lawsuit negotiations rarely motivate those enforcers to refrain from filing lawsuits and shifting the burden to the company to prove that no warning is required. Thus, this option in reality

> means choosing to spend substantial resources and money to establish the defendant's affirmative defense, even in an unmeritorious lawsuit.

Providing warnings as a means to avoid unmeritorious lawsuits has real consequences to ACMI and its members. As ACMI has explained in other submissions to OEHHA, ACMI's certification program prohibits products from bearing both the AP seal and a Proposition 65 warning. Thus, by protecting themselves from enforcement actions, ACMI members lose critical markets, like school districts, which only purchase AP seal-bearing art materials. Accordingly, ACMI has a strong interest in ensuring that the proposed listing of this, or any other chemical, fully meets all statutory and regulatory listing criteria, is based on sound science and comports with Proposition 65's objectives.

As explained further below, a review of the BPA Hazard Identification Materials (HIM) and other studies reveals that the criteria for listing BPA as a female reproductive toxicant are not met. Therefore, BPA must not be listed.

Finally, ACMI wishes to draw the bigger picture from the perspective of businesses in the enforcement litigation trenches. Proposition 65 listing decisions do not occur in a vacuum and are not consequence-free. They are the starting point for warnings and the starting point for enforcement actions. When not based on robust evaluations aligned with statutory and regulatory criteria, listing decisions lead to warnings that do not promote the public health and encourage frivolous litigation. OEHHA has stated repeatedly that it seeks to reduce the proliferation of unnecessary Proposition 65 warnings and litigation. To that end, the agency recently published an extensive revision of the Proposition 65 warning regulations for public notice and comment. The Developmental and Reproductive Toxicant Identification Committee (DARTIC or the Committee) and OEHHA must ensure that their evaluation of the BPA HIM and other studies, and the ultimate listing decision, comport with statutory and regulatory requirements, for failure to do so will undermine the larger program goals that OEHHA seeks to attain.

III. THE STUDIES IDENTIFIED IN THE HAZARD IDENTIFICATION MATERIALS DO NOT MEET APPLICABLE LISTING CRITERIA AND BPA IS NOT *KNOWN* TO CAUSE FEMALE REPRODUCTIVE TOXICITY WITHIN THE MEANING OF PROPOSITION 65

BPA will be considered for listing under Proposition 65 as a chemical known to the state to cause female reproductive toxicity at the meeting of the DARTIC scheduled for May 7, 2015 (which may be extended to May 21, 2015).[1] The proposed listing is being made pursuant to the State's Qualified Experts (SQE) mechanism. Under that mechanism, "a chemical is known to the state to cause…reproductive toxicity if in the opinion of the state's qualified experts it has been clearly shown through scientifically valid testing according to generally accepted principles

[1] *See* Hazard Identification Materials for Consideration of the Female Reproductive Toxicity Of Bisphenol A (February 20, 2015), located at http://www.oehha.ca.gov/prop65/hazard_ident/BPAhazardID2014.html.

to cause…reproductive toxicity….”[2] As explained further in this submission, BPA does not meet this requirement for listing.

Importantly, one new study of BPA exposure conducted according to scientifically sound hazard identification criteria, used by the Food and Drug Administration, determined that BPA does not cause female reproductive toxicity to rats following low dose exposure during gestation and early postnatal development[3]. This recent study conducted by the National Center for Toxicological Research (NCTR) exposed rats orally to BPA at very low doses or high doses during gestation and for 90-days after parturition. There were no effects of BPA on female reproductive organs, estrous cycles, or female hormone levels in rats exposed to BPA during development at doses below those that caused significant systemic toxicity. These results support the determination that BPA does ***not*** cause female reproductive toxicity.

A. The Committee Is Required To Apply Specific Criteria In Evaluating A Chemical for Listing under Proposition 65

The DARTIC, as an advisory body to the Governor and OEHHA, is a committee of OEHHA’s Science Advisory Board and the state’s qualified experts regarding findings of reproductive toxicity. The Committee is empowered to render an opinion as to “whether specific chemicals have been clearly shown, through scientifically valid testing according to generally accepted principles, to cause reproductive toxicity.” [4] This standard is adopted verbatim from the statutory criterion for listing a chemical under the SQE mechanism.

OEHHA has published criteria by which the Committee ***must*** evaluate a chemical being proposed for listing under the SQE mechanism (the DARTIC Criteria).[5] The DARTIC Criteria articulate the requirements that human and animal studies must meet in order for them to deemed sufficient to meet the statutory requirement that a chemical be “clearly shown, through scientifically valid testing according to generally accepted principles, to cause…reproductive toxicity.”[6] Further, “[i]n evaluating the sufficiency of data, a ‘weight-of-evidence’ approach ***shall*** be used to evaluate the body of information available for a given chemical.”[7]

Ultimately, the overarching statutory listing requirement is that BPA be ***known*** to cause reproductive toxicity:

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[2] California Health and Safety Code § 25249.8(b) (hereinafter Section 25249.8(b)).

[3] Delclos et al., 2014. Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90. Tox Sci, 139(1), 174-197.

[4] 27 Cal.Code Regs. § 25305(b)(1).

[5] *See* http://www.oehha.ca.gov/prop65/policy_procedure/pdf_zip/dartCriteriaNov1993.pdf (DARTIC Criteria), attached as Exhibit 1.

[6] Section 25249.8(b).

[7] DARTIC Criteria at Section I.D (emphasis added); see also Section 3.A(3) (weight of evidence considerations must be applied in evaluating human studies); Section 3.C(6) (same for animal studies).

> [C]hemicals may be included on the Proposition 65 list only if there is a ***sufficient*** showing that they ***in fact*** cause cancer or reproductive harm. This interpretation is consistent with the legislative history underlying Proposition 65….[8]

And indeed, this interpretation is entirely consistent with voter intent:

> Proposition 65's new civil offenses focus only on chemicals that are ***known to the state*** to cause cancer or reproductive disorders. Chemicals that are only suspect are not included.[9]

Thus, if BPA is not ***known*** to cause cancer or reproductive harm, it "cannot be properly included" on the Proposition 65 list.[10]

BPA previously was considered for listing under Proposition 65 by the DARTIC on July 15, 2009. At that time, the DARTIC determined that BPA had not been clearly shown to cause developmental, female reproductive or male reproductive toxicity. At the upcoming meeting, the DARTIC will advise OEHHA, pursuant to Section 25249.8(b), whether BPA has been clearly shown, through scientifically valid testing according to generally accepted principles, to cause female reproductive toxicity. The body of scientific data generated and published to characterize the potential effects of BPA exposure on the female reproductive system does not support the listing. As explained further below, a review of the BPA literature focusing on data generated between 2009 and 2014 demonstrates there is not sufficient evidence in humans or in experimental animals to support a causal relationship, meeting the required statutory standard for listing, between BPA exposure and female reproductive toxicity. BPA must not be listed.

B. The Food and Drug Administration, An Authoritative Body under Proposition 65, Has Ruled Out A Causal Connection Between BPA Exposure and Female Reproductive Toxicity Based On Criteria Aligned With Listing Requirements

The U.S. Food and Drug Administration (FDA) BPA Joint Emerging Science Working Group (FDA Working Group) has reviewed the scientific literature on BPA in detail periodically since 2008 and characterized the utility of available study reports for use in hazard identification and risk assessment[11] The FDA, an authoritative body under Proposition 65, has ruled out female reproductive toxicity as an endpoint of potential hazard from BPA exposure, based on the

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[8] *Styrene Information Research Center v. OEHHA* (2012) 210 Cal.App.4th 1082, 1101 (hereinafter "*SIRC*") (emphasis added).

[9] Proposition 65 Ballot Pamphlet, 1986 at p. 54 (emphasis in original).

[10] *See SIRC*, 210 Cal.App.4th at 1101.

[11] U.S. FDA, 2014. 2014 Updated Review of Literature and Data on Bisphenol A (CAS RN 80-05-7), Memorandum from FDA Bisphenol A (BPA) Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council. June 6, 2014. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424071.pdf.

weight of the evidence in the scientific literature [12]. In contrast, a recent review article by Peretz and coauthors[13] presented by OEHHA as the primary new document in the BPA HIM[14] concluded that BPA is both an ovarian and a uterine toxicant.

Significantly for this discussion, the procedures used by the FDA Working Group, on the one hand, and Peretz and coauthors, on the other, to compile, summarize, and review the recent BPA literature are different from one another. Peretz presented all published study reports on BPA without any explicit review to determine relevance to hazard identification; in contrast, the FDA Working Group set specific, scientifically sound, acceptance criteria for use of studies in hazard identification. The rigorous evaluation and presentation of the available data by the FDA Working Group reveals no causal relationship between BPA exposure and female reproductive toxicity sufficient to meet the standard of Section 25249.8(b).

C. The FDA Working Group Used Scientifically Sound Review Criteria That Are Aligned With Applicable Listing Criteria; Peretz And Coauthors Did Not

For the BPA literature review, the FDA Working Group specified scientifically sound criteria to determine acceptability of study report data for hazard identification and risk assessment. That is, reviewed studies had to meet certain acceptance criteria in order for the data to be used for BPA hazard identification (hazard ID).

The criteria used by the FDA Working Group for hazard identification are those identified by the National Toxicology Program's Center for the Evaluation of Risks to Human Reproduction (CERHR), itself an authoritative body under Proposition 65[15]. Different criteria apply to different categories of studies. These and other criteria, which are aligned with the DARTIC Criteria, serve to ensure that the studies accepted for hazard identification are scientifically valid according to generally accepted principles, as required by Section 25249.8(b).

Briefly, acceptance criteria for whole animal studies to be used for hazard ID include:

- Effects related to litter of origin must be accounted for in design and statistical procedures;

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[12] U.S. FDA, 2014. Final report for the review of literature and data on BPA. Memorandum from FDA Bisphenol A Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council. June 6, 2014. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424011.pdf.

[13] Peretz, J., et al., 2014. "Bisphenol A and Reproductive Health: Update of Experimental and Animal Evidence, 2007 – 2013," EHP 122(8): 775-786.

[14] OEHHA, 2015. Hazard Identification Materials for Consideration of the Female Reproductive Toxicity of Bisphenol A. http://oehha.ca.gov/prop65/hazard_ident/BPAhazardID2014.html

[15] U.S. FDA, 2011. 2011 Updated Review of the 'low dose' Literature (Data) on Bisphenol A (CAS RN 80-05-7) and Response to Charge Questions Regarding the Risk Assessment on Bisphenol A. Memorandum from FDA Bisphenol A (BPA) Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council (footnote 5). May 24, 2011. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424074.pdf

- Animals need to be dosed via the dam or directly under individual housing conditions to minimize cross-animal contamination;
- A minimum of 6 animals per treatment group must be used to provide confidence in the results; and
- When similar tests are conducted at multiple ages statistical procedures, accounting for repeated measures, must be used so as to not inflate the degrees of freedom.

The FDA Working Group acceptance criteria for BPA epidemiological studies for hazard ID include[16]:

- Utility of study design (e.g., prospective cohort);
- Sufficiency of size;
- Representativeness of study sample;
- Adherence to proper statistical analyses; and
- Measurement of BPA exposure and outcome, accounting for measurement uncertainty due to diurnal, seasonal and individual variability, and possible contamination from laboratory plastics.

In addition to the criteria outlined here and specified in greater detail in the cited documents, the FDA Working Group noted that the likelihood of sample contamination and inadvertent exposure to trace levels of environmental BPA is high in the low dose studies conducted in recent years[17]. Thus, the procedures to control environmental contamination described in the reviewed articles (or absence thereof) were explicitly considered as acceptance criteria in the review process. Three separate BPA literature reviews have been completed by the FDA Working Group, the two cited above from 2011 and 2014, and a third dated August 2013[18]. The FDA Working Group generally did not review studies on BPA characterized as mechanistic in design. *In vitro* studies except those designed to inform pharmacokinetics were considered to be mechanistic.

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[16] U.S. FDA, 2011. 2011 Updated Review of the 'low dose' Literature (Data) on Bisphenol A (CAS RN 80-05-7) and Response to Charge Questions Regarding the Risk Assessment on Bisphenol A. Memorandum from FDA Bisphenol A (BPA) Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council (footnote 5). May 24, 2011. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424074.pdf.

[17] U.S. FDA, 2014. Final report for the review of literature and data on BPA. Memorandum from FDA Bisphenol A Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council. June 6, 2014. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424011.pdf.

[18] U.S. FDA, 2013. Updated review of literature and data on BPA. Memorandum from FDA Bisphenol A Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council. August 22, 2013. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424073.pdf.

Based on the reviewed studies, and applying scientifically sound review criteria, FDA ruled out female reproductive toxicity as an endpoint of potential hazard from BPA exposure, based on the weight of the evidence in the scientific literature [19].

Peretz and her coauthors took a much different approach in evaluating BPA studies compared to the FDA Working Group. They considered the strength of the evidence that supported effects of BPA described in the literature, categorizing evidence as strong, limited, or inconclusive[20]. In contrast to the scientifically disciplined criteria used by FDA Working Group criteria, Peretz and her coauthors evaluated studies as follows:

- Evidence for an effect was considered strong when multiple studies in multiple species indicated a similar effect of BPA on a reproductive tissue or endpoint, even if there was not complete agreement among studies;
- Evidence for an effect was considered to be limited when half of the studies or less indicated a similar effect of BPA on a reproductive tissue or endpoint, and/or when *in vitro*, animal, and epidemiological study results were discordant; and
- Evidence was considered to be inconclusive when a limited number of studies evaluated the potential effect of BPA on a reproductive tissue or endpoint or when the studies were conducted in only one species or *in vitro* only.

This review by Peretz and coauthors did not take into account the strength of the individual studies. They did not account for the criteria that the DARTIC must consider when evaluating a chemical for possible listing.[21] For example, epidemiological studies were not evaluated for proper control of confounding factors, bias, and effect modifiers, and the animal toxicology studies were not evaluated based on overall protocol, including numbers of animals, presence of appropriate controls, adequate number of dose levels to assess dose response, and consideration of maternal and systemic toxicity. All of these criteria and more are specified as criteria for recommending chemicals for listing as "known to the state to cause reproductive toxicity" by the DARTIC[22]. Because those criteria were not accounted for by Peretz and her coauthors, the studies on which they rely should not be considered by the DARTIC.

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[19] U.S. FDA, 2014. Final report for the review of literature and data on BPA. Memorandum from FDA Bisphenol A Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council. June 6, 2014. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424011.pdf.

[20] Peretz, J., et al., 2014. "Bisphenol A and Reproductive Health: Update of Experimental and Animal Evidence, 2007 – 2013," EHP 122(8): 775-786.

[21] Developmental and Reproductive Toxicant Identification Committee (Nov. 1993) Criteria For Recommending Chemicals For Listing As "Known To The State To Cause Reproductive Toxicity." http://oehha.ca.gov/prop65/policy_procedure/pdf_zip/dartCriteriaNov1993.pdf.

[22] Developmental and Reproductive Toxicant Identification Committee (Nov. 1993) Criteria For Recommending Chemicals For Listing As "Known To The State To Cause Reproductive Toxicity." http://oehha.ca.gov/prop65/policy_procedure/pdf_zip/dartCriteriaNov1993.pdf.

The Peretz et al. review article concludes that strong evidence exists that: 1) BPA is an ovarian toxicant in animal models and women, and 2) BPA is a uterine toxicant in animal models.

- The specific endpoints of ovarian toxicity identified in the Peretz et al. article relevant to listing of BPA for female reproductive toxicity are: 1) adverse effect on early oogenesis and ovarian follicle formation, 2) alteration of steroidogenesis, and 3) reduction in oocyte quality.
- Specific endpoints of uterine toxicity relevant to listing of BPA for female reproductive toxicity identified are: 1) impairment of uterine endometrial cellular proliferation, 2) decrease in uterine receptivity, and 3) increased implantation failure in several strains and species of animals.

Each of these endpoints is discussed further below.

D. A Critical Review of the Evidence Presented by Peretz and Coauthors Demonstrates That Such Evidence Cannot Support Listing BPA As A Female Reproductive Toxicant

The great majority of the epidemiological and laboratory animal studies identified by Peretz that associated BPA exposure with female reproductive toxicity do not meet the DARTIC Criteria for listing BPA as a female reproductive toxicant. In order for an epidemiological study or studies to meet the DARTIC Section 3.A(1) criteria of "Sufficient evidence in humans," the study must be "scientifically valid according to generally accepted principles, and provide convincing evidence to support a causal relationship between exposure to" BPA and the reproductive effect in question[23]. In order for a laboratory animal study or studies to meet the DARTIC Section 3.C criteria of "Sufficient evidence in experimental animals (mammals), such that extrapolation to humans is appropriate," a list of four criteria, found in Section 3.C(1)-(4) of the DARTIC Criteria, must be met. Briefly, these criteria include: appropriate experimental design and number of animals, presence of appropriate controls, relevant exposure timing and route of administration, and an adequate number of dose levels to demonstrate a dose-response effect.

These criteria are virtually the same as those specified in somewhat greater detail by the FDA Working Group for hazard identification of BPA[24]. The epidemiological and animal studies cited by Peretz et al. have been reviewed and, as discussed below, virtually none conform

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[23] Developmental and Reproductive Toxicant Identification Committee (Nov. 1993) Criteria For Recommending Chemicals For Listing As"Known To The State To Cause Reproductive Toxicity." http://oehha.ca.gov/prop65/policy_procedure/pdf_zip/dartCriteriaNov1993.pdf.

[24] U.S. FDA, 2011. 2011 Updated Review of the 'low dose' Literature (Data) on Bisphenol A (CAS RN 80-05-7) and Response to Charge Questions Regarding the Risk Assessment on Bisphenol A. Memorandum from FDA Bisphenol A (BPA) Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council (footnote 5). May 24, 2011. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424074.pdf.

to the DARTIC Criteria for listing BPA as female reproductive toxicant[25,26,27]. The DARTIC should not consider them.

For the convenience of the DARTIC members, TSG has prepared summaries of the studies attached as Exhibits 4-9.

1. Adverse effects on early oogenesis and ovarian follicle formation

Peretz and coauthors[28] purportedly found "strong evidence" that developmental BPA exposure alters the onset of meiosis, interferes with germ cell nest breakdown in the fetal ovary, and accelerates follicle transition. These findings were based on approximately 13 articles cited related to the topic. Five of the articles cited by Peretz were conducted *in vitro* and do not meet the DARTIC Criteria since they are not epidemiological and were not conducted in experimental animals[29]. Two additional studies were cited; one was mechanistic in nature[30] and the route of BPA administration in the other was intraperitoneal injection in 3 week old rats, which is not relevant to expected human exposures[31].

Briefly, the remaining six studies conducted in experimental animals, cited by Peretz as being related to BPA alterations in early oogenesis and follicle formation, were determined to be of ***no utility*** for hazard ID or listing as known to cause female reproductive toxicity [32,33]. The primary reasons across studies were:

- Lack of purity of administered BPA;

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[25] U.S. FDA, 2011. 2011 Updated Review of the 'low dose' Literature (Data) on Bisphenol A (CAS RN 80-05-7) and Response to Charge Questions Regarding the Risk Assessment on Bisphenol A. Memorandum from FDA Bisphenol A (BPA) Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council (footnote 5). May 24, 2011. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424074.pdf.

[26] U.S. FDA, 2013. Updated review of literature and data on BPA. Memorandum from FDA Bisphenol A Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council. August 22, 2013. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424073.pdf.

[27] U.S. FDA, 2014. Final report for the review of literature and data on BPA. Memorandum from FDA Bisphenol A Joint Emerging Science Working Group, to FDA Chemical and Environmental Science Council. June 6, 2014. http://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/FoodAdditivesIngredients/UCM424011.pdf.

[28] Peretz, J., et al., 2014. "Bisphenol A and Reproductive Health: Update of Experimental and Animal Evidence, 2007 – 2013," EHP 122(8): 775-786.

[29] Brieño-Enriquez, 2011, 2012; Trapphoff, 2013; Peretz, 2012; Ziv-Gal, 2013.

[30] Zhang XF, 2012.

[31] Li et al, 2014.

[32] Lawson, 2011 and Rodriguez, 2010, summarized in FDA, 2011; Rivera, 2011 and Veiga-Lopez, 2013 summarized in FDA, 2013; Hunt, 2012 summarized in FDA, 2014.

[33] Zhang HQ, 2012, reviewed by Technology Sciences Group Inc.

- Possible or known phytoestrogens in the diet
- Potential environmental contamination of animals and/or samples with BPA;
- No dose-response characterization;
- A single BPA dose level administered; and
- Small subject number.

Other deficiencies also plagued the studies on which Peretz et al. relied related to purported oogenesis and follicle development alterations. They included:

- In the Hunt et al. study, the route of exposure via implanted silastic capsules was of questionable relevance[34];
- The Lawson et al. study contained statistical deficiencies due to pooling ovaries across litters[35]; and
- The Rodriguez et al. study failed to demonstrate the biological significance of the assessed molecules to follicle development and physiology. [36].

2. Alteration of female steroidogenesis

Peretz and coauthors[37] found purportedly "strong evidence" that developmental BPA exposure alters steroid hormone production in animals and women. These findings were based on approximately 20 articles cited related to the topic, including six epidemiological reports.

All six of the epidemiological studies were reviewed and were determined to be of no utility for hazard ID or listing as known to cause female reproductive toxicity[38]. Of the remaining 14 reports cited by Peretz, four were conducted *in vitro* and are not relevant to DART listing[39]. One article in experimental animals was published prior to the previous DARTIC meeting in 2009 and thus already has been considered (and presumably rejected) by the DARTIC[40]. Seven experimental animal studies were reviewed and only one was determined to be of utility for hazard ID[41]. The last two articles cited by Peretz related to BPA-induced

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[34] Hunt et al., 2012, summarized in FDA, 2014.

[35] Lawson et al., 2011, summarized in FDA, 2011.

[36] Rodriguez et al., 2010, summarized in FDA, 2011.

[37] Peretz, J., et al., 2014. "Bisphenol A and Reproductive Health: Update of Experimental and Animal Evidence, 2007 – 2013," EHP 122(8): 775-786.

[38] Galloway et al., 2010, Kandaraki et al., 2011, and Mok-Lin et al, 2010,summarized in FDA, 2011; Bloom et al., 2011a, summarized in FDA, 2013 and Ehrlich, 2012a and Ehrlich 2013, summarized in FDA, 2014.

[39] Grasselli, 2010, Peretz, 2011, Ziv-Gal, 2013 and Zhou 2008.

[40] Berger, 2008.

[41] Fernandez, 2010 and Mendoza-Rodriguez, 2011, summarized in FDA, 2011; Rivera, 2011, and Varayoud, 2011, summarized in FDA, 2013; and Kobayashi, 2012, Lee SG, 2013, and Tan, 2013, summarized in FDA, 2014.

changes in female steroidogenesis reported exposure to doses of BPA known to be toxic, 100 mg/kg/day or greater (Berger, 2010), or evaluated molecular changes not associated with specific adverse effects[42]. Maternal toxicity must be considered by the DARTIC as a basis for rejecting a study purporting to demonstrate reproductive or developmental effect. The Committee should reject the Berger, 2010 study here.

None of the six epidemiological studies cited by Peretz was determined to have utility for hazard ID or listing as known to cause female reproductive toxicity. The reasons for a lack of utility for hazard ID or listing were different for each study; still, they each establish that these studies do not reflect "scientifically valid testing according to generally accepted principles":

- The Bloom study of 58 women completing a first *in vitro* fertility (IVF) cycle was found to be too small and underpowered to be useful[43];
- The Ehrlich prospective study, intended to detect a correlation between egg implantation failure following IVF treatment and urine BPA levels, was not useful for hazard ID or listing due to the uncontrolled infertility factors and health status of the patients and partners, the timeliness of IVF medication, and the status of previous IVF failures[44];
- The Mok-Lin study of women who intended to use their own oocytes for IVF was not useful for hazard ID or listing currently, based on: the small sample size, limited power for analysis by infertility type, the fact that the findings may not relate to those not using IVF, and the fact that there was no indication of whether the known diurnal variation in urine BPA was accounted for[45];
- The Kandaraki study intended to measure BPA levels in women with polycystic ovary syndrome (PCOS) was not useful for hazard ID or a listing recommendation for a number of reasons: BPA levels reported in blood may be unreliable due to non-specific binding in the enzyme-linked immunoassay (ELISA) used to measure BPA in blood, human subject variability, and variable analytical methods[46]. Additional limitations included a lack of clarity regarding the study period, source and selection of controls, and the impact of laboratory plastics on BPA;
- A second Ehrlich study designed to investigate the relationship between BPA concentrations and CYP19A1 gene expression in granulosa cells collected from a subsample of women enrolled in a larger prospective cohort study, was not useful for hazard identification or listing because of its small subject number and all-Caucasian study sample[47]; and

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42 Berger, 2010, and Xi, 2011.

43 Bloom 2011a, summarized in FDA, 2013.

44 Ehrlich, 2012a, summarized in FDA, 2014.

45 Mok-Lin, 2010, summarized in FDA, 2011.

46 Kandaraki, 2011, summarized in FDA, 2011.

47 Ehrlich, 2013, summarized in FDA, 2013.

- The Galloway study from Italy that presented urine and blood BPA levels in addition to sex hormone levels was not found to be useful for hazard ID or listing recommendation, because it did not clarify the relationship between BPA levels and sex hormone levels[48].

Only one of the seven reports of animal studies cited by Peretz to describe the effects of BPA on female steroidogenesis was identified as useful for hazard ID, and this was by Fernandez and coworkers[49], while one study by Varayoud[50] had limited value for hazard ID but only at a 20 mg/kg dose. The Fernandez study reported a dose-dependent increase in estradiol levels in adult rats in estrus that had been treated subcutaneously with 0.25 to 0.62 mg/kg/day, 2.5-6.2 mg/kg/day, or 25 to 62 mg/kg/day BPA on postnatal days 1 to 10. Limitations of this study include the fact that dosing was approximate since animals were not weighed, and pups in different dose groups were housed in the same cage. Thus, these studies must not be considered by the DARTIC because they do not meet the DARTIC Criteria for listing and they demonstrate that the testing was not undertaken in a scientifically valid manner accordance with generally accepted principles, as Proposition 65 requires.

The remaining five reports of experimental animal studies cited by Peretz to describe the effects of BPA on female steroidogenesis were identified as not useful for hazard ID or listing as known to cause female reproductive toxicity. Limitations for utility of the studies included: small sample size of 3 to 5 per group, potential for BPA contamination in caging, water, food or phytoestrogen in the diet, use of inappropriate test unit (pups instead of litter), use of a single dose level, details regarding estrous cycle not specified, and in the Tan[51] study, the standard deviations were unusually low for the endpoint measured. Like the studies discussed above, these studies also must not be considered by the DARTIC because they do not meet the DARTIC Criteria for listing and they demonstrate that the testing was not undertaken in scientifically valid manner accordance with generally accepted principles, as Proposition 65 requires.

3. Reduction in oocyte quality

Peretz and coauthors[52] found purportedly “strong evidence” that developmental BPA exposure reduced oocyte quality in animals and women. These findings were based on approximately eight articles cited related to the topic, including three epidemiological reports.

Two of the epidemiological reports were cited in relation to the steroidogenesis topic and are addressed in the previous section[53]. All three of the epidemiological studies were reviewed

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48 Galloway, 2010, summarized in FDA, 2011.

49 Fernandez, 2010, summarized in FDA, 2011.

50 Varayoud, 2011, summarized in FDA, 2011.

51 Tan, 2013, summarized in FDA, 2014.

52 Peretz, J., et al., 2014. "Bisphenol A and Reproductive Health: Update of Experimental and Animal Evidence, 2007 – 2013," EHP 122(8): 775-786.

53 Mok-Lin, 2010, summarized in FDA, 2011, and Ehrlich, 2013, summarized in FDA, 2013.

and were determined to be of no utility for hazard ID or listing as known to cause female reproductive toxicity[54].

Five experimental animal study reports cited by Peretz for supporting an endpoint of reduction in oocyte quality were evaluated. One report was a review article and a second one focused on the potential for phytoestrogens to modulate effects on oocytes, so neither is useful for hazard ID or listing as known to cause female reproductive toxicity[55]. Thus, they must not be considered by the DARTIC. Two additional reports were published prior to the previous DART Identification Committee meeting in 2009 and thus already have been considered (and rejected) by the DARTIC[56]. The remaining study report was determined to be of no utility for hazard ID or listing as known to cause female reproductive toxicity[57]. Thus, these too must not be considered by the DARTIC.

The epidemiological study by Fujimoto was cited as purporting to demonstrate an overall correlation between BPA serum concentrations and the developing potential of human oocytes[58]. However, deficiencies in the study demonstrate that the DARTIC and statutory criteria for listing are not met:

- There was only an association between BPA and oocyte maturation in the nine Asian women in the intracytoplasmic sperm injection (ICSI) group, but no association when all the cases were considered, though there was an association between blood BPA level and decreased fertility among the 26 cases of ICSI or conventional insemination;
- The findings might not be applicable to the general population;
- The study was a part of a larger hypothesis-generating study and the subject number is small; and
- There was no investigation of another possible confounding exposure associated with elevated BPA that could be related to the decreased fertility[59].

The experimental animal study by Chao and coworkers was intended to evaluate potential effects of BPA on several genes in mice and to determine if alterations in gene expression influenced oocyte development. Limitations in this study[60] included

- A lack of clarity in the experimental design and methods description, including dosing route described as by “hypodermical injection”;

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54 The third study was Fujimoto et al., 2011, summarized by FDA in 2011.

55 Hunt et al., 2009 and Muhlhauser et al., 2009.

56 Eichenlaub-Ritter, 2008 and Pacchierotti et al, 2008.

57 Chao, 2012, summarized in FDA, 2014.

58 Fujimoto, 2011, summarized in FDA in 2011.

59 Fujimoto, 2011, summarized in FDA in 2011.

60 Chao, 2012, summarized in FDA, 2014.

- A vague study protocol description of female pups grouping with dams;
- An unknown number of female pups assigned to treatment groups and selected for measurements or pooled for molecular studies; and
- There were only two treatment groups so dose-response could not be assessed, route of exposure was not clearly described and no information was presented on housing conditions, diet or source or purity of BPA[61].

4. Impairment of uterine endometrial cellular proliferation

Peretz and coauthors found purportedly "strong evidence" that BPA exposure impairs uterine endometrial cellular proliferation in animals. These findings were based on approximately thirteen cited articles related to the topic, including two epidemiological reports[62]. Three of the articles cited by Peretz were conducted *in vitro* and do not meet the DARTIC Criteria since they are not epidemiological and were not conducted in experimental animals[63]. Eight experimental animal studies were evaluated for utility in hazard ID and listing as known to cause female reproductive toxicity, but three of them reported only mechanistic endpoints not useful for identification of a causal relationship between BPA exposure and female reproductive toxicity[64], and one was published prior to the previous DARTIC meeting in 2009 (and thus already has been considered and presumably rejected)[65]. The remaining four experimental animal studies were evaluated for utility in hazard ID and listing as known to cause female reproductive toxicity[66].

The epidemiological study by Cobellis that suggested BPA may be associated with endometriosis did not meet the criteria for hazard ID or listing because it lacked proper study design[67]. The lack of BPA detection in control sera in the Cobellis study suggests possible selection bias or measurement error, and since no comparison could be made between control and case BPA levels, no case could be made for a statistically significant correlation between BPA and endometriosis. Thus, the study does not meet the DARTIC or statutory criteria for listing.

The epidemiological study by Buck did not meet the criteria for hazard ID or listing because no significant correlations between BPA and endometriosis were found and there was uncertainty in the diagnosis of endometriosis, as well as an endometriosis finding only in a

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61 Chao, 2012, summarized in FDA, 2014.

62 Cobellis, 2009, summarized in FDA, 2011 and Buck, 2013, summarized in FDA, 2014.

63 Aghajanova, et al., 2011, Bredhult, et al., 2009, An, et al., 2013.

64 Bromer, 2010, summarized in FDA, 2011, Hiyama, 2011 and Berger, 2010.

65 Varayoud, 2008.

66 Aldad, 2011, reviewed in FDA, 2013, Bosquiazzo, 2010, reviewed in FDA, 2011, Mendoza-Rodriguez, 2011, and Signorile, 2010, reviewed in FDA, 2011.

67 Cobellis, 2009, summarized in FDA, 2011.

population cohort with a small sample size of 14 with endometriosis compared to 113 without[68]. Again, because this study does not meet the DARTIC or statutory criteria for listing, it should be rejected.

One of the four experimental animal studies reviewed for the endpoint of impairment of endometrial cellular proliferation by Aldad[69] was determined to have limited utility for hazard ID and relevancy for listing. This study in non-human primates demonstrated that BPA could inhibit estrogen-induced progesterone receptor gene expression in the endometrium of the African green monkey after oophorectomy and estrogen supplementation. The significant limitations of the study, which reveal the study's failure to meet the relevant Proposition 65 listing criteria, included:

- Administration of only a single dose level of BPA;
- A description of methods inadequate to determine the validity of the system;
- Dosing was by implanted Alzet minipumps (BPA) and subcutaneous silastic implants (estradiol), which may not be relevant to human exposures;
- Systemic levels of BPA were not provided; and
- Potential sources of BPA contamination were not characterized.

Two of the experimental animal studies reviewed for the endpoint of impairment of endometrial cellular proliferation were determined to have no utility for hazard ID or relevancy for listing[70]. The limitations in these two studies included: statistical analysis that did not address the litter effects adequately, lack of a dose response in measured BPA levels, lack of description of contamination control, small sample size, and use of inappropriate test unit (pups instead of litter). The third study in experimental animals was identified as useful only as a supporting study[71]. This study evaluated vascular endothelial growth factor (VEGF) expression and endothelial cell proliferation which could be important in understanding a mechanism of BPA action, but alone the effects are not considered adverse and cannot be used as a basis for listing.

5. Decrease in uterine receptivity

Peretz and coauthors found purportedly "strong evidence" that BPA exposure impairs uterine receptivity, primarily in animals. These findings were based on approximately nine articles cited related to the topic, including one epidemiological report. Three of the articles did not relate to female reproductive toxicity because the effects of BPA exposure on implantation in ***unexposed*** female rats mated to exposed males were evaluated[72]. Two of the studies were

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68 Buck Louis, 2013, summarized in FDA, 2013.

69 Aldad, 2011, reviewed in FDA, 2013.

70 Mendoza-Rodriguez, 2011, and Signorile, 2010, summarized in FDA, 2011.

71 Bosquiazzo, 2010, summarized in FDA, 2011.

72 Salien 2009a, 2009b, summarized in FDA, 2011, and Tiwari & Vanage, 2013, summarized in FDA, 2014.

conducted with high doses known to be associated with systemic toxicity, the lowest dose being 100 mg/kg/day[73]. Systemic toxicity is a factor for rejecting a study as supporting reproductive or developmental toxicity, and the DARTIC should reject this study on that basis. One study cited was published prior to the previous DART Identification Committee meeting in 2009 and therefore already has been considered and presumably rejected[74].

The epidemiological study by Ehrlich suggesting BPA may be associated with decreased uterine receptivity did not meet the criteria for hazard ID or listing because it was limited by variability in urine BPA measurements and had a small sample size[75]. In addition, the study did not consider possible contributions from the male partner or the possibility that data from IVF-treated women may not apply to the larger population. These deficiencies demonstrate that the DARTIC and statutory criteria for listing are not met, and the DARTIC should reject this study.

One of the two experimental animal studies reviewed for the endpoint of decreased uterine receptivity had limited utility for hazard identification and listing as mentioned above for the steroidogenesis endpoint[76]. This study by Varayoud demonstrated a long-lasting decrease in implantation sites in rats that received 20 mg/kg BPA by subcutaneous injection on postnatal days 1, 3, 5 and 7. A limitation of this study is that the uterine environment of rats dosed with 20 mg/kg BPA as neonates is not normal. The other experimental animal study reviewed for the endpoint of decreased uterine receptivity was primarily a mechanistic study with one non-mechanistic component[77] . This study had no utility for hazard identification or listing, based on a non-statistically significant decrease in implantation on gestation days 4.5 to 5.5 in mice exposed to 40 mg/kg/day BPA on gestation days 0.5 through 3.5.

6. Increased implantation failure in several strains and species

The Peretz article acknowledges that “many experimental studies have reported that low-dose BPA does not affect the number of live pups or total number of delivered pups in mice and rats” but also indicates that: “In a few studies, however, low-dose BPA exposure was reported to decrease the number of live pups born to prenatally plus neonatally exposed CD-1 mice and Holtzman rats.”[78] Three studies were cited to support the observations of a decrease in pup number, suggesting implantation failure[79].

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[73] Berger, 2010, Hiyama, 2011.

[74] Berger et al, 2008.

[75] Ehrlich 2012b, summarized in FDA, 2014.

[76] Varayoud, 2011, summarized in FDA, 2013.

[77] Xiao, 2011, summarized in FDA, 2013.

[78] Peretz, J., et al., 2014. "Bisphenol A and Reproductive Health: Update of Experimental and Animal Evidence, 2007 – 2013," EHP 122(8): 775-786.

[79] Cabaton, 2001, summarized in FDA, 2011, and Salien 2009a, 2009b, summarized in FDA, 2011.

As noted above, two of the cited articles did not relate to female reproductive toxicity because the effects of BPA exposure on implantation in ***unexposed*** female rats mated to exposed males were evaluated[80]. The third study cited as reporting an increase in implantation failure associated with BPA exposure was not suitable for hazard ID or listing[81]. In this study by Cabaton and coworkers, doses of BPA (0, 25, 250, and 25,000 ng/kg/day) were administered to rat dams in a vehicle of 50% DMSO via osmotic minipump from gestation day 8 through lactation day 16. F1 females were continually bred beginning at 8 weeks of age, with pups removed on the day of delivery. The cumulative number of pups delivered was decreased compared to controls in the low and high dose groups, but not the mid-dose group. The high dose group had fewer pregnancies and a more rapid decline in pups delivered over time. Limitations in this study included the inappropriate solvent vehicle, the unusual dose-response, and the fact that forced breeding alters maternal hormones and may alter the response to BPA.

E. Summary and Conclusions of the Critical Review of Peretz Evidence

Of the approximately 38 epidemiological and experimental animal study reports on BPA identified by Peretz and coauthors published between 2009 and 2014 as providing "strong evidence" that BPA is an ovarian and uterine toxicant, two[82] were found scientifically suitable for hazard ID and listing recommendation, two[83] provided limited utility and one[84] provided some utility for hazard ID and listing recommendation. Taken together, these studies do not support listing of BPA for female reproductive toxicity.

None of the studies identified as scientifically useful for hazard ID and listing actually supports the listing of BPA as known by the state to cause female reproductive toxicity. One of the two studies found to be of utility for hazard ID and listing was by Kobayashi, who reported that low dose BPA did not alter gestation length in gestationally plus neonatally exposed mice[85]. In this study there was no effect of dietary BPA at 0.05, 0.5 or 5.0 mg/kg/day on reproduction and development in the C57BL/6J mouse. The second study found to be of utility for hazard ID and listing by Fernandez reported a dose-dependent increase in estradiol levels in adult rats that had been treated on postnatal days 1 to 10 by subcutaneous injection with 0, 0.25 to 0.62 mg/kg/day, 2.5-6.2 mg/kg/day, or 25 to 62 mg/kg/day BPA.

The studies that provide some, or limited utility for hazard ID and listing do not support the listing of BPA as known by the state to cause female reproductive toxicity. The Varayoud study[86] provided some utility for hazard ID and listing, showing a long-lasting reduction in

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80 Salien 2009a, 2009b, summarized in FDA, 2011.

81 81 Cabaton, 2001, summarized in FDA, 2011.

82 Fernandez, 2010, and Kobayashi, 2010, both summarized in FDA, 2011.

83 Aldad, 2011, summarized in FDA, 2013, and Kobayashi, 2012, summarized in FDA, 2014.

84 Varayoud, 2011, summarized in FDA, 2013.

85 Kobayashi, 2010, summarized in FDA, 2011.

86 Varayoud, 2011, summarized in FDA, 2013.

implantation sites in rats that received 20 mg/kg injections of BPA subcutaneously on postnatal days 1, 3, 5, and 7. However, a critical deficiency in this study is that the uterine environment of rats that receive this dose of BPA neonatally is not normal. The Aldad study provided data that demonstrated BPA could inhibit estrogen-induced progesterone receptor gene expression in the endometrium of the African green monkey[87]. Limitations to this study included use of a single dose level, dosing by osmotic minipumps and subcutaneous silastic implants (a system that was not validated), and potential sources of contamination were not characterized. The third study that provided limited utility for hazard ID and a listing recommendation is another study by Kobayashi and coworkers that demonstrated that low dose BPA did not alter gestation length in gestationally plus neonatally exposed rats[88].

F. A New BPA Study Conducted by the NCTR Not Reviewed by Peretz and Coauthors, Delclos et al, 2014, Demonstrates That There Is No Causal Effect Between BPA Exposure and Female Reproductive Toxicity

A new comprehensive study by Delclos et al., 2014 (attached as Exhibit 2 to this submission) was conducted to identify effects caused by oral administration of BPA to rats by gavage from gestation day 6 through postnatal day 90[89]. BPA was administered to groups of Sprague-Dawley rats by gavage at seven equally-spaced doses below the current no observed adverse effect level (2.5 to 2,700 μg/kg/day), and at two high doses (100,000 and 300,000 μg/kg/day). Two positive control groups received ethinyl estradiol (0.5 and 5.0 μg/kg/day), and there were two negative control groups, a vehicle control that received 0.3% carboxymethyl cellulose and a naïve group. Various measurements were taken from pups on postnatal days (PNDs) 15 and 21, but the focus of the study was on the measurements from animals maintained until PND 90.

Endpoints measured included: blood levels of aglycone (active) and conjugated BPA, markers of sexual development (e.g., timing of vaginal opening), vaginal cytology for evaluation of estrous cycling, organ weights, and histopathology. The number of female subjects in each dose group evaluated at the 90-day time point was between 18 and 23. One animal per sex per litter was tested and euthanized for necropsy and histology.

The Delclos study reported clear systemic adverse effects of BPA in female rats at doses of 100,000 and 300,000 μg/kg/day, including significant decrease in body weight in the high dose group. Potential effects in the low-dose BPA groups could not be linked to treatment as these occurred sporadically across dose groups and were not consistent across organs as the effects of ethinyl estradiol (EE2) and high dose BPA were. Small ovaries were associated with treatment in both EE2 groups and the 300,000 μg BPA/kg/day group, with incidences of 8/20, 16/20, and 10/19 in the 0.5 EE2, 5.0 EE2, and 300,000 μg/kg/day BPA groups, respectively. The

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87 Aldad, 2011, summarized in FDA, 2013.

88 Kobayashi, 2012, summarized in FDA, 2011.

89 Delclos et al., 2014. Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90. Tox Sci139(1) 174-197.

results of the study supported the authors' conclusion that "BPA in the "low dose" region from 2.5 to 2700 µg/kg bw/day did not produce effects in the evaluated endpoints that differ from normal background biological variation."

The Delclos study meets all the criteria identified by the DARTIC, the CERHR, and the FDA Working group as scientifically valid and conducted according to generally accepted scientific principles. Briefly, the study meets the following scientific criteria:

- The effects related to litter of origin were accounted for in the experimental design, as one female pup per litter was designated for blood chemistry and histopathology on PND 90, the main focus of the study;
- The routes of exposure were relevant for extrapolation to humans; rats received BPA during gestation via the dam, and by direct oral gavage exposure beginning on PND 1;
- The female subject number in each dose group evaluated at PND 90 was between 18 and 23, sufficient to provide confidence in the results;
- The study design had sufficient negative and positive control groups, including a vehicle control, a naïve control, and two positive control groups that received ethinyl estradiol;
- An appropriate number and range of BPA doses to demonstrate a dose-response effect were included: seven low doses of BPA, and two high doses; and,
- The study was conducted under Good Laboratory Practices (GLP) as specified in 21 CFR, Part 58.

A study by Churchwell[90] and coworkers evaluated the internal dosimetry for orally administered BPA throughout perinatal and adult life stages in rats that corresponds to the exposures achieved in the companion paper by Delclos et al. The evaluation showed that only the two highest doses of BPA (100 and 300 mg/kg/day) produced a pattern of internal dosimetry and estrogen receptor occupancy similar to the positive reference estrogen compounds, and a similar pattern of estrogenic effects in vivo. In addition, this study demonstrated that despite great care taken by the researchers to minimize contamination of serum samples during collection and storage, trace level measurements of free BPA were compromised at the lower doses, and an unidentified source of BPA in the animal room lead to levels of metabolized BPA in control rats similar to those in the low dose BPA groups. This internal dosimetry and the detailed analysis of BPA contamination strengthen the Delclos study results.

IV. BPA MUST NOT BE LISTED AS A FEMALE REPRODUCTIVE TOXICANT

The weight of the evidence on BPA female reproductive toxicity generated since the DARTIC last considered BPA does not support listing BPA for female reproductive toxicity. Approximately 38 epidemiological and experimental animal study reports were identified by Peretz and coauthors as supporting BPA as a female reproductive toxicant, but a scientific review of these reports indicates that the body of work does not meet the statutory and regulatory

[90] Churchwell MI, et al., 2014. Comparison of Life-Stage-Dependent Internal Dosimetry for Bisphenol A, Ethinyl Estradiol, a Reference Estrogen, and Endogenous Estradiol to Test an Estrogenic Mode of Action in Sprague Dawley Rats. Toxicological Sciences 139(1), 4–20.

criteria for listing: they do not clearly show, through scientifically valid testing according to generally accepted principles, that BPA causes female reproductive toxicity. The FDA Working Group review of recent scientific studies, using scientifically sound criteria and a weight of the evidence approach aligned with the DARTIC Criteria, concludes that BPA does not cause female reproductive toxicity. Further, a comprehensive new study of oral exposure to BPA during and after gestation demonstrates that the substance does not cause female reproductive toxicity at doses that do not also cause significant systemic toxicity.

Thus, BPA is not ***known*** to cause female reproductive toxicity within the meaning of Proposition 65, and must not be listed. Listing BPA in these circumstances will lead to Proposition 65 warnings that will not promote the public health, and will lead to enforcement actions that are unconnected to the public interest. Such consequences are directly contrary to OEHHA's larger goals of improving Proposition 65 by reducing unnecessary warnings and lawsuits.

Respectfully,

Grimaldi Law Offices

By:

Ann G. Grimaldi
Counsel for The Art and Creative Materials Institute

Encls. (References; Exhibits 1-9)

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EXHIBITS 1- 9

TO THE SUBMISSION OF

THE ART AND CREATIVE MATERIALS INSTITUTE

REGARDING

BPA HAZARD IDENTIFICATION MATERIALS

April 20, 2015

EXHIBIT 1

(DARTIC Criteria)

(Nov. 1993) CRITERIA FOR RECOMMENDING CHEMICALS FOR LISTING AS "KNOWN TO THE STATE TO CAUSE REPRODUCTIVE TOXICITY"

1. General Principles

   A. The criteria included herein shall be utilized by the Office of Environmental Health Hazard Assessment Science Advisory Board Developmental and Reproductive Toxicant (DART) Identification Committee to identify chemicals to be recommended as known to the State to cause reproductive toxicity, for purposes of the Safe Drinking Water and Toxic Enforcement Act of 1986 ("Proposition 65").

   B. These criteria shall be updated periodically, as appropriate, to incorporate contemporary scientific views dealing with the evaluation of reproductive toxicity.

   C. These criteria are intended to give the DART Identification Committee maximal flexibility in evaluating all pertinent scientific information in determining whether a chemical is known to the State to cause reproductive toxicity. These criteria are not intended to limit the scope of the Committee's consideration of appropriate scientific information, nor to limit its use of best scientific judgment.

   D. In evaluating the sufficiency of data, a "weight-of-evidence" approach shall be used to evaluate the body of information available for a given chemical.

2. Definitions

   A. Chemicals known to the State to cause reproductive toxicity include those that have been clearly shown to be toxic by "scientifically valid testing according to generally accepted principles" (Health and Safety Code, Section 25249.8(b)).

   B. For purposes of these criteria, "reproductive toxicity" includes "developmental toxicity", "female reproductive toxicity", and "male reproductive toxicity".

   C. "Developmental toxicity" is defined to include adverse effects on the products of conception (i.e., the conceptus), including but not limited to:

(1) Embryo/fetal mortality (including resorption, miscarriage/spontaneous abortion, or stillbirth), malformations, structural abnormalities and variations, altered fetal growth, and change in gestational age at delivery.

(2) Postnatal parameters including growth and development, physiological deficits and delay, neurological, neurobehavioral and psychological deficits, altered sex ratio, abnormal sexual development or function, and morbidity or mortality.

(3) Transplacental carcinogenesis.

(4) Somatic or genetic (germ cell) mutations in the conceptus.

D. "Female reproductive toxicity" is defined to include effects on the adult or, where appropriate, developing female organism, including, but not limited to:

(1) Adverse effects on reproductive structure or function including:

a. Genetic damage to the ovum or its precursors.

b. Alterations in ovulation, menstrual (estrous) cycle and/or menstrual (estrous) disorders.

c. Impaired or altered endocrine function.

d. Complication of pregnancy.

(2) Impaired reproductive performance (e.g., sub fertility or infertility, including:

a. Increased pregnancy wastage (e.g., miscarriage/spontaneous abortion or stillbirth).

b. Inability or decreased ability to conceive (e.g., time to conception).

c. Adverse effects observed in sexual behavior, onset of puberty, fertility, gestation, parturition, lactation, or premature reproductive senescence.

E. "Male reproductive toxicity" is defined to include effects on the adult or, where appropriate, developing male organism, including, but not limited to:

(1) Adverse effects on reproductive structure or function including:

a. Genetic damage to the spermatozoon or its precursors.

b. Impaired sperm and/or seminal fluid production, including alterations in sperm number, morphology, motility, and ability to fertilize.

c. Impaired or altered endocrine function.

(2) Impaired reproductive performance (e.g., sub fertility, infertility, or impotence).

3. Developmental, and female and male reproductive effects shall meet at least one of the following criteria for recommendation as known to the State to cause reproductive toxicity.

A. Sufficient evidence in humans.

(1) Includes any of a variety of epidemiological studies, so long as the study or studies are scientifically valid according to generally accepted principles and provide convincing evidence to support a causal relationship between exposure to the chemical, and the developmental or reproductive effect in question. This requires accurate exposure and toxicity endpoint classification and proper control of confounding factors, bias, and effect modifiers.

(2) Clinical cases can be used if carefully delineated with respect to the presence of a specific syndrome (or developmental/reproductive toxicity endpoint) and if the reports consistently show an association between exposure to the agent and the occurrence of the particular endpoint of developmental or reproductive toxicity. Exposure to the agent should have occurred at the developmental or reproductive stage relevant to the endpoints identified.

(3) Weight of evidence considerations.

a. Data from multiple studies increase the confidence for classification of an agent as a developmental or reproductive toxicant, and unless there is an exceptionally strong study (see below), effects should occur in more than one human study for a chemical to be recommended for listing on the basis of epidemiologic evidence alone.

b. Data from a single well conducted epidemiologic developmental or reproduction toxicity study showing a clear relationship between exposure and effect may be sufficient to classify an agent as a developmental or reproductive toxicant, provided there are not equally well conducted studies which do not show an effect and which have sufficient power to call into question the repeatability of the observation in the positive study.

B. Limited evidence or suggestive evidence in humans, supported by sufficient experimental animal (mammalian) data, as described below.

C. Sufficient evidence in experimental animals (mammals), such that extrapolation to humans is appropriate. "Sufficient" animal data would, in most cases, be based on the adequacy of the following:

(1) The experimental design, including overall protocol and numbers of animals, and presence of appropriate controls.

(2) The exposure, in terms of route of administration, is relevant to expected human exposures, and in terms of timing, with regard to critical periods of development for developmental toxicity, sexual maturation, stage of pregnancy, or other critical periods for female reproductive toxicity, and sexual maturation, spermatogenesis, or other critical periods for male reproductive toxicity.

(3) Number of dose levels, so that the presence of a dose-response relationship can be evaluated. It is desirable that the high dose level should elicit maternal toxicity in developmental studies, and systemic toxicity in female and male reproductive studies, and that the low dose should elicit no observable adverse effect for adult and offspring.

(4) Consideration of maternal and systemic toxicity.

Differentiating between (a) the effects of a toxic agent on the conceptus or reproduction and (b) the effects on the conceptus or reproduction that are secondary to the maternal or systemic toxic effects is sometimes difficult and may require special attention, on a case by case basis.

(5) Number of tests or experimental animal species.

a. In general, effects should occur in multiple studies or multiple species for a substance to be recommended for listing.

b. Weight of evidence considerations.

1. Data on a single species from a well conducted developmental or reproduction study may be sufficient to classify an agent as a reproductive toxicant provided there are not equally well conducted studies which do not show an effect and which have sufficient power to call into questions the repeatability of the observation in the positive study.

2. Data on more than one species or from more than a single study increase the confidence for classification of an agent as a reproductive toxicant.

(6) Other considerations, including, but not limited to those listed below, which can increase or decrease the confidence for classification of an agent as a reproductive toxicant.

a. Severity or consistency of findings.

b. Metabolic and pharmacokinetic data.

c. Time course of events.

4. Statistical considerations and biological plausibility.

A. Statistical analyses are important in determining the effect of a particular agent; however, the biological significance of the data should not be overlooked. Given the number of endpoints that can be quantified in developmental and reproduction studies, a few statistically significant

differences may occur by chance alone. Conversely, apparent dose-related trends may be biologically relevant even though statistical analyses do not indicate a significant effect.

B. In determining whether a chemical is to be recommended to be listed as known to the State to cause reproductive toxicity, the biological plausibility of the association between the adverse reproductive effects observed and the chemical in question should be considered. Confidence is increased when, based on known principles of developmental and reproductive biology, physiology, and toxicology, a sound scientific basis exists for the observed adverse effects and the known characteristics of the particular chemical. Conversely, confidence is decreased if the observed adverse effects are contradictory to the known characteristics of the particular chemical.

EXHIBIT 2

(Delclos, 2014)

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Toxicity Evaluation of Bisphenol A Administered by Gavage to Sprague Dawley Rats From Gestation Day 6 Through Postnatal Day 90

K. Barry Delclos,[*,1] Luísa Camacho,[*] Sherry M. Lewis,[†] Michelle M. Vanlandingham,[*] John R. Latendresse,[‡] Greg R. Olson,[‡] Kelly J. Davis,[‡] Ralph E. Patton,[‡] Gonçalo Gamboa da Costa,[*] Kellie A. Woodling,[*] Matthew S. Bryant,[*] Mani Chidambaram,[*] Raul Trbojevich,[*] Beth E. Juliar,[§] Robert P. Felton,[§] and Brett T. Thorn[§]

**Division of Biochemical Toxicology, 3900 NCTR Road, Jefferson, Arkansas 72079; †Office of Scientific Coordination, 3900 NCTR Road, Jefferson, Arkansas 72079; ‡Toxicologic Pathology Associates, Jefferson, Arkansas 72079; and §Division of Bioinformatics and Biostatistics, National Center for Toxicological Research, 3900 NCTR Road, Jefferson, Arkansas 72079*

[1]To whom correspondence should be addressed. Fax: 870-543-7136. E-mail: barry.delclos@fda.hhs.gov.

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Bisphenol A (BPA) is a high production volume industrial chemical to which there is widespread human oral exposure. Guideline studies used to set regulatory limits detected adverse effects only at doses well above human exposures and established a no-observed-adverse-effect level (NOAEL) of 5 mg/kg body weight (bw)/day. However, many reported animal studies link BPA to potentially adverse effects on multiple organ systems at doses below the NOAEL. The primary goals of the subchronic study reported here were to identify adverse effects induced by orally (gavage) administered BPA below the NOAEL, to characterize the dose response for such effects and to determine doses for a subsequent chronic study. Sprague Dawley rat dams were dosed daily from gestation day 6 until the start of labor, and their pups were directly dosed from day 1 after birth to termination. The primary focus was on seven equally spaced BPA doses (2.5–2700 μg/kg bw/day). Also included were a naïve control, two doses of ethinyl estradiol (EE_2) to demonstrate the estrogen responsiveness of the animal model, and two high BPA doses (100,000 and 300,000 μg/kg bw/day) expected from guideline studies to produce adverse effects. Clear adverse effects of BPA, including depressed gestational and postnatal body weight gain, effects on the ovary (increased cystic follicles, depleted corpora lutea, and antral follicles), and serum hormones (increased serum estradiol and prolactin and decreased progesterone), were observed only at the two high doses of BPA. BPA-induced effects partially overlapped those induced by EE_2, consistent with the known weak estrogenic activity of BPA.

Key words: **Bisphenol A; ethinyl estradiol; Sprague Dawley rat; 90-day study.**

Bisphenol A (BPA) is a high production volume industrial chemical used as a monomer for polycarbonate plastics and epoxy resins that have broad applications in consumer products, including medical devices and storage containers for foods and beverages (Shelby, 2008; Willhite *et al.*, 2008). Biomonitoring studies support widespread exposure to BPA (Calafat *et al.*, 2005, 2008). Regulatory agencies and expert panels have concluded that the primary route of exposure of humans to BPA is through the diet and that, whereas levels of exposure vary somewhat across age groups, they are generally below 1 μg/kg body weight (bw)/day (Health Canada, 2012; USFDA, 2010; WHO, 2010). The toxicity of BPA has been debated in recent years, particularly with regard to effects in rodents that have been reported at doses claimed to produce internal exposures in the range reported in humans and/or exhibit nonmonotonic dose-response curves (reviewed in Richter *et al.*, 2007; NTP, 2008a; Vandenberg *et al.*, 2012). These reported effects include central nervous system and behavioral alterations, preneoplastic lesions in prostate, mammary gland, and uterus, altered development of the prostate, male urinary tract, and female mammary gland, obesity and insulin resistance. Studies have similarly shown epidemiological associations of urinary BPA levels with a variety of adverse human health effects (e.g., Carwile and Michels, 2011; LaKind *et al.*, 2012; Lang *et al.*, 2008; Melzer *et al.*, 2010, 2012; Silver *et al.*, 2011; Spanier *et al.*, 2012; Teppala *et al.*, 2012; Trasande *et al.*, 2012; Wang *et al.*, 2012, 2013). The reports of "low dose" effects in animal studies have provided support for the concerns raised by these association studies; however, other large rodent studies, including those conducted according to currently accepted regulatory guidelines, have failed to demonstrate such effects (e.g., Ema *et al.*, 2001; Howdeshell *et al.*, 2008; Ryan *et al.*, 2010; Stump *et al.*, 2010; Tyl *et al.*, 2002, 2008).

Many of the studies that report low dose effects of BPA utilized an insufficient number of dose groups to establish a clear dose-response relationship, used small numbers of animals, and/or failed to appropriately account for possible litter effects in the analysis of the data (Shelby, 2008). Also there is a general lack of information, even from guideline studies, on potential background exposures and internal dosimetry of BPA. Altogether, these issues have led to difficulties in coming to a consensus on the interpretation of the large body of data in experimental animals with regard to the risk of BPA in humans. The present study was designed to address some of

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these uncertainties. Although the focus of the present study was on the reproductive toxicity of BPA, other endpoints that have been associated with BPA in animal and/or human studies (see above) were also evaluated, including fat pad weights, serum levels of glucose, triglycerides, insulin, cardiac troponin, and histopathology of the heart and blood vessels.

The "low" [below the current no-observed-adverse-effect level (NOAEL)] BPA doses in this study (2.5–2700 μg/kg bw/day) were selected to cover a range where effects of BPA have been reported in various research studies (Shelby, 2008) and were spaced at an interval (approximately a half-log dose progression) such that a dose response could be established. This is the BPA dose range of regulatory concern. The two high doses of BPA (100,000 and 300,000 μg/kg bw/day) were selected as doses in the range where the "high dose" effects of BPA have been observed, such as delayed puberty in both sexes in rats and systemic toxicity (Tan *et al.*, 2003; Tyl *et al.*, 2002). Because many of the reported effects of BPA involve estrogen signaling pathways, the use of a reference estrogen control has been recommended in BPA toxicity studies (e.g., Richter *et al.*, 2007). Thus, two doses of ethinyl estradiol (EE_2) were included in the study. The higher dose (5.0 μg EE_2/kg bw/day) was selected based on our earlier dietary studies (Delclos *et al.*, 2009; Latendresse *et al.*, 2009) and a 10-fold lower dose of EE_2 was also used, based on the differing exposure of the neonates in the present study.

MATERIALS AND METHODS

BPA, EE_2, and Vehicle

BPA [CAS no. 80-05-7, TCI America Lot no. 111909/AOHOK (air-milled)] and EE_2 (CAS no. 57-63-6, Sigma Lot no. 028K1411) were used. An extensive chemical analysis of the BPA was conducted at Battelle Laboratory (Columbus, OH). The Battelle analysis concluded that the BPA purity was consistent with the manufacturer's Certificate of Analysis value of 99.9%. The identities and purities of both BPA and EE_2 were confirmed to be > 99% pure by the Chemistry Support Group and Mass Spectrometry Laboratory of the Division of Biochemical Toxicology, NCTR, prior to dosing of animals and at the end of the period of animal dosing.

The vehicle used to deliver the BPA and EE_2 was 0.3% aqueous carboxymethylcellulose (CMC). CMC was obtained from Sigma-Aldrich (St. Louis, MO; catalog no. C5013, lot no. 048K0023).

Diet and Assessment of Background Levels of BPA in Diet and Other Study Materials

The study diet was soy- and alfalfa-free to minimize phytoestrogen content [5K96 verified casein diet 10 IF, round pellets, γ-irradiated (catalog no. 1810069), Test Diets, Purina Mills, Richmond, IN]. Extracts of each lot of diet were monitored for daidzein, genistein, and BPA content by liquid chromatography/mass spectrometry. The mean levels of daidzein and genistein in the six diet lots utilized in the study were 0.249 ± 0.064 (SD) ppm and 0.374 ± 0.118 ppm, respectively. The mean level of BPA measured in the diet was 2.6 ± 0.8 ppb. Other study materials screened for BPA levels included animal bedding, polysulfone cage leachates, silicone water bottle stoppers, and drinking water. None of these materials had BPA levels detectable above the average analytical blanks.

Preparation and Analysis of Dose Formulations

BPA and EE_2 doses were prepared in the vehicle, 0.3% CMC in water, and administered to the animals seven days a week at a rate of 5 ml/kg body weight. Homogeneity was established for the highest BPA dose level suspension. Stability was determined to be acceptable (100 ± 10%) for at least 35 days for the lowest BPA dose solution (0.5 μg/ml) and both EE_2 doses. A suspension of 100 mg/ml BPA, higher than the actual dose used, was determined to be stable for at least 47 days.

The vehicle solutions were certified to have undetectable (below the analytical blank) levels of BPA. Every batch of every dosing solution/suspension was assayed for BPA or EE_2 concentration prior to delivery to the animal rooms and certified to be within ± 10% of the target dose with a coefficient of variation of 10% or less. Doses were administered by oral gavage with a modified Hamilton Microlab 500 series pump (Lewis *et al.*, 2010). Separate dosing stations were used for vehicle control, low dose BPA (2.5–2700 μg/kg bw/day), high dose BPA (100,000 and 300,000 μg/kg bw/day), and EE_2 doses (0.5 and 5.0 μg/kg bw/day). Dosing was always conducted from low dose to high dose on a given pump, with flushing of the Teflon tubing between dose levels. At the end of each day, the dosing pump, tubing, and syringes were flushed with Millipore-filtered water, followed by 70% ethanol, and again with filtered water; this flushing procedure was certified to reduce BPA to less than 10 ng/ml following use of a 100 mg/ml BPA suspension in 0.3% CMC.

Source and Specification of Animals

All animal use and procedures for this study were approved by the NCTR Laboratory Animal Care and Use Committee and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Throughout the study, animal rooms were maintained at 23 ± 3°C with a relative humidity of 50 ± 20% and a 12 h light/dark cycle, with lights on at 6 A.M., and food and water were available *ad libitum*. There were at least 10 room air changes per hour. Four hundred male and 400 female weanling [*circa* postnatal day (PND 21)] NCTR Sprague Dawley cesarean-derived (CD) rats (strain code 23) were obtained from the NCTR breeding colony in four loads of 100 of each sex spaced three weeks apart. Although in the breeding colony, these F_0 breeders were maintained with their dams on NIH-41-irradiated feed pellets (IRR. NIH-41, catalog no. 7919C, Harlan Laboratories, Madison, WI) and housed in polycarbonate cages with hardwood chip bedding (P.J. Murphy,

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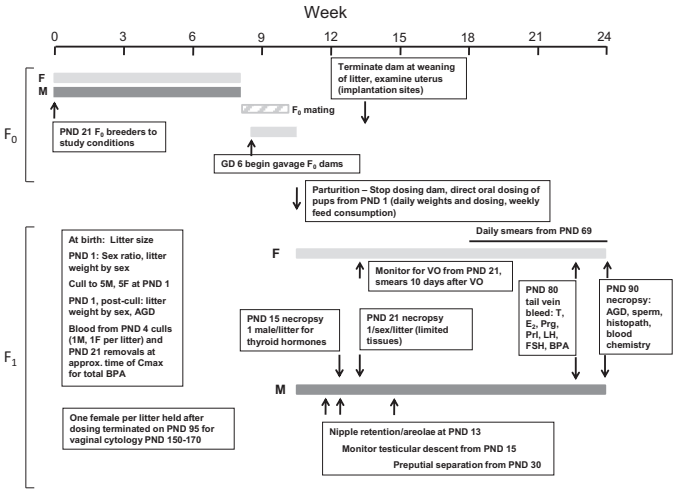

FIG. 1. Schematic representation of the study experimental design. A detailed description of the protocol is found in the Materials and Methods section. Blood draws and terminal necropsies were conducted in PND 80 ± 3 and PND 90 ± 5 windows, respectively. The indicated measurements of serum BPA levels are reported (Churchwell *et al.*, 2014 in addition to the animals indicated here, male and female pups from each litter were maintained until termination at PND 90 ± 5 and tissues were transferred to other studies (results not reported here). Data obtained from those animals were included in the analyses of AGD and pubertal landmarks.

Montville, NJ) and polycarbonate water bottles. Once assigned to the study at weaning, they were fed pelleted-irradiated Purina 5K96 feed (Test Diets, Purina Mills), housed in polysulfone cages fitted with microisolator tops (Ancare, Corp, Bellmore, NY) with hardwood chip bedding, and given Millipore-filtered water from glass water bottles with silicone stoppers. The rats were held under these conditions until they were mated (at 10–14 weeks of age for females and 11–15 weeks of age for males). The health of the animals was monitored during the study in accordance with NCTR's Sentinel Animal Program and the sentinel animals were determined to be free of pathogenic organisms.

Animal Breeding and Maintenance

A summary of the experimental design is presented in Figure 1. Approximately two weeks prior to mating, female breeders were randomized to treatment groups stratified by body weight to give approximately equivalent mean body weights in each group. The premating assignment of dams to dose groups was necessitated by the use of randomly cycling females and a 14-day mating period described below. Male breeders were assigned to breeding pairs with the stipulation that no sibling or first cousin mating was permitted. Animals were mated in four loads spaced three weeks apart. The number of dams assigned to each treatment group and the number of animals (up to one per sex per litter) evaluated at necropsy are shown in Supplementary table 1. The number of pairs assigned to treatment groups was adjusted in later loads based on the number of litters enrolled in the study from prior loads. Throughout the study, treatment groups were randomized to racks within each room. Racks and cages within racks were rotated every three weeks to minimize any environmental/room effects.

Males were placed in wire-bottomed breeding cages for acclimation approximately 48 h prior to introduction of the female. The breeding pairs were housed together and the females were examined daily until evidence of mating had occurred or for 14 days, whichever occurred first. Either the presence of an *in situ* copulation plug or sperm in the vaginal smear was considered evidence of mating [gestation day (GD) 0] and triggered separation of the breeding pair. The males were removed from the study and euthanized after a successful mating and the female was placed in a separate solid-bottomed polysulfone cage with hardwood chip bedding. Except for the naïve control group, daily gavage dosing of the dams started on GD 6 and continued until the initiation of labor. The dose volume was determined by the daily body weight. Neither dams nor pups were dosed on the day of birth (PND 0). Litters were randomly culled to a maximum of five males and five females on PND 1. No fostering was conducted, and a minimum of three males and three females in the litter was required to keep the litter on study. Direct oral gavage dosing of the pups started on PND 1, after the litter was culled. Pups were weighed and dosed daily until the scheduled day of removal (PND 15 or PND 21) or until the day prior to their scheduled removal (PND 90 ± 5). For pups younger than PND 5, the gavage needle did not enter the esophagus. Naïve control dams and F_1 pups were weighed daily and returned to their cages after any scheduled observations were conducted. Pups were housed individually after weaning, consistent with the Food and Drug Administration (FDA) guidance in place at the time the experiment was initiated (USFDA, 2007).

In-Life Data Collection

Morbidity/mortality checks were performed twice daily and clinical observations once daily. Daily body weights were obtained prior to dosing for dams from GD 6 through parturition and similarly for pups from PND 1. Naïve control animals were similarly weighed but not dosed. Feed consumption was measured weekly. On the day of birth (PND 0), the numbers of pups alive and dead were recorded, but there were no other manipulations of the dam or litter. On PND 1, the number of pups alive and dead, sex ratio, and live litter weight by sex were determined and litters were randomly culled as described above. Anogenital distance (AGD), the distance from the anterior edge of the anal opening (closest to genitalia) to the midpoint of the genital tubercle, was measured on PND 1 pups retained after culling. The pups were manually restrained, with care taken not to stretch the pup, and the measurement was made in triplicate using a dissecting microscope fitted with an ocular micrometer. The mean of the three determinations for each pup was used in the statistical analysis of AGD and was divided by the cube root of body weight for the analysis of AGD index (AGDI).

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Males were evaluated for nipple retention on PND 13, and daily for testicular descent and for preputial separation from PND 15 and 30, respectively, until these events were noted. Females were monitored daily for vaginal opening from PND 22. Females (one per litter) designated for histopathology evaluation were monitored by daily vaginal smears from the day of vaginal opening up to 10 days, to determine the day of first estrus, and again daily from PND 69 until the day prior to termination at PND 90 ± 5, to evaluate treatment effects on estrous cyclicity and to attempt to stage animals for necropsy in estrus. A subset of females (one per litter, from litters with three females available at the time of weaning) was removed from dose at PND 90 ± 5 and maintained in a separate room to evaluate delayed treatment effects on estrous cyclicity by daily vaginal cytology from PND 150 to PND 170.

At PND 80 ± 3, a blood sample was taken from the lateral tail vein of animals designated for histopathology for measurement of luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, estradiol, and progesterone in females and LH, FSH, prolactin, and testosterone in males. This sampling occurred after dosing, between 7:30 and 11:00 A.M., and on days when females were predicted to be in estrus based on the vaginal smear of the previous day or on PND 83 for animals not cycling normally. Serum was aliquoted and frozen in a −80°C freezer until batch testing. Rat-specific LH (IDS, Liege, Belgium), FSH (TSZ ELISA, Framingham, MA), estradiol, and prolactin (Calbiotech, Spring Valley, CA) assays were performed using ELISA methods according to the manufacturers' instructions. Cross-reactivity of the test agents, BPA and EE_2, in the estradiol assay was not evaluated. The plates were read on an ELx800 Universal Microplate Reader (Bio-Tek, Winooski, VT). Testosterone and progesterone quantification assays ("Coat-a-Count," Siemens, Los Angeles, CA) were performed using radioimmunoassay (RIA) methods according to the manufacturer's instructions. The tubes were counted on a Wizard2 gamma counter (Perkin Elmer, Shelton, CT).

Data Collected at Termination

F_0 dams. F_0 dams that were observed to be sperm positive or had an *in situ* vaginal plug observed during mating were removed after litters were weaned, after delivery of litters that did not meet study criteria (i.e., less than three males and three females born or litter born before GD 20), or after GD 26 if no litter was delivered. The animals were euthanized with gaseous carbon dioxide, the uterus was removed, examined for visible implantation sites, and submerged in 10% ammonium sulfide for a minimum of 15 min to reveal early implantation sites. The total number of implantation sites (i.e., visible plus early) was used for statistical analysis. The number of resorptions was calculated by subtracting the number of pups born from the total number of implantation sites.

PND 15. On PND 15, in litters with four male pups, one male per litter was removed, decapitated, and trunk blood collected for measurement of serum T3, T4, and thyroid stimulating hormone (TSH). Euthanasia occurred after dosing and was completed between 7 and 11 A.M.. Serum was aliquoted and frozen in a −80°C freezer until batch testing. Total T3, total T4 (both "Coat-a-Count" Siemens), and TSH (IDS) were measured using RIA according to the manufacturers' instructions.

PND 21. On PND 21, in litters with three pups of a given sex, one animal per sex per litter was euthanized, blood collected by cardiac puncture for preparation of serum, and inguinal mammary glands, fat pads (retroperitoneal, epididymal, ovarian/parametrial), prostate, brain, ovaries, and uterus were collected. Tissues were weighed and flash frozen [except for the right ovary and fifth mammary glands that were fixed in 10% NBF (neutral buffered formalin)] for transfer to other studies. The fifth left mammary glands were processed for histopathology and the right for whole mount. Only the histopathology results from the fifth left mammary gland are reported.

PND 90. The main focus of this experiment was on the animals maintained until PND 90 ± 5 for necropsy and histopathology (designated the "histopathology study arm"). A littermate of each sex was also maintained after weaning. These animals were similarly treated in the same animal room as the histopathology arm animals and were necropsied at PND 90 ± 5 for harvest of tissues that were provided to other studies (designated the "frozen tissue study arm"). Only limited data from these littermates will be reported here. However, in cases where an animal that had been designated for histopathology was lost to the study for any reason, the same sex littermate was reassigned to be utilized for histopathology at PND 90 ± 5. This occurred in only five cases.

On PND 90 ± 5, one animal per sex per litter was euthanized after an overnight fast. Females were euthanized when predicted to be in estrus, based on daily vaginal smears collected up to the day before sacrifice, or on the last day of the specified time period that was not a holiday or weekend, if not cycling normally. Blood was collected from the retro-orbital sinus and serum was prepared. AGD, the distance between the inner edge of the anal opening closest to the genitalia and the inner edge of the prepuce or vaginal opening closest to the anal opening, was measured on the carcass with a caliper. At sacrifice, all tissues of interest (see below) were examined grossly and removed. Gross findings on all tissues were captured electronically and gross lesions were removed for histological evaluation. The following organs were dissected and weighed: adrenals, brain, dorsolateral and ventral prostate (dissected and weighed after fixation), epididymides, heart, kidneys, liver, ovaries, pituitary (after fixation), seminal vesicles with coagulating gland, spleen, testes, thymus, thyroid (after fixation), uterus (blotted), and fat pads (retroperitoneal, epididymal, and ovarian/parametrial). The left epididymis was dissected from the left testis and both processed for sperm evaluation (see below). If gross lesions or abnormalities were noted at necropsy with either the left testis or epididymis, the right or-

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gan was used for sperm motility and count studies and the left was used for histopathology. The following organs were evaluated microscopically: adrenals, aorta (thoracic), bone marrow (femur), brain, right epididymis, heart, kidneys, liver, fifth left mammary gland (inguinal, both sexes), ovaries, oviduct, pancreas, pituitary, prostate (dorsolateral and ventral), seminal vesicles with coagulating gland, spleen, right testis, thymus, thyroid, uterus, and vagina. The left fifth (inguinal) mammary gland and fat pad were removed as a unit to include the inguinal lymph node. It was placed in a cassette in a "flattened" dorsoventral orientation, similar to the orientation of the unit before it was removed from the carcass. This orientation provided a histological section with a similar profile to that of a routine mammary whole mount. All tissues except testes were fixed in 10% NBF; testes were fixed in modified Davidson's fixative (Latendresse *et al.*, 2002). Fixation time for all tissues except brain was limited to 48 h. Brain remained in fixative until processing. Tissues were trimmed, processed, and embedded in infiltrating media (Formula R, Leica, Buffalo Grove, IL), sectioned at 5 μm, stained with hematoxylin and eosin (except for testes), and examined by light microscopy by a Study Pathologist (one for female tissues and one for male tissues). The testes were stained with periodic acid-Schiff stain. When applicable, nonneoplastic lesions were graded for severity as minimal (grade 1), mild (grade 2), moderate (grade 3), or marked (grade 4).

Serum clinical chemistry analyses were conducted on an Alfa Wassermann ALERA (West Caldwell, NJ). Alfa Wassermann reagents were used for glucose, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, gamma glutamyl transpeptidase, total protein, albumin, cholesterol, triglycerides, blood urea nitrogen, and creatinine. Catachem (Bridgeport, CT) reagents were used for sorbitol dehydrogenase and total bile acids. All testing for these endpoints was completed on the day of collection. Rat-specific troponin I, troponin T (both Biotang, Waltham, MA), and leptin (Millipore, St. Charles, MO) were measured by ELISA in serum that had been frozen and stored in a −80°C freezer. The plates were read on an ELx800 Universal Plate Reader. Total T3, total T4, and TSH were measured by RIA as described above. Insulin was also measured by RIA ("Coat-a-Count," Siemens).

Epididymal (left) sperm evaluations were conducted using an integrated visual optical system (IVOS)-automated sperm analyzer (Hamilton Thorne Inc., Beverly, MA). The cauda section was dissected and immediately placed in 40 ml of a prewarmed (37°C) solution consisting of 1% bovine serum albumin dissolved in phosphate buffered saline. A minimum of 2 min was allowed for sperm to swim out of the cauda. Five fields on an 80 μm deep slide were automatically selected by the analyzer to calculate the percent motility for each animal. After the motility sample was acquired, the left cauda was minced and approximately 1 ml of the suspension was frozen on dry ice and stored in a −80°C freezer for later determination of the total cauda sperm count. The left testis was weighed, placed on dry ice, and stored in a −80°C freezer until determination of testicular spermatid head counts. For sperm counting, the tunic was removed from the thawed testis, and the testis was weighed and homogenized in a solution of 1% bovine serum albumin in phosphate buffered saline. The suspensions were transferred to a vial containing a fluorescent DNA-specific stain (IDENT, Hamilton Thorne Inc.) to stain the sperm heads. The sperm counts were calculated from 20 automatically selected fields. For sperm morphology evaluation, two eosin-stained slides were prepared from the caudal epididymis suspension and a minimum of 200 sperm/animal was examined for morphological abnormalities (amorphous, small head, enlarged head, double head, and tail coiled or bent).

Statistical Methods

The statistical methods used varied by endpoint and are described in Supplementary materials.

The dose range of primary interest for the present study was between 2.5 and 2700 μg BPA/kg bw/day and a major purpose of the study was to provide information for a subsequent chronic study. To minimize false negatives within the dose range of interest due to over correction, comparisons of each dose group to vehicle controls were made in four analysis subgroups (naïve controls, low dose BPA, high dose BPA, and EE_2) for each sex. Trends were also examined within the low BPA (2.5–2700 μg/kg bw/day) subgroup.

The tests of interest in this report are pairwise comparisons to vehicle control. Unless otherwise noted, pairwise tests are corrected for multiple comparisons using Dunnett's method (Dunnett, 1955) for those doses within the analysis subgroup. Any reported linear trends were performed using orthogonal linear contrasts. Simple means and standard errors are reported in tables.

Reported tests are two-sided at the 0.05 significance level with the exception of comparisons to vehicle control for histopathology data, which were conducted as one-sided tests at the 0.05 significance level per NTP convention.

Quality Assurance Procedures

This study was conducted in compliance with the FDA Good Laboratory Practice Regulations (21 CFR, Part 58).

For the PND 90 ± 5 pathology results, a Quality Assessment (QAS) Review and a Pathology Working Group (PWG) Review of the data and pathology slides were conducted by a group of external veterinary pathologists not associated with the original reading of the study slides. These reviews thoroughly evaluated the PND 90 ± 5 naïve and vehicle control groups and all BPA dose groups, but not the two EE_2 dose groups. The final pathology report and the data reported here reflect the resulting consensus.

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RESULTS

Selected data are presented in tables and figures or as Supplementary data, whereas all data collected on the study are available in the NCTR Study Report (available upon request).

Treatment Effects on Dams During Gestation and Litter Endpoints

BPA doses ≤ 2700 μg/kg bw/day did not affect gestational body weight gain, but the 100,000 and 300,000 μg BPA/kg bw/day doses reduced body weight gain over the period from GD 6 to parturition by 16% and 11%, respectively (Table 1). Similarly, both 0.5 and 5.0 μg EE_2/kg bw/day reduced gestational body weight gain by 7% and 14%, respectively (Table 1). No treatment significantly affected gestation length or gestational metabolic efficiency (i.e., the change in body weight divided by food consumption for a given time interval) determined from the time that dosing started on GD 6 (data not shown).

Neither BPA nor EE_2 at any dose tested affected the number of implantation sites measured in the uterus of the F_0 dams nor any other endpoints evaluated for the litters, including the AGD and AGDI (data not shown). At PND 90, the mean AGD and AGDI values of the male naïve control were 6–7% greater than those of the vehicle control. The AGDI of males in the 300,000 μg BPA/kg bw/day group was also approximately 6.5% greater than that of the vehicle control group (data not shown).

F_1 Pup Preweaning Survival

There was a significant difference in survival between the naïve and the vehicle males (100% vs. 88.3%, respectively). The only significant BPA effect on pup preweaning survival was a decrease by 35%, both in males and females, in the 300,000 μg/kg bw/day dose group. EE_2 did not significantly affect this endpoint (data not shown).

Body Weights

Growth curves over the entire study period for female and male animals are shown in Figures 2 and 3, respectively. There were no differences in body weights between the naïve and vehicle controls or the low dose BPA groups and vehicle control in either sex. Body weights of females and males in the highest BPA dose group (300,000 μg/kg bw/day) of females and males were consistently lower than vehicle control throughout the study, varying from approximately 6–13% (average across weeks approximately 10%) lower. For the 100,000 μg BPA/kg bw/day group, body weights were significantly lower by approximately 8–9% than vehicle control in the last two weeks (weeks 12 and 13) in females only. However, females used for the delayed vaginal cytology assessment did not show a high dose BPA body weight effect (Supplementary fig. 1). In females, low dose EE_2 significantly increased body weight (approximately 6–9%) on weeks 5–9 and the high dose EE_2 sig-

TABLE 1
Gestational Body Weight Gain (from GD 6)[a]

	Treatment													
	Negative controls		BPA (μg/kg bw/day)										EE_2 (μg/kg bw/day)	
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
n	25	23	26	18	24	24	23	22	25	24	25	20	21
Body weight gain (g) from GD6 – parturition	163.6 ± 3.7	164.9 ± 4.2	162.0 ± 3.8	175.3 ± 6.1	164.2 ± 4.2	164.1 ± 3.5	172.3 ± 4.0	168.0 ± 5.8	164.3 ± 3.5	138.7 ± 4.0 ***	147.2 ± 3.9 ***	153.3 ± 3.6 *	141.4 ± 3.8 ***

[a]Results are presented as simple means ± SEM. Results presented for dams with pups on study. The statistical significance of the results did not change when all dams producing litters were analyzed. Significant differences from the vehicle control are indicated: * $p < 0.05$ and *** $p < 0.001$.

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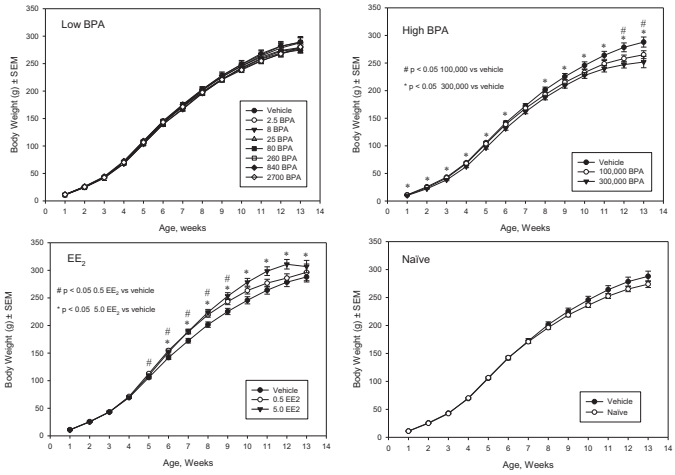

FIG. 2. F_1 female body weights. Mean weekly body weights (g) are shown for the study-defined subgroups (low BPA, high BPA, EE_2, and naïve) versus vehicle.

nificantly increased (average across weeks approximately 10%) body weights from week 6 (Fig. 2). In the delayed vaginal cytology arm, the significant body weight difference in the high EE_2 dose group persisted, whereas the low dose of EE_2 did not show a significant body weight effect versus vehicle control (Supplementary fig. 1). Male body weight was not affected by EE_2 (Fig. 3).

PND 15 Clinical Chemistry Measurements

In PND 15 males, total T3 was increased by 13% and 30%, respectively, at 100,000 and 300,000 μg BPA/kg bw/day, whereas TSH was decreased by approximately 28% at 300,000 μg BPA/kg bw/day (Table 2). There were no other significant effects among the low BPA doses, the EE_2 doses, or naïve versus vehicle controls.

Markers of Sexual Development

Table 3 summarizes the results for age and body weights at the time of vaginal opening, testicular descent, and preputial separation. None of the BPA doses affected the time of vaginal opening or the body weight at which the landmark was achieved. Both doses of EE_2 affected the time of vaginal opening, with average delays of approximately 6.1 and 23.2 days at 0.5 and 5.0 μg EE_2/kg bw/day, respectively. Consistent with this timing delay, the body weights at which the landmark was attained were 32% and 108% higher than vehicle controls at the low and high EE_2 doses, respectively. Time to first estrus was significantly delayed by 5.0 μg EE_2/kg bw/day, but neither the lower dose of EE_2 nor any dose level of BPA affected this parameter. Naïve controls did not differ from vehicle controls for these endpoints.

No male in any dose group had retained nipples when examined at PND 13. Testicular descent was significantly delayed by approximately 1 and 2 days, respectively, in the 260 and

TABLE 2
Male PND 15 Clinical Chemistry: Serum T3, T4, and TSH Levels[a]

	Treatment												
	Negative controls		BPA (μg/kg bw/day)								EE2 (μg/kg bw/day)		
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
n	21	15	23	16	21	24	20	20	20	16	7	11	12
T3, ng/dL	86.88 ± 3.01	88.70 ± 5.52	90.05 ± 3.34	96.63 ± 3.88	89.79 ± 3.21	80.34 ± 2.26	88.46 ± 3.90	87.25 ± 3.27	85.80 ± 2.60	100.09 ± 4.45**	115.26 ± 7.75***	92.74 ± 3.06	89.06 ± 4.53
n	21	15	23	16	21	24	20	20	20	16	7	11	13
T4, μg/dL	4.97 ± 0.22	5.31 ± 0.21	5.26 ± 0.18	4.92 ± 0.21	4.77 ± 0.22	4.81 ± 0.19	5.07 ± 0.19	4.87 ± 0.14	5.26 ± 0.25	5.31 ± 0.22	5.89 ± 0.62	4.93 ± 0.36	5.37 ± 0.32
n	21	15	21	16	19	24	20	19	20	15	6	10	13
TSH, ng/ml	5.84 ± 0.54	5.93 ± 0.61	5.22 ± 0.32	6.17 ± 0.59	4.85 ± 0.36	7.17 ± 1.01	5.50 ± 0.44	5.36 ± 0.31	4.68 ± 0.52	5.13 ± 0.44	4.24 ± 0.28*	7.63 ± 0.62	6.17 ± 0.82

[a]Results are given as simple means ± SEM. Significant differences from the vehicle control are indicated: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

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TABLE 3
Markers of Sexual Development[a]

	Treatment												
	Negative controls		BPA (μg/kg bw/day)									EE_2 (μg/kg bw/day)	
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
Vaginal opening													
No. of litters	21	22	26	18	24	23	23	22	24	23	19	20	21
Age, days	34.0 ± 0.8	32.5 ± 0.5	33.1 ± 0.5	32.2 ± 0.5	32.6 ± 0.6	32.9 ± 0.4	33.1 ± 1.2	32.8 ± 0.6	32.4 ± 0.4	33.2 ± 0.8	35.2 ± 1.8	38.6 ± 1.7**	56.2 ± 2.7***
Body weight, g	118.9 ± 4.2	108.8 ± 3.0	113.3 ± 2.6	106.3 ± 2.0	108.3 ± 2.7	110.8 ± 2.9	113.4 ± 8.3	112.1 ± 3.4	111.4 ± 3.1	111.6 ± 3.4	109.6 ± 7.1	142.5± 7.1***	227.4 ± 9.8***
Testicular descent													
No. of litters	20	20	23	18	21	20	20	21	21	23	18	20	20
Age, days	23.5 ± 0.3	23.6 ± 0.3	23.9 ± 0.2	23.7 ± 0.3	23.8 ± 0.3	24.0 ± 0.3	24.7 ± 0.3*	23.8 ± 0.3	23.4 ± 0.2	24.3 ± 0.4	25.8 ± 0.6**	24.5 ± 0.3	29.4 ± 0.8***
Body weight, g	67.7 ± 2.1	67.5 ± 2.0	70.6 ± 1.5	69.8 ± 1.9	70.2 ± 1.6	68.1 ± 1.7	72.5 ± 1.6	71.2 ± 1.9	68.0 ± 1.4	72.0 ± 1.9	74.5 ± 3.5	74.8 ± 2.1*	102.9 ± 3.8***
Preputial separation													
No. of litters	20	20	23	18	21	20	20	21	21	23	18	19[b]	20
Age, days	43.6 ± 0.5	44.0 ± 0.6	44.8 ± 0.8	43.3 ± 0.8	42.9 ± 0.8	45.4 ± 1.2	44.7 ± 1.1	42.9 ± 0.9	44.7 ± 0.6	44.6 ± 0.6	47.8 ± 1.7	45.8 ± 1.0	56.6 ± 2.3***
Body weight, g	220.0 ± 6.1	214.9 ± 4.7	230.4 ± 5.8	215.9 ± 5.4	207.1 ± 7.2	230.2 ± 12.5	221.8 ± 9.0	215.7 ± 8.7	228.8 ± 6.1	217.0 ± 4.7	217.9 ± 10.0	240.3 ± 9.0	309.0 ± 14.7***

[a]Results are presented as simple litter means ± SEM. Significant differences from the vehicle control are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.
[b]In one litter in the 0.5 μg EE_2/kg bw/day dose group, neither male pup was observed to have preputial separation by the time of termination.

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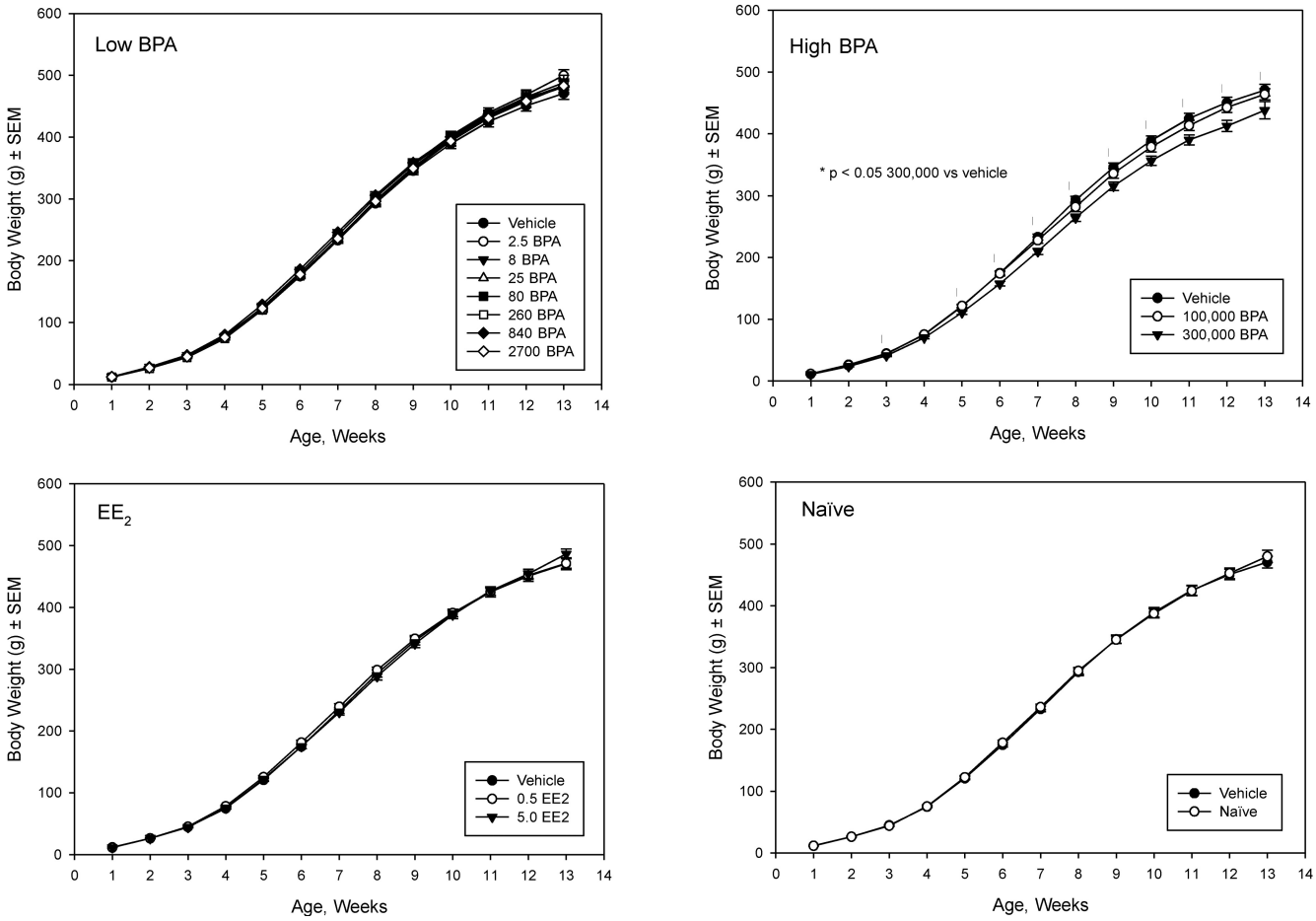

FIG. 3. F_1 male body weights. Mean weekly body weights (g) are shown for the study-defined subgroups (low BPA, high BPA, EE_2, and naïve) versus vehicle.

300,000 μg BPA/kg bw/day dose groups. There were no differences in body weights at the time of testicular descent in these groups. EE_2 at 5.0 μg/kg bw/day delayed testicular descent by 5.8 days, and the body weights at the time of testicular descent were approximately 11% and 52% higher than that of vehicle controls in the 0.5 and 5.0 μg EE_2/kg bw/day dose groups, respectively. None of the BPA doses differed significantly from the vehicle control in the time of preputial separation or body weight at which preputial separation was achieved. The high dose of EE_2 significantly delayed the average time of preputial separation (approximately 11.5 days) and, accordingly, the mean body weight at which this landmark was achieved was 42% greater for this dose group than for the vehicle control. The naïve and vehicle control groups did not differ in the timing of testicular descent or preputial separation.

Vaginal Cytology

Vaginal smears were taken daily from PND 69 to termination at PND 90 ± 5 for evaluation of the estrous cycle. A summary of the occurrence of animals with abnormal cycles across treatment groups is shown in Table 4. Between 20% and 35% of vehicle control animals showed at least one abnormal cycle over the evaluation period, and there was no significant difference between naïve and vehicle controls. The majority of the abnormal cycles were due to extended diestrus. There was a clear effect of both doses of EE_2 on estrous cyclicity, with 95–100% of the animals showing abnormal cycles, primarily due to extended estrus (strings of estrus alone or proestrus, proestrus/estrus, and estrus combined) and extended estrus/diestrus (Table 4). Low BPA doses had no effect on the percentage of animals showing abnormal cycles versus vehicle control. Similarly, 100,000 μg BPA/kg bw/day did not affect the proportion of animals showing abnormal cycles, whereas 300,000 μg BPA/kg bw/day significantly increased the proportion of animals showing abnormal cycles in a manner similar to that of EE_2.

A set of females was also maintained after PND 95 without further dosing and vaginal smears were monitored from PND 150 through PND 170. Results were similar to those observed in the younger animals in that only the two EE_2 groups and the

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TABLE 4
Estrous Cycle Evaluations Summary, PND 69 to PND 90 ± 5

Pattern[a]	Estrous cycle	Treatment												
		Negative controls		BPA (μg/kg bw/day)									EE_2 (μg/kg bw/day)	
		Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
Extended estrus	Total examined	20	20	23	18	21	20	21	20	20	21[b]	19	19[c]	19[c]
	Present	0	0	0	0	0	0	0	0	0	0	6**[d]	10***	10***
Extended combined estrus	Total examined	20	20	23	18	21	20	21	20	20	21	19	19	19
	Present	0	0	0	0	0	0	0	0	0	0	5*	9***	11***
Extended diestrus	Total examined	20	20	23	18	21	20	21	20	20	21	19	19	19
	Present	5	7	5	7	9	5	8	14	10	7	7	3	8
Extended E/D	Total examined	20	20	23	18	21	20	21	20	20	21	19	19	19
	Present	0	0	0	0	0	0	0	0	1	0	5*	11***	6**
Any abnormal	Total examined	20	20	23	18	21	20	21	20	20	21	19	19	19
	Present	5	7	5	8	10	5	8	14	11	7	17**	18***	19***

[a]The responses that were evaluated were: extended estrus (three or more consecutive days of E), extended combined estrus (four or more consecutive days of P, E, or P/E), extended diestrus (four or more consecutive days of D), extended E/D (two or more consecutive days of E/D), and any abnormal cycling (including patterns other than those listed, such as extended P (two or more in a row), extended P/E (three or more in a row), extended D/P (two or more in a row)). P/E, E/D, and D/P are transitional stages.

[b]In the 100,000 μg BPA/kg bw/day, data from two littermates were inadvertently included in the analysis. An animal in the histopathology study arm died early and smears from its littermate in the frozen tissue arm were collected and included.

[c]For one animal in each of the EE_2 dose groups, vaginal opening was not recorded by the time of study termination and no vaginal cytology data were available.

[d]Significant differences from the vehicle control are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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BPA doses above 2700 μg/kg bw/day increased abnormal cycling relative to the vehicle controls (data not shown).

PND 80 Clinical Chemistry (Serum Hormones)

The data for hormones analyzed in PND 80 ± 3 female and male serum are summarized in Supplementary tables 2 and 3, respectively. The vaginal cytologies collected on the day of the blood draws indicated that 60–90% of the females in any given dose group had profiles consistent with estrus or proestrus. The vaginal cytology data discussed above, as well as the PND 90 ± 5 histopathology data discussed below, indicated that the high dose BPA and EE_2 groups were not cycling normally. There were no significant treatment-related effects in the low BPA dose range in females, but mean serum estradiol was significantly increased by 67% and 113%, respectively, in the 100,000 and 300,000 μg BPA/kg bw/day groups. Progesterone was significantly decreased by 51% in the highest BPA dose group. In the EE_2 groups, estradiol was significantly increased by 34% and 29% and progesterone significantly decreased by 70% and 80% in the 0.5 and 5.0 μg EE_2/kg bw/day groups, respectively. Both doses of EE_2 and the highest dose of BPA had significantly elevated prolactin levels, although prolactin did not differ significantly from vehicle control in a two-factor analysis of treatment and cycle stage with interaction (Supplementary table 2). The observed differences in hormone levels are consistent with the ovarian histological changes (see below). There were no significant differences from vehicle controls for FSH and LH (data not shown) for any BPA or EE_2 dose group, and there were no significant differences between vehicle and naïve controls for any serum hormone examined.

In males, there were no treatment-related differences between any treatment group and vehicle control for the measured hormones FSH, LH, testosterone, and prolactin (Supplementary table 3). Both doses of EE_2 and the highest BPA dose had mean serum testosterone levels that were 16–29% lower than vehicle control means, but these differences were not statistically significant. The high variability of the testosterone measurements and insufficient group size may have reduced the power to detect potential changes in this endpoint.

Organ Weights

In PND 21 females, both doses of EE_2 increased uterine weights and decreased ovarian weights (Supplementary table 4). These effects were observed for absolute organ weights as well as organ weights adjusted for brain or body weights. The highest BPA dose (300,000 μg/kg bw/day) also decreased ovarian weights (absolute and adjusted for brain weight).

The female organ weights measured at PND 90 ± 5 are shown in Supplementary table 5. Absolute weights and weights adjusted for brain and body weights were analyzed with the results of the body and brain weight adjustments in general agreement. The only significant difference between the naïve and vehicle controls was for the heart adjusted for body weight, with the naïve weight 7% higher than the vehicle ($p = 0.047$). There were no significant treatment effects of low dose BPA (2.5–2700 μg/kg bw/day) on any organ weighed from the females. At 100,000 μg BPA/kg bw/day, absolute spleen weight was approximately 10% lower than the vehicle control weight. All other statistically significant effects of BPA on organ weights were confined to the 300,000 μg BPA/kg bw/day group. Brain, heart, fat pads, and ovary weights, either absolute and/or adjusted to body or brain weights, were reduced by the highest dose of BPA tested. Liver weight adjusted for body weight was increased by 10%. EE_2 significantly affected multiple organs, in most cases whether absolute weights or weights adjusted for brain or body weight were analyzed, at one or both doses. Both doses of EE_2 increased the weight of the adrenal gland, heart, kidney, and liver. The low dose, but not the high dose, EE_2 increased pituitary gland weight, whereas the spleen and thymus weights were significantly elevated only by the high dose EE_2. Ovarian/parametrial fat and ovaries were significantly lighter than vehicle controls in both EE_2 dose groups, whereas uterine weight was significantly lower only at the high dose. Retroperitoneal fat was significantly reduced in weight relative to vehicle control at the low EE_2 dose (when analyzed with brain or body weight as covariates) and at the high EE_2 dose (when analyzed with body weight as covariate).

The organ weight data for males at PND 90 ± 5 are summarized in Supplementary table 6. There were no statistically significant differences between the vehicle and naïve controls for any organ. The sole significant effect in the low dose BPA groups was a decreased (approximately 10%) heart weight adjusted for body weight relative to vehicle control at 2700 μg BPA/kg bw/day. Significant effects of high dose BPA were restricted to the 300,000 μg/kg bw/day group. Absolute brain weight was approximately 5% lower than vehicle. Absolute and brain weight-adjusted epididymal fat pad, heart, kidney, liver, and spleen weights were all significantly less than vehicle control weights. None of these effects were significant when the body weight-adjusted weights were analyzed, suggesting that the effects were secondary to the body weight reduction observed in the highest BPA dose group. All significant effects of EE_2 were confined to the high dose group (5.0 μg/kg bw/day). Adrenal gland, pituitary gland, and spleen weights were significantly increased by the high EE_2 dose over vehicle controls whether absolute or brain- or body-adjusted weights were analyzed. Epididymis, epididymal fat pad, dorsolateral and ventral prostate, seminal vesicles with coagulating gland, and testis were significantly decreased by the high EE_2 dose. Liver weight adjusted for body weight was increased by approximately 9% in the high EE_2 dose group.

PND 90 Clinical Chemistry

Females. Table 5 summarizes selected clinical chemistry parameters measured in serum collected at necropsy from PND 90 ± 5 females after an overnight fast. There were no significant differences between the naïve and vehicle control groups for any of the analytes. In the low BPA dose range, the only sta-

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tistically significant effect was an increase (10%) in aspartate aminotransferase at 2700 μg BPA/kg bw/day. The 100,000 μg BPA/kg bw/day dose significantly depressed cholesterol and triglycerides by 16% and 30%, respectively, relative to vehicle controls, but there was no effect on these endpoints at 300,000 μg BPA/kg bw/day. TSH was significantly increased by 43% and 62% at 100,000 and 300,000 μg BPA/kg bw/day, respectively. Leptin was significantly decreased (56%) in the highest BPA dose group. Both doses of EE_2 significantly affected cholesterol, with a 19% increase at the 0.5 μg/kg bw/day dose and a 17% decrease at the 5.0 μg/kg bw/day dose. TSH was also significantly increased in both EE_2 dose groups, with elevations of 66% and 62% at 0.5 and 5.0 μg EE_2/kg bw/day, respectively. T4 was significantly depressed (22%) in the low, but not the high, EE_2 dose group. Total protein was significantly higher (7%) in the low EE_2 dose group. In the high EE_2 dose group, alkaline phosphatase, alanine aminotransferase, blood urea nitrogen, and sorbitol dehydrogenase were significantly elevated.

Males. Table 6 summarizes selected clinical chemistry parameters measured in serum collected at necropsy from PND 90 ± 5 males after an overnight fast. There were no statistically significant differences between the naïve and vehicle control for any measured parameter. BPA had limited statistically significant effects on the clinical chemistry parameters measured in PND 90 ± 5 males. 100,000 and 300,000 μg BPA/kg bw/day reduced cholesterol by 16% and 21%, respectively. The 100,000 μg BPA/kg bw/day dose group had a glucose level 8% lower than vehicle controls, whereas the 300,000 μg BPA/kg bw/day dose reduced leptin and total bile acids by 33% and 25%, respectively, and elevated creatinine and T4 by 16% and 18%, respectively. There were no statistically significant differences between the low EE_2 dose group and vehicle control. For the high dose of EE_2 (5.0 μg/kg bw/day), cholesterol was significantly lower (16%) than in the vehicle control and creatinine and T3 were elevated by 20% and 18%, respectively, over the vehicle control group.

PND 90 Sperm Parameters

There were no statistically significant differences between the naïve and vehicle control groups for any sperm parameter evaluated or between any treatment group and the vehicle controls for sperm motility or morphology (data not shown). The only treatment-related difference between a dosed group and the vehicle control was a reduction (33%) of the cauda sperm count in the 5.0 μg EE_2/kg bw/day dose group.

Necropsy Observations and Histopathology

The lesions discussed below are selected lesions of interest based primarily on statistically and/or biologically significant effects. Statistical results from the Poly-3 test on lesion incidences are presented in the summary tables (Tables 7–9). For lesions for which subjective severity scores were assigned by the Study Pathologists, results of two additional statistical tests incorporating these scores, the Jonckheere-Terpstra/Shirley-Williams (JT/SW) and Relative Treatment Effect (RTE) tests, were also performed. The rationale for preferring the Poly-3 test is presented in the Supplementary material. In general, the three statistical tests applied gave similar results for strong effects (i.e., high incidence lesions), whereas low incidence lesions gave occasional positives in the alternate tests, but did not show a consistent dose dependency (e.g., positive only in one or two intermediate and nonadjacent doses). Such lesions were judged unlikely to be biologically significant treatment-related lesions.

PND 21. Results of the microscopic evaluation of the PND 21 mammary glands in both sexes are summarized in Table 7. There were no significant differences between naïve and vehicle controls in either sex. No significant effects were observed in females for either dose of EE_2. Intraductal hyperplasia, i.e, focal, segmental, or diffuse proliferation (thickening or piling up) of epithelial cells lining the mammary ducts, was not observed as a treatment-related lesion. Ductal hyperplasia of the mammary gland in the context of this study indicates a relative increase in the number (density) of branching ducts and alveolar buds per unit area of mammary gland. There was a statistically significant trend in mammary gland ductal hyperplasia in the low dose BPA subgroup and the 2700 and 100,000 μg BPA/kg bw/day dose group differed significantly from the vehicle (Table 7). The incidence (4/19, or 21%) at 25 μg BPA/kg bw/day was significant by the RTE test. In all cases, the severity grade for the hyperplasia was minimal. The vehicle control incidence of mammary gland ductal hyperplasia was 0/16, whereas the naïve control incidence was 2/17, which diminishes the likelihood of the biological significance of the statistical findings in the dosed groups.

There was a significantly increased incidence of ductal hyperplasia in the male mammary glands of PND 21 pups treated with 0.5 and 5.0 μg EE_2/kg bw/day, with severity as well as incidence increased at the highest EE_2 dose level (Table 7). There were no statistically significant effects of BPA on the male mammary gland ductal hyperplasia at any dose level.

PND 90

Naïve versus vehicle control. Significant differences in nonneoplastic lesions between the naïve and vehicle control groups for both sexes at PND 90 were few. In females, renal cysts were higher in naïve controls (5/20) than in the vehicle controls (1/20). As discussed below, the low incidence of renal cysts in the vehicle control females relative to females in other dose groups affected the interpretation of apparent treatment effects. In males, degeneration of acinar cells (12/20 in naïve and 4/20 in vehicle) and infiltration of lymphocytic cells (8/20 in naïve and 2/20 in vehicle) in the pancreas were significantly different, but no other group differed significantly from the vehicle control (data not shown).

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TABLE 5
Clinical Chemistry, Females PND 90 ± 5[a]

	Treatment												
	Negative controls		BPA (μg/kg bw/day)									EE_2 (μg/kg bw/day)	
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
n	20	20	23	18	21	20	20	20	20	20	19	20	20
Aspartate aminotransferase, U/L	86.6 ± 2.5	81.2 ± 2.3	81.9 ± 2.7	88.2 ± 3.8	85.5 ± 2.9	88.6 ± 2.2	89.6 ± 2.5	79.2 ± 2.6	89.0 ± 2.4*	81.1 ± 2.8	87.3 ± 7.7	79.3 ± 3.6	91.9 ± 6.4
Blood urea nitrogen, mg/dL	17.5 ± 0.5	16.5 ± 0.8	16.6 ± 0.7	17.1 ± 0.7	16.7 ± 0.6	16.8 ± 0.7	15.8 ± 0.4	15.4 ± 0.6	16.0 ± 0.8	16.1 ± 0.8	17.3 ± 0.6	17.6 ± 1.0	19.6 ± 0.7**
Cholesterol, mg/dL	104.9 ± 6.0	99.0 ± 2.5	106.3 ± 4.0	100.3 ± 4.2	98.9 ± 2.1	103.1 ± 3.6	107.1 ± 4.5	101.0 ± 4.3	97.3 ± 4.5	83.2 ± 3.9*	94.3 ± 4.8	117.5 ± 6.3*	82.3 ± 5.0**
Glucose, mg/dL	120.0 ± 6.3	122.2 ± 5.3	116.0 ± 3.5	125.4 ± 7.5	115.9 ± 4.7	124.7 ± 6.3	120.0 ± 6.3	115.1 ± 4.3	108.4 ± 4.3	112.5 ± 3.9	110.9 ± 4.6	121.4 ± 6.2	122.5 ± 7.3
Insulin, mIU/ml	7.7 ± 1.7	8.9 ± 1.8	7.1 ± 1.0	9.2 ± 1.9	7.4 ± 1.0	7.7 ± 1.2	5.7 ± 0.6	5.8 ± 0.6	7.2 ± 1.5	5.4 ± 0.8	4.9 ± 0.6	7.8 ± 1.4	9.4 ± 1.6
Leptin, ng/ml	2.5 ± 0.3	2.7 ± 0.4	2.6 ± 0.3	3.0 ± 0.3	2.1 ± 0.2	2.3 ± 0.3	2.6 ± 0.4	2.3 ± 0.2	2.1 ± 0.3	1.8 ± 0.2	1.2 ± 0.1***	1.9 ± 0.3	2.4 ± 0.4
Sorbitol dehydrogenase, U/L	43.2 ± 4.8	39.8 ± 4.0	37.3 ± 2.4	43.4 ± 3.9	46.6 ± 4.5	52.2 ± 5.7	40.3 ± 4.2	43.1 ± 6.0	44.6 ± 4.9	50.3 ± 5.8	31.3 ± 2.7	36.4 ± 2.9	60.8 ± 6.4*
T3, ng/dL	68.7 ± 2.7	72.2 ± 3.1	76.4 ± 3.0	63.9 ± 3.0	68.2 ± 1.7	71.9 ± 3.6	72.3 ± 3.0	74.9 ± 4.2	67.3 ± 3.1	65.8 ± 2.7	74.4 ± 4.2	73.3 ± 4.8	73.9 ± 4.0
T4, μg/dL	4.8 ± 0.2	4.9 ± 0.3	5.0 ± 0.3	4.7 ± 0.2	4.5 ± 0.2	4.7 ± 0.3	4.7 ± 0.3	5.0 ± 0.4	4.6 ± 0.3	5.2 ± 0.3	5.8 ± 0.5	3.8 ± 0.2**	5.4 ± 0.2
TSH, ng/ml	6.4 ± 0.5	6.1 ± 0.5	7.4 ± 0.6	6.0 ± 0.6	6.3 ± 0.6	7.9 ± 0.9	7.6 ± 1.0	6.5 ± 0.6	6.4 ± 0.8	8.7 ± 0.9*	9.9 ± 1.4*	10.1 ± 1.1**	9.9 ± 0.7***
Triglycerides, mg/dL	81.9 ± 10.6	85.2 ± 7.6	81.2 ± 7.2	76.3 ± 5.1	93.7 ± 9.2	75.5 ± 5.2	83.7 ± 9.5	82.5 ± 7.4	75.8 ± 5.8	59.8 ± 2.6**	68.9 ± 7.8	102.3 ± 7.9	147.8 ± 21.3

[a]Results are given as mean ± SEM. Significant differences from vehicle control are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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TABLE 6
Clinical Chemistry, Males PND 90 ± 5[a]

	Treatment												
	Negative controls		BPA (μg/kg bw/day)									EE_2 (μg/kg bw/day)	
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
n	20	20	23	18	21	20	20	20	20	20	18	20	20
Cholesterol, mg/dL	83.5 ± 5.0	77.5 ± 3.8	81.0 ± 4.5	77.7 ± 3.4	86.1 ± 4.3	81.1 ± 2.6	76.9 ± 5.3	85.5 ± 4.2	84.1 ± 3.7	65.3 ± 2.9*	61.2 ± 3.9**	69.3 ± 4.4	65.1 ± 3.3*
Creatinine, mg/dL	0.470 ± 0.016	0.455 ± 0.022	0.452 ± 0.020	0.489 ± 0.024	0.486 ± 0.021	0.480 ± 0.022	0.515 ± 0.017	0.485 ± 0.017	0.415 ± 0.020	0.490 ± 0.040	0.528 ± 0.027*	0.510 ± 0.018	0.555 ± 0.020***
Glucose, mg/dL	110.8 ± 2.9	116.3 ± 3.1	123.5 ± 6.1	113.4 ± 4.3	126.9 ± 8.6	111.8 ± 4.0	117.6 ± 4.6	127.1 ± 6.4	118.3 ± 5.5	107.6 ± 4.0*	111.4 ± 3.7	110.0 ± 3.3	120.9 ± 5.0
Insulin, mIU/ml	10.5 ± 1.0	12.5 ± 3.6	10.7 ± 2.0	10.4 ± 1.1	11.5 ± 0.9	9.3 ± 1.1	9.7 ± 1.4	12.7 ± 1.9	9.9 ± 1.2	7.3 ± 0.7	8.7 ± 0.8	7.9 ± 0.7	10.7 ± 2.0
Leptin, ng/ml	4.1 ± 0.4	3.9 ± 0.4	3.7 ± 0.3	4.3 ± 0.5	4.7 ± 0.4	4.5 ± 0.6	4.4 ± 0.5	4.4 ± 0.3	4.6 ± 0.5	3.2 ± 0.2	2.6 ± 0.3**	3.6 ± 0.4	3.3 ± 0.3
T3, ng/dL	75.9 ± 3.7	68.1 ± 3.4	73.7 ± 2.9	75.4 ± 3.2	68.0 ± 3.6	69.7 ± 3.7	76.0 ± 3.4	66.7 ± 3.8	73.1 ± 3.6	73.1 ± 4.1	74.4 ± 3.9	71.9 ± 3.4	80.5 ± 3.0**
T4, μg/dL	6.4 ± 0.2	6.6 ± 0.3	6.2 ± 0.3	6.4 ± 0.3	6.9 ± 0.3	6.4 ± 0.4	6.8 ± 0.4	6.5 ± 0.3	6.4 ± 0.2	7.2 ± 0.3	7.8 ± 0.3*	6.7 ± 0.3	7.2 ± 0.3
TSH, ng/ml	8.0 ± 0.7	7.6 ± 0.9	8.2 ± 0.8	8.2 ± 1.0	7.8 ± 0.6	8.4 ± 0.6	7.0 ± 0.6	7.8 ± 0.5	7.9 ± 1.0	7.9 ± 0.6	6.9 ± 0.6	7.1 ± 0.5	9.2 ± 1.0
Total bile acids, μmol/L	49.1 ± 4.0	54.2 ± 4.8	58.7 ± 5.3	60.3 ± 5.7	54.4 ± 5.3	52.9 ± 5.9	63.8 ± 6.7	53.9 ± 4.1	49.9 ± 4.5	44.7 ± 4.8	40.5 ± 2.7*	57.1 ± 5.2	55.3 ± 5.1
Triglycerides, mg/dL	130.7 ± 10.5	119.0 ± 8.5	105.7 ± 8.5	118.4 ± 6.3	137.2 ± 10.5	125.1 ± 14.4	125.4 ± 12.7	126.8 ± 10.4	126.2 ± 10.4	120.6 ± 7.9	138.2 ± 15.3	129.1 ± 11.5	142.1 ± 13.2

[a]Results are given as mean ± SEM. Significant differences from vehicle control are indicated: $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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TABLE 7
Histological Evaluation of Female and Male PND 21 Mammary Glands[a]

	Treatment												
	Negative controls		BPA (μg/kg bw/day)									EE_2 (μg/kg bw/day)	
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
Females													
Duct, hyperplasia													
Significance	-		-	-	-	-	-	-	*	**	-	-	-
Incidence	2/17	0/16	2/19	1/13	4/19	1/20	1/13	2/18	5/17	6/17	3/12	0/20	1/20
Severity profile	2/0/0/0	0/0/0/0	2/0/0/0	1/0/0/0	4/0/0/0	1/0/0/0	1/0/0/0	2/0/0/0	5/0/0/0	6/0/0/0	3/0/0/0	-	-
Males													
Duct, hyperplasia													
Significance	-		-	-	-	-	-	-	-	-	-	***	***
Incidence	2/20	1/19	0/22	0/17	0/21	0/20	1/20	0/20	3/20	2/20	2/10	11/19	14/17
Severity profile	1/1/0/0	0/1/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	1/0/0/0	0/0/0/0	2/1/0/0	2/0/0/0	2/0/0/0	9/2/0/0	1/8/5/0

[a]Data are presented as incidence (the number of animals with lesion/the number of animals examined). The severity profile is also shown, i.e, the number of animals with lesions graded as minimal/mild/moderate/marked or grades 1/2/3/4, respectively. Statistical significance based on incidence, or lack thereof, is indicated in the row above the incidence row. A "-" indicates no significance. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

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TABLE 8
Female Histopathology, PND 90 ± 5[a,b]

	Treatment												
	Negative controls		BPA (μg/kg bw/day)									EE_2 (μg/kg bw/day)	
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
Ovary													
Cyst, follicle	–		-	-	-	-	-	-	-	-	***	***	***
Incidence	2/20	4/20	3/23	1/18	7/21	5/20	2/20	5/20	7/20	0/21	14/19	16/19	19/20
Severity profile	0/2/0/0	0/4/0/0	1/2/0/0	1/0/0/0	3/4/0/0	1/4/0/0	0/2/0/0	3/2/0/0	3/4/0/0	0/0/0/0	1/5/4/4	0/2/4/10	1/1/5/12
Corpora lutea, depletion	-		-	-	-	-	-	-	-	-	***	***	***
Incidence	0/20	0/20	2/23	2/18	0/21	1/20	1/20	2/20	2/20	1/21	14/19	17/19	18/20
Severity profile	0/0/0/0	0/0/0/0	0/2/0/0	2/0/0/0	0/0/0/0	0/2/0/0	1/0/0/0	0/2/0/0	1/1/0/0	0/1/0/0	3/6/4/1	0/5/5/7	0/0/1/17
Antral follicles, depletion	-		-	-	-	-	-	-	-	-	*	***	***
Incidence	1/20	3/20	1/23	1/18	0/21	2/20	0/20	1/20	2/20	2/21	10/19	16/19	19/20
Anestrus[c]													
Incidence	2/20	3/20	2/23	0/18	1/21	1/20	1/20	3/20	3/20	1/21	13/19	18/19	19/20
Uterus, endometrium													
Hyperplasia	-		-	-	-	-	-	-	-	-	*	-	-
Incidence	1/20	0/20	0/23	4/18	2/21	0/20	0/21	2/20	1/20	3/21	7/19	5/20	5/20
Severity	0/1/0/0	0/0/0/0	0/0/0/0	4/0/0/0	2/0/0/0	0/0/0/0	0/0/0/0	2/0/0/0	1/0/0/0	3/0/0/0	3/4/0/0	4/1/0/0	2/3/0/0
Uterus, endometrium (continued)													
Metaplasia	-		-	-	-	-	-	-	-	-	-	***	***
Incidence	2/20	1/20	3/23	2/18	3/21	3/20	1/21	0/20	3/20	4/21	2/19	11/20	13/20
Severity	2/0/0/0	1/0/0/0	3/0/0/0	2/0/0/0	3/0/0/0	3/0/0/0	1/0/0/0	0/0/0/0	3/0/0/0	4/0/0/0	2/0/0/0	6/3/2/0	5/7/0/1
Mammary gland													
Duct, hyperplasia	-		-	-	-	-	-	-	-	-	**	-	-
Incidence	9/20	7/20	11/23	6/18	11/21	8/20	8/20	9/20	11/20	13/20	14/19	13/20	7/20
Severity profile	7/2/0/0	7/0/0/0	7/4/0/0	4/2/0/0	10/1/0/0	1/7/0/0	3/5/0/0	5/4/0/0	4/7/0/0	8/5/0/0	13/1/0/0	8/5/0/0	4/3/0/0
Kidney													
Renal tubule, cyst	-		-	-	*	*	*	-	*	**	***	*	-
Incidence	5/20	1/20	5/23	5/18	8/21	6/20	6/21	5/20	6/20	8/21	12/19	7/20	2/20
Severity profile	5/0/0/0	1/0/0/0	2/2/0/1	4/0/1/0	5/3/0/0	4/0/2/0	5/1/0/0	3/0/2/0	5/1/0/0	7/1/0/0	5/3/3/1	5/2/0/0	2/0/0/0

[a]Data are presented as incidence (the number of animals with lesion/the number of animals examined). The severity profile is also shown, i.e, the number of animals with lesions graded as minimal/mild/moderate/marked or grades 1/2/3/4, respectively. Statistical significance based on incidence, or lack thereof, is indicated in the row above the incidence row. A "-" indicates no significance. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

[b]The number of animals examined includes early death animals. One female in the 260 μg BPA/kg bw/day dose group was accidentally killed and one in the 100,000 μg BPA/kg bw/day dose group was sacrificed early in a moribund condition (diagnosed with nephropathy associated with polyarteritis). Cases where the total number of tissues examined differs among tissues reflect insufficient or missing tissue.

[c]For this study, the term "anestrus" designates a likely abnormal state of the estrous cycle, either arrested or prolonged based on the accompanying histopathological changes in the ovary that are consistent with inactivity or abnormal activity.

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TABLE 9
Male Histopathology, PND 90 ± 5[a, b, c]

	Treatment												
	Negative controls		BPA (μg/kg bw/day)									EE_2 (μg/kg bw/day)	
	Naïve	Vehicle	2.5	8	25	80	260	840	2700	100,000	300,000	0.5	5.0
Mammary gland													
Duct, hyperplasia	-		-	-	-	-	-	-	-	-	-	*	***
Incidence	1/20	0/20	3/23	0/18	1/21	0/20	0/20	2/21	1/20	1/23	1/18	5/20	14/20
Severity profile	1/0/0/0	0/0/0/0	3/0/0/0	0/0/0/0	1/0/0/0	0/0/0/0	0/0/0/0	1/1/0/0	0/1/0/0	1/0/0/0	1/0/0/0	3/2/0/0	3/4/5/2
Alveolus, hyperplasia	-		-	-	-	-	-	-	-	-	-	**	**
Incidence	6/20	5/20	3/23	7/18	5/21	7/20	7/20	4/21	8/20	10/23	6/18	14/20	13/20
Severity profile	3/3/0/0	4/1/0/0	2/1/0/0	7/0/0/0	5/0/0/0	3/4/0/0	6/0/1/0	3/1/0/0	6/2/0/0	7/2/1/0	3/3/0/0	5/5/3/1	1/3/4/5
Duct or alveolus, hyperplasia	-		-	-	-	-	-	-	-	-	-	***	***
Incidence	6/20	5/20	6/23	7/18	6/21	7/20	7/20	6/21	9/20	11/23	7/18	16/20	20/20
Severity profile	3/3/0/0	4/1/0/0	5/1/0/0	7/0/0/0	6/0/0/0	3/4/0/0	6/0/1/0	4/2/0/0	6/3/0/0	8/2/1/0	4/3/0/0	5/7/3/1	1/3/9/7
Coagulating gland													
Epithelium, hyperplasia	-		-	-	-	-	-	-	-	-	-	-	**
Incidence	0/20	0/20	0/23	0/18	0/21	0/20	0/19	0/21	0/20	0/20	0/18	0/20	6/20
Severity profile	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/5/1/0
Testis													
Germinal epithelium, degeneration	-		-	-	-	-	-	-	-	-	-	-	**
Incidence	1/20	1/20	0/23	0/18	0/21	0/20	2/20	0/21	0/20	0/20	0/18	2/20	9/20
Severity profile	1/0/0/0	0/0/0/1	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/1/1	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/2	2/2/1/4
Seminiferous tubule, giant cell	–		*	-	-	-	-	-	-	-	-	0/20	2/20
Incidence	0/20	0/20	5/23	1/18	4/21	2/20	2/20	1/21	3/20	0/20	2/18	-	-
Severity profile	0/0/0/0	0/0/0/0	5/0/0/0	1/0/0/0	4/0/0/0	2/0/0/0	2/0/0/0	1/0/0/0	3/0/0/0	0/0/0/0	2/0/0/0	0/0/0/0	2/0/0/0
Epididymis													
Hypospermia	-		-	-	-	-	-	-	-	-	-	-	*
Incidence	0/20	1/20	0/23	0/18	0/21	0/20	2/20	0/21	0/20	0/20	0/18	2/20	7/20
Severity profile	0/0/0/0	0/0/0/1	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/1/0/1	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/2	0/4/0/3
Exfoliated germ cell	-		-	-	-	-	-	-	-	-	-	-	*
Incidence	0/20	1/20	0/23	0/18	0/21	0/20	3/20	0/20	0/20	0/20	0/18	2/20	6/20
Severity profile	0/0/0/0	1/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	1/2/0/0	0/0/0/0	0/0/0/0	0/0/0/0	0/0/0/0	2/0/0/0	3/3/0/0

[a]Data are presented as incidence (the number of animals with lesion/the number of animals examined). The severity profile is also shown, i.e, the number of animals with lesions graded as minimal/mild/moderate/marked, or grades 1/2/3/4, respectively.

[b]The number of animals examined includes early death animals. One male in each of the 840 and 100,000 μg BPA/kg bw/day dose group was sacrificed early in moribund condition and both were diagnosed with nephropathy associated with polyarteritis. Two males in the 100,000 μg BPA/kg bw/day dose group were removed early due to dosing accidents. Cases where the total number of tissues examined differs among tissues reflect insufficient or missing tissue.

[c]Statistical significance, or lack thereof, is indicated in the row above the incidence row. A "-" indicates no significance. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

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Females

Females were sacrificed on a day they were predicted to be in estrus based on the vaginal cytology of the previous day. Histological evaluation of the female reproductive tract (ovary, uterus, and vagina) indicated that the percentage of females in proestrus, estrus, or transition from estrus to metestrus at the time of necropsy ranged from 75% to 100% across dose groups, except for animals in both EE_2 groups and the 300,000 μg BPA/kg bw/day group, which had high numbers of anestrus animals (see below).

Bilaterally small ovaries and abnormal genitalia and thick vagina were common treatment-related gross observations. Small ovaries were noted in both EE_2 groups and the 300,000 μg BPA/kg bw/day group, with incidences of 8/20, 16/20, and 10/19 in the 0.5 EE_2, 5.0 EE_2, and 300,000 BPA groups, respectively. The small ovaries phenotype correlated microscopically with depletion of corpora lutea and antral follicles. Abnormal genitalia and gross thickening of the vaginal wall were observed in the 5.0 μg EE_2/kg bw/day treatment group (14/20) and were manifested microscopically as hypertrophy of the vaginal muscular wall (muscularis) and mucosal hyperplasia.

A mammary gland ductal adenocarcinoma was observed in the 2.5 μg BPA/kg bw/day group and this was the only neoplasm observed in the PND 90 ± 5 histopathology animals. Although there are limited historical data for this lesion in the NCTR Sprague Dawley rat at this age, a similar neoplasm was recently diagnosed in a PND 90 female NCTR Sprague Dawley rat on an unrelated NCTR study on silver nanoparticles (Boudreau, personal communication).

Non-neoplastic lesions in the mammary gland, ovary, uterus, and kidney are summarized in Table 8. The incidence of mammary gland ductal hyperplasia was variable across the dose range, including naïve and vehicle controls, but was significantly increased in the 300,000 μg BPA/kg bw/day group relative to vehicle control. There were no other BPA-induced significant effects on the mammary gland in females as determined by the Poly-3 test, although the RTE and JT/SW analyses, which incorporate severity information, indicated significant increases of mammary gland ductal hyperplasia at 2700 and 100,000 μg BPA/kg bw/day. Non-neoplastic lesions in females in the low BPA dose range that were significantly different from those in vehicle controls were few and limited to the kidney (Table 8). Renal tubular cysts were elevated above vehicle control in multiple low dose BPA groups, in the naïve controls, in both high BPA groups, and in the low EE_2 group. Unlike with the other groups, the higher incidence and severity of renal cysts at 300,000 μg BPA/kg bw/day suggest a possible treatment effect.

There was a low incidence of cystic endometrial hyperplasia in the uterus of the naïve control (5%) and several BPA dose groups (Table 8). The Poly-3 test indicated a significant increase in the incidence of cystic endometrial hyperplasia in the uterus of animals dosed with 300,000 μg BPA/kg bw/day versus vehicle control. The RTE test further indicated a significant increase in the 8 μg BPA/kg bw/day dose group. In the 300,000 μg BPA/kg bw/day group, there were high and statistically significant incidences of corpora lutea depletion, cystic follicles, and antral follicle depletion (Table 8 and Fig. 4).

Asynchrony, defined as a difference of at least two stages of the estrous cycle in either the uterus or vagina compared with the ovary or when the ovary was designated as anestrus, was prevalent in the 300,000 μg BPA/kg bw/day group compared with the vehicle control (63% vs. 12%). All other BPA dose groups had a percentage of asynchronous animals lower than the vehicle controls.

Lesions in the EE_2 dose groups in the ovary, uterus, mammary gland, and kidney are also shown in Table 8. As indicated in the Materials and Methods section, the histopathology data of the EE_2 groups were not evaluated in the QAS and PWG reviews and therefore cannot be directly compared with the BPA groups.

Animals in both EE_2 dose groups had increased follicular cysts, depletion of corpora lutea, and depletion of antral follicles (Table 8) consistent with the altered cycling observed (see above). In addition, high incidences of uterine endometrial metaplasia were diagnosed in both EE_2 dose groups that, in contrast to the low incidence endometrial metaplasia observed in control and BPA groups, was luminal rather than glandular. In the low and high EE_2 dose groups, 94% and 100% of the animals, respectively, were determined to be asynchronous versus 12% in the vehicle control group.

Males

The most commonly recorded gross pathology observation in males was decreased testicular size. This was noted in the 0.5 and 5.0 μg EE_2/kg bw/day groups, with incidences of 4/20 and 16/20, respectively, and in the 260 and 300,000 μg BPA/kg bw/day groups, with incidences of 5/20 in each group. Less than half of the animals with small testis showed degeneration of the seminiferous tubular epithelium at a microscopic level (2/5, 0/5, 2/4, and 7/16 in the 260 BPA, 300,000 BPA, 0.5 EE_2, and 5.0 EE_2, respectively).

The incidences and severities of histopathological lesions observed in the male reproductive tract, mammary glands, and kidney are shown in Table 9. The Poly-3 test indicated a significantly elevated incidence of seminiferous tubule giant cells in the 2.5 μg BPA/kg bw/day group. The RTE test also indicated the incidence at 25 μg BPA/kg bw/day (4/21, 19%) was significant ($p = 0.022$). The incidence of seminiferous cell giant cells was variable (ranging from 5% to 22%) across the low BPA dose groups, was present at 2/18 (11%) in the 300,000 μg BPA/kg bw/day group, and was not observed in the vehicle or naïve control groups. This diagnosis was characterized by a multinucleated syncytial cell present along the apical epithelial border, and while this is often associated with degeneration, most of these observations featured only one or two syncytial cells and were thus not coded as degeneration. Unlike the sit-

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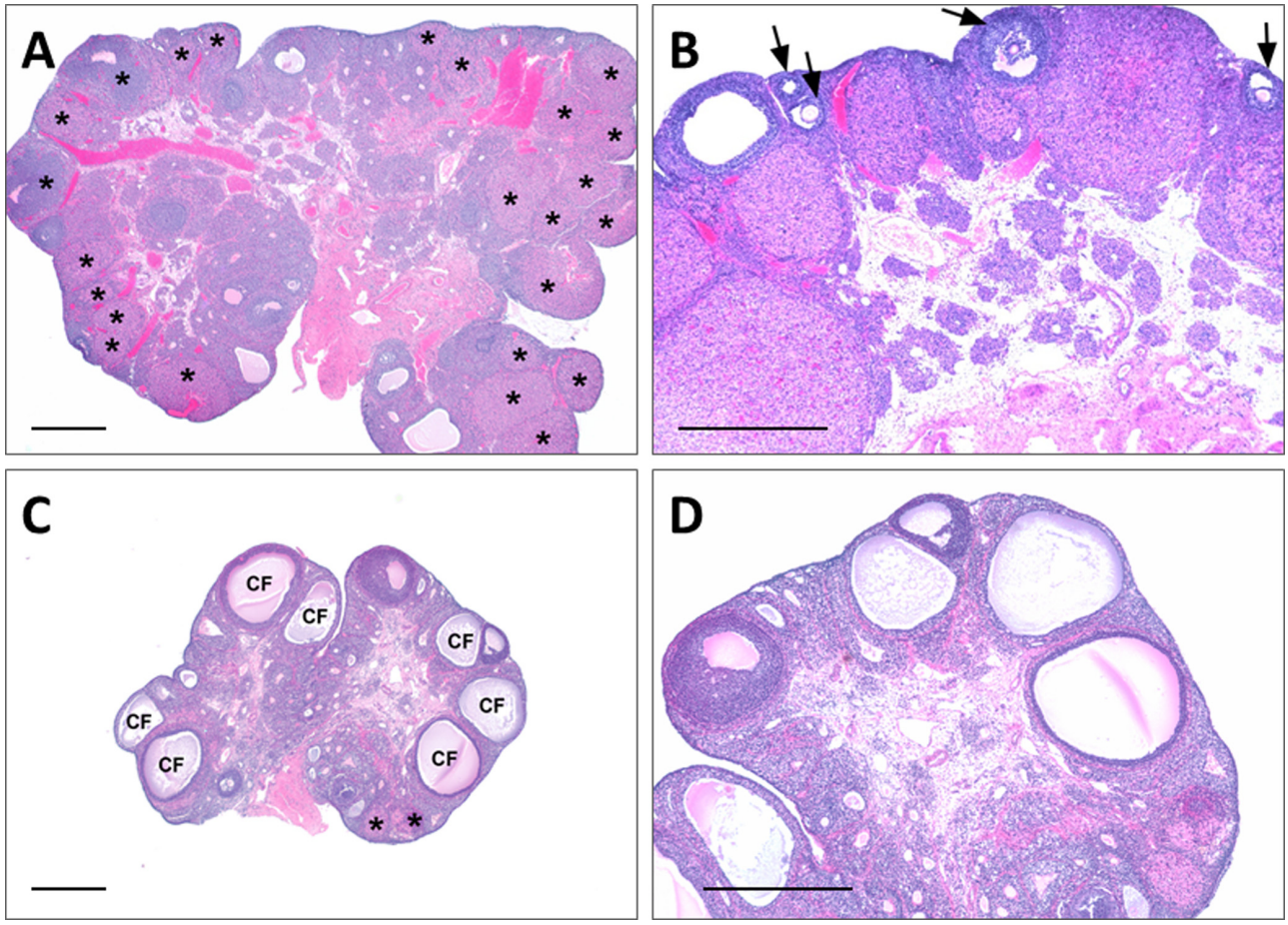

FIG. 4. Ovary histology of animals dosed with the vehicle [(A) and (B)] or 300,000 μg BPA/kg bw/day [(C) and (D)]; tissue sections stained with hematoxylin and eosin. (A) Ovary from a normal cycling rat. A minimum of three generations (sets) of corpora lutea (*) from current and previous consecutive estrous cycles are present. The sets were determined morphologically by the relative size, cellularity, and lipochrome pigment content typically present in various stages of corpora luteal growth, degeneration, and regression. (B) Normal appearing antral follicles (arrows) in various stages of maturation from early to late. (C) Numerous cystic follicles (CF) and depletion of corpora lutea [*, compare with panel (A)]. Follicular cysts and fewer sets of corpora lutea than normal imply that either the estrous cycle has been arrested or prolonged. (D) Lack of normal appearing antral follicles [compare with panel (B)]. Depletion of antral follicles was defined as less than two to three normal appearing antral follicles and/or a relative increase in atretic ones. Severity for cystic follicles was subjectively based on the relative number of cysts present in sections; severity for corpora lutea depletion was based on the number of sets present (three sets only = grade 1; two sets = grade 2; one set = grade 3; and no sets = grade 4); depletion of antral follicles coded as present or absent. Bar, 50 μm.

uation in the 5 μg EE_2/kg bw/day group, where degeneration of the seminiferous epithelium was diagnosed and associated with hypospermia and exfoliated germ cells in the epididymides along with reduced epididymal sperm counts (Table 9), there were no such related lesions in the BPA groups. There was a slightly elevated incidence of epididymal exfoliated germ cells in the 260 μg BPA/kg bw/day group (Table 9) that was not significant by the Poly-3 test, but was significant by the RTE test.

The high dose of EE_2 significantly increased the incidence of germinal epithelium degeneration in testes with hypospermia and exfoliated germ cells noted in the epididymides. Epithelial hyperplasia was induced in the coagulating gland in this same group.

Histopathologic assessment of the male mammary gland indicated that ductal hyperplasia occurred at low incidence (ranging from 0% to 13%) across the BPA doses. There were no significant treatment effects by the Poly-3 method, but the RTE test indicated significance at 2.5 and 840 μg BPA/kg bw/day. Ductal hyperplasia showed a clear EE_2 dose-dependent increase in both incidence and severity (Table 9). Alveolar hyperplasia was also elevated by both EE_2 doses, with an apparent increased severity in the high dose group.

DISCUSSION

Under the conditions of this study, BPA had clear adverse effects at doses of 100,000 and 300,000 μg/kg bw/day, with

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the majority of these effects observed in females. In the study-defined "low BPA" dose range of 2.5–2700 μg/kg bw/day, which was the primary focus of this study, potential effects could not be clearly linked to treatment as they were observed sporadically across the dose groups and did not occur in a consistent grouping across organs as did effects of EE_2 (0.5 and 5.0 μg/kg bw/day) or "high BPA" (100,000 and 300,000 μg/kg bw/day).

Differences in sensitivity of animal strains to BPA and other agents that may act to disrupt estrogen signaling pathways have been much debated (e.g., Beronius *et al.*, 2013; EFSA, 2010; Richter *et al.*, 2007). The animal model used in the current study, the Sprague Dawley rat from the NCTR colony, has been used previously for assessments of dietary EE_2 and genistein (Delclos *et al.*, 2009; Latendresse *et al.*, 2009; NTP, 2008a,b, 2010a,b), and shown to be sensitive to estrogenic compounds. In addition, a comprehensive evaluation of the pharmacokinetics of BPA across life stages has been reported in this strain (Doerge *et al.*, 2010a,b, 2011; Twaddle *et al.*, 2010).

BPA is a ubiquitous environmental contaminant, making the assessment of its toxicity at low levels problematic. Safety assessments of other ubiquitous environmental contaminants or common dietary components [e.g., phthalates, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), acrylamide, and soy isoflavones] present common issues in study conduct and interpretation, given the potential presence of these agents in study materials that may lead to exposures in negative control animals (Kondo *et al.*, 2010; Vanden Heuvel *et al.*, 1994; Twaddle *et al.*, 2004; NTP, 2008a,b). The presence of additional environmental or dietary components that might affect pathways common to the BPA also needs to be taken into account (e.g., Thigpen *et al.*, 2013). In the current study, low-BPA study materials (including polysulfone cages, glass water bottles, and hardwood chip bedding) were used and the levels of BPA in these materials were monitored regularly. Because many of the effects of BPA are thought to occur through interference with estrogen signaling pathways, a soy- and alfalfa-free diet was used and the dietary levels of the phytoestrogens genistein and daidzein were monitored, in addition to BPA. All study materials, except the feed, had BPA levels below the average analytical blanks. A cutoff of 5 ppb BPA was established for the diet, because ingestion of feed containing 5 ppb or more of BPA would have resulted in a BPA dietary exposure of approximately 0.25 μg/kg bw/day, a 10-fold lower dose than the lowest BPA dose used in the study; a single lot of feed was rejected based on this criterion. Blood samples were collected from study animals at various time points to evaluate systemic exposures to aglycone (bioactive) and conjugated BPA (Churchwell *et al.*, 2014). Despite our efforts to minimize the unintentional exposure of animals to BPA and the precautions taken to physically separate vehicle dosing from the BPA and EE_2 doses (see the Materials and Methods section), BPA-glucuronide was detected in the serum of vehicle and naïve control animals at levels similar to those detected in animals dosed with 2.5 μg BPA/kg bw/day (Churchwell *et al.*, 2014). The presence of BPA-glucuronide, which is formed by metabolism of aglycone BPA in the body, in the serum of control animals implies unintentional exposure of the animals to BPA rather than postsampling contamination of the serum samples. The source of this exposure has not been definitively identified (Churchwell *et al.*, submitted). Thus, the 2.5 μg BPA/bw/day dose group is not distinguishable from the two negative control groups.

The current study involved daily oral gavage dosing of pregnant dams from GD 6 until the start of labor followed by direct dosing of their pups from PND 1 until PND 90 ± 5. This exposure regimen complements many of the existing studies of BPA that have been conducted for regulatory purposes, in which BPA was administered by diet or gavage to lactating dams (Ema *et al.*, 2001; Stump *et al.*, 2010; Tyl *et al.*, 2002, 2008). Pharmacokinetic data from the animal model used in the current study have demonstrated marked differences in BPA metabolism with age (Doerge *et al.*, 2010a) and also indicated limited lactational transfer of BPA to pups (Doerge *et al.*, 2010b). Thus, it is important to recognize that, whereas the BPA dose rate in terms of μg/kg bw/day was constant across our study, the internal dose of BPA varied markedly with age. A very similar pattern of age-dependent serum concentrations was obtained for EE_2 (Churchwell *et al.*, submitted). The direct dosing of pups better mimics the exposure of concern in infants and young children than does exposure through the dam's milk and bypasses the low lactational transfer of BPA, allowing a more substantial and controlled neonatal exposure. The magnitude of exposure during the early postnatal period is of particular concern because this is a sensitive age for rodent reproductive tract development (e.g., Barraclough, 1961; Foster *et al.*, 2004; Gorski, 1963; McPherson *et al.* 2008; Newbold *et al.*, 2004) and that of other organs (e.g., Burri, 2006; Eriksson, 1997; Schreuder *et al.*, 2011).

Although evidence exists to suggest that the low dose effects of BPA reported in the literature would not be mediated through classical estrogen receptors alone (Hugo *et al.*, 2008; Alonso-Magdalena *et al.*, 2008; NTP, 2008a; Thayer and Belcher, 2010), many of the reported BPA effects do involve estrogen-related pathways and inclusion of a reference estrogen control has been recommended for low dose BPA studies (e.g., Richter *et al.*, 2007). Both dose levels of EE_2 tested in the current study induced multiple adverse effects in females that are consistent with other studies on EE_2 (Andrews *et al.*, 2002; Ferguson *et al.*, 2011; Howdeshell *et al.*, 2008; Sawaki *et al.*, 2003a,b; Shiorta *et al.*, 2012), including the depression of gestational body weight gain, disruption of estrous cyclicity, induction of cystic ovarian follicles, and depletion of corpora lutea and antral follicles. The effects on the estrous cycle and the ovary are consistent with the anovulatory syndrome induced by neonatal treatment with estrogens or androgens (Barraclough, 1961; Gorski, 1963). The masculinizing organizational effect of perinatal estrogen exposure has also been reported for body weight gain in females (e.g., Bell and Zucker, 1971; Dono-

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hoe and Stevens, 1983), an effect induced by the high EE_2 dose used here. Partially overlapping effects were produced by one or both high doses of BPA used in this study, with depression of gestational body weight gain at 100,000 and 300,000 μg/kg bw/day, and ovarian and estrous cycle effects only at the highest BPA dose. The depression of gestational body weight gain by BPA is consistent with other reports of BPA in rats (Tyl *et al.*, 2002; Zoeller *et al.*, 2005). The dosing method used in the current study, with high exposure to young neonates, likely accounts for the observed histopathological effects in the reproductive tract of females dosed with 100,000 and 300,000 μg BPA/kg bw/day, in contrast to the lack of such effects in the dietary study of Tyl *et al.* (2002), in which doses near 500,000 μg BPA/kg bw/day were administered in the diet without notable reproductive tract lesions in either sex.

Unexpectedly, the EE_2 dosing regimen used in the present study produced a significant dose-dependent delay in the time of vaginal opening relative to vehicle controls. The delay in vaginal opening was also noted in a parallel study conducted at NCTR under identical conditions, in which animals were orally dosed by gavage with 5 or 10 μg EE_2/kg bw/day from GD 6 through PND 21 (Ferguson, in preparation). Although exposure to neonatal androgen has been reported to delay or accelerate the timing of vaginal opening depending on timing of neonatal exposure and dose (Tramezzani *et al.*, 1963; Rossano *et al.*, 1973), oral administration (diet, water, or gavage) of diethylstilbestrol (DES) or EE_2 to the dam during gestation and/or lactation has been reported mostly to accelerate the time of vaginal opening or to have no effect, depending on timing and dose level (e.g., Delclos *et al.*, 2009; Odum *et al.*, 2002; Rasier *et al.*, 2006; Ryan *et al.*, 2010). Sawaki *et al.* (2003a,b), evaluated the effects of gavage doses of 0.5, 5, and 50 μg EE_2/kg bw/day to dams from GD 7 through PND 18 on the development of female pups and reported a delay of vaginal opening at the lower doses, but not the high dose (Sawaki, 2003a), but this was not reproducible (Sawaki, 2003b). Hines and Goy (1985) and Hines *et al.* (1987) reported that subcutaneous or intramuscular administration of 1–3 μg DES/day to guinea pigs during gestation significantly delayed vaginal opening. Although vaginal opening in rodents is known to occur with the rise of estrogen levels near puberty, the precise mechanism whereby estrogens and androgens affect the timing of vaginal opening has not been established, although direct actions on vaginal epithelial cells to open the vaginal canal are likely (Silbergeld *et al.*, 2002). In discussing the exposure time-dependent delay of vaginal opening resulting from neonatal treatment of female mice with the estrogenic fungal product zearalenone, Ito and Ohtsubo (1994) suggested the potential involvement of inhibition of mitosis resulting from multiple neonatal doses of estradiol prior to PND 5 in vaginal epithelial cells as described by Forsberg (1969). Further investigation would be required to examine the molecular changes induced in the vaginal epithelium that might explain the EE_2-induced delay in vaginal opening observed in the current study. In any case, BPA did not alter the timing of puberty in females at any dose.

The effects of the highest BPA dose on the ovary and estrous cycles were similar to the effects of EE_2, suggesting an estrogenic effect at 300,000 μg BPA/kg bw/day. The observation of estrogenic effects of BPA only at high oral dose levels is consistent with observations made in several uterotrophic assays (e.g., Ashby and Tinwell, 1998; Diel *et al.*, 2004; Laws *et al.*, 2000; Yamasaki *et al.*, 2000). On the other hand, the interpretation of the high dose BPA effects are confounded by apparent systemic toxicity indicated by the significant body weight depression observed at this dose. This is also true for the organ weight effects of BPA, which appeared to be secondary to the treatment-induced body weight depression, particularly in males. Body weight decrements have been reported previously with high doses of BPA in rats and mice (Tyl *et al.*, 2002, 2008).

High EE_2 adversely affected PND 90 male reproductive organ weights and sperm production, whereas none of the BPA doses induced these effects. Induction of male mammary hyperplasia by EE_2 and other estrogenic agents has been reported previously by us and others to be a very sensitive indicator of estrogen exposure (Latendresse *et al.*, 2009 and references therein). In the current study, both doses of EE_2 increased the incidence of mammary gland hyperplasia in males; however, BPA did not induce a similar effect at any tested dose.

F_1 females and males had body weights that differed from vehicle controls across the study period only at the highest BPA dose tested (300,000 μg /kg bw/day), with a treatment-induced depression of body weight in both sexes. Effects of BPA on body weight and fat deposition at much lower doses (<5000 μg/kg bw/day) have been investigated in multiple experimental models with mixed results including increases, decreases, or no effect (discussed in Thayer *et al.*, 2012; Harley *et al.*, 2013). In the present study, the low dose range of BPA did not significantly affect body or fat pad weights nor the serum levels of glucose, triglycerides, insulin, or leptin. Likewise, no effects on thyroid hormones, thyroid weight, or thyroid histology were observed in the low BPA dose region. At PND 15, elevated T3 was observed at both 100,000 and 300,000 μg BPA/kg bw/day with depressed TSH observed only at the highest BPA dose; both high doses of BPA depressed the TSH levels in PND 90 females. Zoeller *et al.* (2005) had reported hyperthyroid effects in PND 15 offspring of dams dosed orally (dosed wafer) daily from GD 6 at 1000 μg BPA/kg bw/day, although later consequences of these effects were not evaluated in that study.

Our interpretation of the results of the present study is that BPA in the "low dose" region from 2.5 to 2700 μg/kg bw/day did not produce effects in the evaluated endpoints that differ from normal background biological variation. The experimental model was sufficiently sensitive to detect clear effects of EE_2 at both dose levels tested, as well as of the high BPA doses. Some endpoints, such as the PND 80 hormone assessments, although showing effects in females dosed with high BPA and EE_2 that were consistent with the histopathology data, were not

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performed under optimal conditions and may have been underpowered to detect subtle effects in some cases (e.g., testosterone; Chapin and Creasy, 2012). As a follow-up to this study, we are currently pursuing a collaborative research project under the auspices of the NTP involving multiple academic investigators funded by NIEHS extramural grants (Schug *et al.*, 2013). This project combines a chronic guideline-compliant oral BPA toxicity study, conducted in the animal model and with the dosing regimen described here, with in-depth evaluations of multiple functional, morphological, and molecular endpoints previously reported to be affected by BPA doses below 5 mg/kg bw/day (the current NOAEL). This study is an attempt to initiate a more comprehensive interagency exchange in order to resolve ultimately issues surrounding the toxicity of BPA and other potential hormonally active agents.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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EXHIBIT 3

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Comparison of Life-Stage-Dependent Internal Dosimetry for Bisphenol A, Ethinyl Estradiol, a Reference Estrogen, and Endogenous Estradiol to Test an Estrogenic Mode of Action in Sprague Dawley Rats

Mona I. Churchwell, Luísa Camacho, Michelle M. Vanlandingham, Nathan C. Twaddle, Estatira Sepehr, K. Barry Delclos, Jeffrey W. Fisher, and Daniel R. Doerge[1]

U.S. Food and Drug Administration, National Center for Toxicological Research, Division of Biochemical Toxicology, 3900 NCTR Road, Jefferson, Arkansas 72079

[1]To whom correspondence should be addressed. E-mail daniel.doerge@fda.hhs.gov.

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Bisphenol A (BPA) was administered by gavage (2.5–300,000 μg/kg body weight (bw)/day) to pregnant Sprague Dawley dams, newborn pups, and continuing into adulthood. Aglycone (i.e., unconjugated and active) and conjugated (i.e., inactive) BPA were evaluated by liquid chromatography electrospray tandem mass spectrometry (LC-ES/MS/MS) in serum to better interpret toxicological endpoints measured in the study. Ethinyl estradiol (EE2, 0.5 and 5 μg/kg bw/day) and the endogenous hormones, 17β-estradiol (E2) and testosterone, were similarly evaluated. Mean BPA aglycone levels in vehicle and naïve control rat serum (0.02–0.5 ng/ml) indicated sample processing artifact, consistent with literature reports of a propensity for postexposure blood contamination by BPA. Direct measurements of BPA-glucuronide in vehicle and naïve control serum (2–10nM) indicated unintentional exposure and metabolism at levels similar to those produced by 2.5 μg/kg bw/day BPA (7–10nM), despite careful attention to potential BPA inputs (diet, drinking water, vehicle, cages, bedding, and dust) and rigorous dosing solution certification and delivery. The source of this exposure could not be identified, but interpretation of the toxicological effects, observed only at the highest BPA doses, was not compromised. Internal exposures to BPA and EE2 aglycones were highest in young rats. When maximal serum concentrations from the two highest BPA doses and both EE2 doses were compared with concurrent levels of endogenous E2, the ERα binding equivalents were similar to or above those of endogenous E2 in male and female rats of all ages tested. Such evaluations of estrogenic internal dosimetry and comprehensive evaluation of contamination impact should aid in extrapolating risks from human BPA exposures.

Key words: **bisphenol A; ethinyl estradiol; pharmacokinetics; estrogen receptor; mass spectrometry.**

Bisphenol A (BPA) is an industrial chemical used extensively in the manufacture of polycarbonate plastic products and epoxy resins used for food can liners (National Toxicology Program, 2008). The U.S. Food and Drug Administration regulates BPA-containing products including food-contact materials and medical devices. Regulatory risk assessments of endocrine-active chemicals like BPA often incorporate data from animal toxicology studies that include explicit evaluation of hazards from perinatal exposure because aberrant developmental programming can induce irreversible adverse changes in phenotype.

The potential for toxicity of BPA exposure was evaluated in a subchronic study that included fetal, neonatal, and adult life stages (see Delclos *et al.*, 2014). Pregnant Sprague Dawley rats were exposed by daily gavage to doses of BPA ranging from 2.5 to 300,000 μg/kg body weight (bw)/day starting on gestation day (GD) 6 until parturition. Direct daily gavage dosing of pups continued with starting on postnatal day (PND) 1 through termination of the study. For a point of reference, the lowest BPA dose tested in this study is approximately 70-fold above median American aggregate daily exposure (0.037 μg/kg bw/day; Lakind *et al.*, 2012).

An important element of this study, which was jointly sponsored by the National Toxicology Program and the U.S. Food and Drug Administration (Birnbaum *et al.*, 2013), was an evaluation of BPA internal dosimetry because its pharmacokinetics are well known to change during fetal and neonatal development in rodents (Doerge *et al.*, 2010a), a phenomenon not observed in nonhuman primates (Doerge *et al.*, 2010b). The critical role of phase II metabolism in detoxification made it important to measure both the aglycone (i.e., unconjugated and active) and conjugated (i.e., inactive glucuronide and sulfate) forms of BPA. Although many molecular and cell-based studies suggest the potential for interaction of BPA with many receptor systems and cellular targets (e.g., thyroid hormone, aryl hydrocarbon, androgen, and aromatase) as possible toxic modes of action, a prominent hypothesis for adverse potential of BPA centers on estrogenic mechanisms, via classical nuclear and nonclassical membrane-bound receptors (Richter *et al.*, 2007). Therefore, it

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was also important to evaluate the internal dosimetry from concurrent treatment with a reference estrogen, EE2, as well as endogenous levels of the major steroid hormones, 17β-estradiol (E2) and testosterone, both of which are known to vary throughout life stages. This paper describes the analytical strategies used to determine these internal exposures and uses this information to compare relative estrogen receptor (ER) occupancy as a contributor to dose-dependent estrogenic effects reported in the companion paper (Delclos *et al.*, 2014).

A goal of these two studies was to evaluate whether life-stage-dependent internal dosimetry of estrogenic compounds could be used to evaluate critically the hypothesis that aberrant estrogenic signaling during critical developmental windows could manifest as phenotypical changes in the adult animal. This goal is important for human risk assessment because consistency between internal dosimetry, receptor-mediated signaling, and demonstrably adverse effects in animal models would facilitate the interpretation of estrogenic mechanisms in the endocrine disruption debate.

EXPERIMENTAL

Materials

All high pressure liquid chromatography (HPLC) solvents including water were Optima LC/MS grade purchased from Fisher Scientific (Pittsburgh, PA). Native BPA, EE2, β-glucuronidase/sulfatase (*Helix pomatia*, H1, 16 units/mg), testosterone, and all other chemical reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO). The isotopically labeled internal standards $^{13}C_{12}$-BPA, $^{13}C_6$-E2, and d4-EE2 were obtained from Cambridge Isotope Labs (Andover, MA). The d6-BPA (99.5 atom%) was obtained from CDN Isotopes (Pointe-Claire, Quebec). The native and d3-testosterone (99.5 atom%) were purchased from Sigma-Aldrich Co. The unlabeled BPA-glucuronide (BPA-G) and $^{13}C_{12}$-BPA-G were kind gifts from the National Toxicology Program. Sprague Dawley rat serum (unfiltered) was purchased from Bioreclamation LLC (Westbury, NY; lot no. 187248) and analyzed using LC/MS/MS. Only uncontaminated lots of serum [i.e., BPA levels below limit of blank (LOB)] were used subsequently as negative control samples and for spiking with known amounts of authentic standards. Certified reference standards of frozen male and female human serum were obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD).

Animal Handling Procedures

Procedures involving care and handling of rats were reviewed and approved by the NCTR Laboratory Animal Care and Use Committee. Sprague Dawley rats, obtained from the NCTR colony, were maintained on a soy-free basal diet (irradiated Purina 5K96 pellets; Test Diets, Purina Mills, Richmond, IN).

For the d6-BPA pharmacokinetic study, Sprague Dawley pups were delivered and culled on PND 1 to 3–5 males and 3–5 females per litter to provide one pup per sex from each litter for blood collection at each of the postdose time points (body weights 10.5 ± 0.79 g). Pups were treated with a single gavage dose of d6-BPA in 0.3% CMC at 100 μg/kg bw on PND 4 and blood was collected by cardiac puncture at various times after dosing (untreated controls, 0.25, 0.5, 1, 2, 4, 8, and 24 h). At each postdose time point, groups of four pups (two males and two females) were sacrificed by CO_2 asphyxiation after the dose administration. The concentration of the dosing solution was verified immediately prior to dosing by LC/MS/MS analysis to be within 10% of target concentration (data not shown).

In the 90-day subchronic study, pregnant Sprague Dawley rats were exposed by daily gavage starting on gestation day (GD) 6 until the initiation of labor. Direct daily gavage dosing of pups continued with starting on postnatal day (PND) 1 through termination of the study. The 13 treatment groups in this study included a vehicle control of 0.3% carboxymethyl-cellulose in water (CMC), an untreated group (naïve control), two reference estrogen groups (ethinyl estradiol, EE2, at 0.5 and 5.0 μg/kg bw/day), and nine BPA doses (2.5, 8, 25, 80, 260, 840, 2700, 100,000, and 300,000 μg/kg bw/day). The PND 80 females were staged to be in estrus at the time of blood collection, which occurred within a time period of 7:30–11:00 A.M. The pup body weights for the respective studies were: PND 4 dosimetry, 10.8 ± 3.1 g; PND 21 dosimetry, 53.5 ± 5.8 g; PND 80 dosimetry, 361.0 ± 102.2 g. Total serum volumes collected varied but were typically 100–200 μl at PND 4 and >200 μl for PND 21 and PND 80.

The PND 4 and PND 21 blood samples were collected by cardiac puncture using a 1 ml tuberculin syringe with a 25 G × 5/8″ needle (BD, Franklin Lakes, NJ, no. 309626), and the PND 80 blood samples were collected from the lateral tail vein using a SurSaver 1 ml syringe with 23 G × 1/2″ fixed needle (Terumo Medical Corporation, Elkton, MD, no. SS01D2313). PND 4 blood samples were transferred into Fisherbrand disposable culture tubes, lime glass, 6 × 50 mm (Fisher Scientific, no. 14-958-A), whereas PND 21 and PND 80 blood samples were collected into Fisherbrand disposable culture tubes, borosilicate glass, 12 × 75 mm (Fisher Scientific, no. 14-961-26). In all cases, serum was transferred from the glass tube to an Eppendorf Safe-lock polypropylene tube (Eppendorf) using a Fisherbrand Pasteur pipette, 53/4 disposable, borosilicate glass (Fisher Scientific, no. 13-678-20B). The syringes, needles, polypropylene Eppendorf tubes, and all glass tubes used were evaluated for contamination by rinsing with either methanol or water (1 ml), to which was added a known amount of $^{13}C_{12}$-BPA internal standard (1 ng) before analyzing for BPA using LC/MS/MS as described below. The syringes, needles, glass, and polypropylene tubes selected for use in blood collection and processing were found to contain levels of BPA at or below the daily LOB (see below). It should be noted that these procedures did identify consistent contamination in rinsates from tubes that were sub-

sequently rejected: 0.1–14 ng/ml in 2.0 ml screwcap cryo-vials (United Laboratory Plastics, St. Louis, MO, no. UP 2031); and 0.2–1.2 ng/ml in CryoElite vials, Wheaton, Millville, NJ, part no. 985868; 0.1–13 ng/ml in Microtainer tubes (BD, Franklin Lakes, NJ, no. 365957); 0.2–1.2 ng/ml in Sample cup blue Roche-USA, part no. 06789).

The volume of blood collected from PND 4 pups was often limited and the numbers of pups with adequate sample volumes for analysis of both aglycone and total BPA varied but were between 4 and 10 per sex, with roughly balanced numbers of males and females in each dose group. Based on the initial PK study where C_{max} was determined to be 0.25 h for aglycone d6-BPA and 1 h for total d6-BPA, blood was collected in the time window between 0.25 and 0.75 h postdosing as required to accommodate the large numbers of pups being treated. The sampling times were recorded, and the mean was 0.47 ± 0.15 h on PND 4, 0.53 ± 0.17 h on PND 21, and 0.43 ± 0.03 h on PND 80. Blood samples were allowed to clot at room temperature, serum was obtained by centrifugation, and samples were stored at –80°C until analyzed.

Input media that could potentially contribute background native BPA exposure to the rats were evaluated. Analysis of BPA in the diet, CMC vehicle, drinking water, bedding, and cage materials is detailed in the companion paper (Delclos *et al.*, 2014). A post hoc evaluation of other possible sources of exposure within the animal rooms was conducted to see whether aerosol/dust distribution could contribute. In a subsequent ongoing BPA dosing study, replicate cages fitted with sealed isolator tops were sampled using cotton swabs by rubbing over the entire surfaces, either interior or exterior, from control, 250 μg BPA/kg bw/day and 25,000 μg BPA/kg bw/day dose group cages. The replicate cages were selected from shelves located on the top of the cage rack, the middle, and the bottom ($n = 3$ per dose group). The used cotton swab was inserted directly into a silanized glass vial and eluted using methanol (1 ml total volume), to which was added a known amount of $^{13}C_{12}$-BPA internal standard (1 ng) before analysis using LC/MS/MS as described below. The respective control/medium/high dose group cage mean total swab levels ($n = 3$) samples on the inside were 0.1, 0.1, and 0.6 ng BPA and on the outside 0.2, 0.4, and 2.3 ng total. The methanol blank swab contained 0.2 ng total and the blank mean + 2 SD was subtracted from each of the cage values. Levels of BPA glucuronide (BPA-G) were similarly evaluated and only the inside swab from the 25,000 μg/kg bw/day dose group cages contained above 0.1 ng (0.06 ng BPA-equivalent). The methanol blank swab contained 0.1 ng BPA-G that was subtracted from each of the cage values.

Pharmacokinetic Analysis

Plots of serum concentrations of total and aglycone d6-BPA versus time following gavage administration to PND 4 rats were analyzed using model-independent pharmacokinetic analysis (PK Solutions 2.0 software, Summit Research Services, Montrose, CO). Log-linear plots of group mean time point values were fit to three kinetic phases corresponding to elimination, distribution, and absorption. The first-order rate constants were determined from the slope of the respective phases after subtracting the contribution from the terminal elimination phase of the respective curve (i.e., feathering). Internal exposures to BPA were determined either as $AUC_{0-\infty}$, determined by using the trapezoidal rule, or as C_{max}, which was determined by visual inspection and occurred at T_{max}.

Statistical Analysis

Pharmacokinetic parameters for d6-BPA from PND 4 rats were determined from plots of mean values at each time point ($n = 4$, two males and two females). Serum concentration measurements for each native BPA dose group were comprised of roughly equal numbers of males and females and because no significant pharmacokinetic sex differences were observed previously (Doerge *et al.*, 2010a), the sexes were combined. Data are expressed as means ± standard deviation (SD).

LC/MS/MS Quantification Methodologies

Native (unlabeled) BPA aglycone. The $^{13}C_{12}$-BPA internal standard was validated as previously described (Twaddle *et al.*, 2010). LC and tandem MS parameters for online column switching analysis of native BPA are those reported previously (Teeguarden *et al.*, 2011). Calibration curves were linear over the ranges of 0 to 300/3000/300,000 ng/ml (1.3μM/13μM/1.3mM), based on the serum volume used, with a slope of 1.02. Pools of control rat serum and incurred rat serum were prepared for use, in duplicate, as daily quality control checks. In addition, four enzyme blanks (*H. pomatia* glucuronidase/sulfatase mixture) or four aglycone procedural blanks were also prepared with each sample set to establish background BPA levels from sample preparation (i.e., LOB). The method was validated over two days using negative control serum (i.e., BPA < LOB), spiked negative control serum, and incurred serum. The use of the incurred serum that contained both aglycone and conjugated BPA was used to validate completeness of enzyme hydrolysis for analysis of total BPA. Overnight incubation (>16 h) at 37°C was used throughout to ensure total enzymatic hydrolysis even though hydrolysis was typically complete after times <4 h. Control rat serum was spiked at 1.0 and 1.8 ng/ml (2.3 and 7.9nM) for analysis of aglycone and total BPA, respectively, in 100 μl serum samples. For 10 μl serum samples, control serum was spiked at 10 ng/ml, and for total BPA analysis an incurred serum sample containing 30 ng/ml was used.

Method validation and quality control. The validation of the online column switching LC/MS/MS method was reported previously (Teeguarden *et al.*, 2011). Measurable responses for BPA were observed in all procedural blanks because trace level contamination by native BPA is difficult to avoid (Ye *et al.*, 2013; Teeguarden *et al.*, 2011; Twaddle *et al.*, 2010). Accordingly, four replicate procedural blanks were analyzed with each

sample set to determine a daily LOB. These samples replaced serum with water and went through the entire sample preparation process. The LOB was defined as the mean value + 2 SD of the replicates and the daily LOB was subtracted from each serum sample concentration (with enzymatic hydrolysis, 0.5–1.8nM; without enzyme, 0.3–1.1nM). In addition, daily limits of detection (LOD) were estimated from the amount of BPA producing a signal/noise ratio >3 above the LOB (with enzymatic hydrolysis, 0.2–1.1nM; without enzyme, 0.1–0.4nM). If the sample quantification value after subtraction of the LOB was not higher than the daily calculated LOD, it was reported as <LOD. Validation was performed on both 10 and 100 μl aliquots of serum. Intra- and interday precision ranged from 0.6 to 5.3% RSD. Intra- and interday accuracy ranged from 98 to 105%.

d6-BPA. Analysis of d6-BPA was performed by using a validated high throughput LC-ES/MS/MS method as previously described (Twaddle *et al.*, 2010). Briefly, labeled internal standard ($^{13}C_{12}$-BPA) was added to each thawed serum sample (10–100 μl), then samples were purified using supported liquid extraction in 96-well plates and analyzed using LC-ES/MS/MS in the multiple reaction monitoring mode of specific negative ion transitions for d6- and $^{13}C_{12}$-BPA. Total serum concentrations of d6-BPA (i.e., aglycone + conjugates) were quantified following incubation with a *H. pomatia* glucuronidase/sulfatase mixture. Special precautions, similar to those previously published (Twaddle *et al.*, 2010), were used to minimize adventitious hydrolysis of conjugates during sample preparation and no evidence for hydrolysis was observed. Method validation included replicate analysis of spiked serum at levels from 0.4 to 40nM with accuracy and precision ranging from 89 to 105% and 2.8 to 18%, respectively, as previously described (Twaddle *et al.*, 2010). The method detection limit (LOD) for d6-BPA, defined as a signal/noise ratio of greater than or equal to 3, was approximately 0.2nM (0.05 ng/ml) in 100 μl serum. When calculating group mean values, samples <LOD were replaced with a value of 1/2LOD (0.1nM).

BPA-glucuronide. The standard method of quantifying conjugated BPA as the aglycone after an enzymatic hydrolysis step (i.e., indirect) was confirmed by developing and validating an LC-ES/MS/MS method for direct quantification of the major BPA metabolite in rats, BPA-G, using an isotopically labeled internal standard. The purity of isotopically labeled $^{13}C_{12}$-BPA-G internal standard and unlabeled BPA-G was characterized by liquid chromatography with ultraviolet detection (LC-UV) (280 nm) and full-scan LC-ES/MS/MS. The transitions and voltages used to analyze labeled and unlabeled BPA-G are listed in Supplementary table 1. The labeled BPA-G was found to contain 0.02% unlabeled BPA-G and 0.01% $^{13}C_{12}$-BPA. The time course (1–16 h) and extent of enzymatic hydrolysis of the unlabeled BPA-G (5–5.7 ng/ml, 12–14nM) were evaluated in spiked negative control serum and water using the procedures described previously (Twaddle *et al.*, 2010). By 1 h, the reaction was ~90% complete, and by 16 h (the overnight incubation time used throughout), 94 ± 1.2 to 102 ± 1.9% (mean ± SD, $n = 3$ at each time point) of the expected BPA was observed on two separate days of analysis. The acetonitrile extraction and online LC/MS/MS procedures used were modifications of those described above for native BPA (not shown) and the method was validated using negative control rat serum for spiking and blank determinations. BPA-G was <LOD in the rat serum sample tested, and serum spiked at 1 ng/ml (2.5nM) showed 101 ± 2.7% recovery ($n = 20$ over two separate days). The LOD for 100 μl serum was approximately 0.4-0.6 nM.

E2 and EE2

Preparation of standards and quality control samples. The purities of labeled E2 and EE2 were evaluated by LC-UV (220 nm detection) and full scan LC/MS (negative ion ES detection). Labeled EE2 and E2 contained undetectable levels of the unlabeled compound (<0.04%). Concentrations of labeled and unlabeled E2 and EE2 solutions were matched by using LC-UV (220 nm detection). Calibration curves were generated by derivatizing varying concentrations of unlabeled E2 and EE2, while keeping the internal standard concentrations constant. The curves were linear over the ranges of 0–1000 pg/ml with slopes of 0.97 and 1.15 for E2 and EE2, respectively. Pools of negative control rat serum and negative control rat serum spiked at 10 pg/ml were prepared for use as daily quality control checks.

Sample preparation. Published procedures using liquid-liquid extraction and dansyl chloride derivatization were adapted for the analysis of E2 and related phenolic compounds (Kushnir *et al.*, 2008 ; Xiong *et al.*, 2010; Xu *et al.*, 2007). Briefly, 100 μl of serum plus 10 μl internal standards were extracted with an equal volume of methyl-*t*-butyl ether (MTBE), the organic layer was separated using a glass pipet, transferred to a 96-well plate, and then evaporated to dryness. The residue was derivatized with dansyl chloride (1 mg/ml in acetone) and sodium bicarbonate (0.1M in water) by heating at 60°C for 10 min. A 20 μl aliquot was injected into the UPLC column. For analysis of total E2 and EE2, serum was first enzymatically deconjugated at 37°C overnight with β-glucuronidase/sulfatase (3.2 units). Then 200 μl of water was added to each sample and the samples were extracted using supported liquid extraction plate with MTBE as the elution solvent (Biotage, Charlotte, NC). The MTBE was evaporated and the samples were derivatized as described above.

Liquid chromatography was conducted with an Acquity UPLC system (Waters, Inc., Milford, MA), a 1260 Infinity HPLC pump (Agilent, Santa Clara, CA), and an automated six port switching valve (Rheodyne, Cotati, CA). The switching valve was used to divert the HPLC column effluent either to waste or the mass spectrometer. The Agilent pump was used to provide continuous flow to the mass spectrometer when the column effluent was diverted to waste. The analytes were sepa-

rated using a Luna Phenyl-Hexyl column (2.0 × 150 mm, 3 μm particle size, Phenomenex, Torrance, CA). The mobile phase consisted of 0.01% formic acid in both water and acetonitrile. The gradient program consisted of 60% acetonitrile for 2 min and then a linear gradient up to 95% acetonitrile from 2 to 12 min. The column was held at 95% acetonitrile for an additional 3 min before resetting to starting conditions. The total run time was 19 min and the flow rate was 0.3 ml/min with a column temperature of 30°C. The analytical column was inline with the mass spectrometer from 7 to 9.7 min and diverted to waste for all remaining time. The Agilent pump ran isocratically with 0.01% formic acid in 20% water and 80% acetonitrile at 0.3 ml/min. An Acquity CSH Phenyl-Hexyl column (2.1 × 100 mm, 1.7 μm particle size, Waters, Inc.) was used for separation of enzymatically deconjugated samples. The gradient program consisted of 55% acetonitrile for 2 min followed by a linear gradient up to 75% acetonitrile from 2 to 7 min and then stepped up to 95% acetonitrile. The column was held at a temperature of 50°C and eluted with 95% acetonitrile for an additional 3 min before resetting to starting conditions. The total run time was 13.5 min. The flow rate was 0.5 ml/min for 2 min and then a linear flow gradient from 0.5 to 0.55 was used over the 2 to 7 min time period. The analytical column was inline with the mass spectrometer from 5.3 to 7 min and diverted to waste for all remaining time. The Agilent pump ran isocratically with 0.01% formic acid in 20% water and 80% acetonitrile at 0.5 ml/min.

A Xevo TQ-S triple quadrupole mass spectrometer (Waters, Inc.) equipped with an electrospray interface was used in selected reaction monitoring mode for analysis of positive ions. The transitions monitored for E2 and EE2 and the corresponding voltages and energies are shown in Supplementary table 1. Other MS parameters included source and desolvation temperatures of 150°C and 500°C, respectively, argon as collision gas (0.15 ml/min) and nitrogen as the desolvation (1000 l/h) and cone gas (150 l/h).

Method Validation and Quality Control. Various extraction, cleanup, and separation parameters were optimized for quantification of E2 and EE2 in serum. The methods were validated for 100 μl aliquots of negative control rat serum over two days using replicates of commercial charcoal-stripped male rat serum, spiked at two levels (10 and 100 pg/ml), pooled control serum, and spiked pooled control serum (10 pg/ml). Intra- and interday precision was expressed as relative standard deviation (RSD), which ranged from 2.1 to 13% for E2 and from 0.6 to 5.5% for EE2. Intra- and interday accuracy ranged from 83 to 94% for E2 and from 89 to 106% for EE2. The LOD for both E2 and EE2 was 1.5 pg/ml (5pM). When calculating group mean values, samples <LOD were replaced with a value of 1/2LOD (0.8 pg/ml). Levels of EE2 were determined in control rats (combined naïve and vehicle-treated) and EE2 dosed rats with group sizes of five males and five to seven females at each age. Levels of E2 were determined from control rats (combined naïve and vehicle-treated) with group sizes of 10 males and 10–14 females at each age.

Testosterone

Testosterone was analyzed by isotope dilution LC/MS/MS using procedures based on those published previously (Hsing *et al.*, 2007). Labeled testosterone contained undetectable levels of the unlabeled compound (<0.04%). Concentrations of labeled and unlabeled testosterone solutions were matched by using LC-UV (260 nm detection). Calibration curves were generated by varying the concentrations of unlabeled testosterone, although keeping the internal standard concentration constant. The transitions and voltages used to analyze labeled and unlabeled testosterone are listed in Supplementary table 1. Calibration curves were linear over the ranges of 0 to 1000 pg/ml with a slope of 1.0. Pools of control female rat serum, incurred serum containing 0.77 ng/ml, and control rat serum spiked at 5 ng/ml were prepared for use as daily quality control checks. Method validation included analysis of incurred and spiked female serum samples on two separate days and the analysis of standard reference materials (NIST frozen male and female human serum containing 6.43 and 0.28 ng/ml, respectively). Accuracy was 90% for the low range and 95% for the high range NIST standards and intra- and interday precision were 2.7–3.8% RSD on three separate days. The LOD for analysis of 100 μl serum was approximately 0.05 ng/ml (0.17nM). When calculating mean values, samples containing <LOD were replaced with a value of 1/2LOD (0.025 ng/ml).

RESULTS

Pharmacokinetics of Oral d6-BPA in PND 4 Rats

The serum concentration-time profiles for total and aglycone d6-BPA following gavage administration of 100 μg/kg bw to PND 4 pups are shown in Figure 1. Aglycone d6-BPA concentration was maximal at the initial sampling point (C_{max}, 14nM at $T_{max,}$15 min), whereas the T_{max} for total d6-BPA was reached at 1 h. At T_{max} (15 min), aglycone d6-BPA represented 4.9% of the total. The $AUC_{0-\infty}$ for aglycone d6-BPA was 26.3 nmol × h × l^{-1}, which represented 0.97% of the total AUC (2698 nmol × h × l^{-1}). The half-time for absorption, distribution, and terminal elimination of aglycone d6-BPA were 0.15, 2.6, and 4.1 h, respectively.

Analysis of BPA in Serum from Vehicle and Naïve Control Rats

Analysis of serum samples from naïve and vehicle control rats of all ages consistently contained measurable levels of native aglycone BPA above the respective daily LOB with mean values in the range of 0.1–2nM (0.02–0.5 ng/ml; Figs. 2–4). The mean percentages of total BPA present as aglycone in the control serum samples (17–58%) exceeded those measured under controlled conditions in the oral PK study with d6-BPA (i.e., maximally 4.2% at C_{max} in PND 4 pups; Fig. 1) and are best

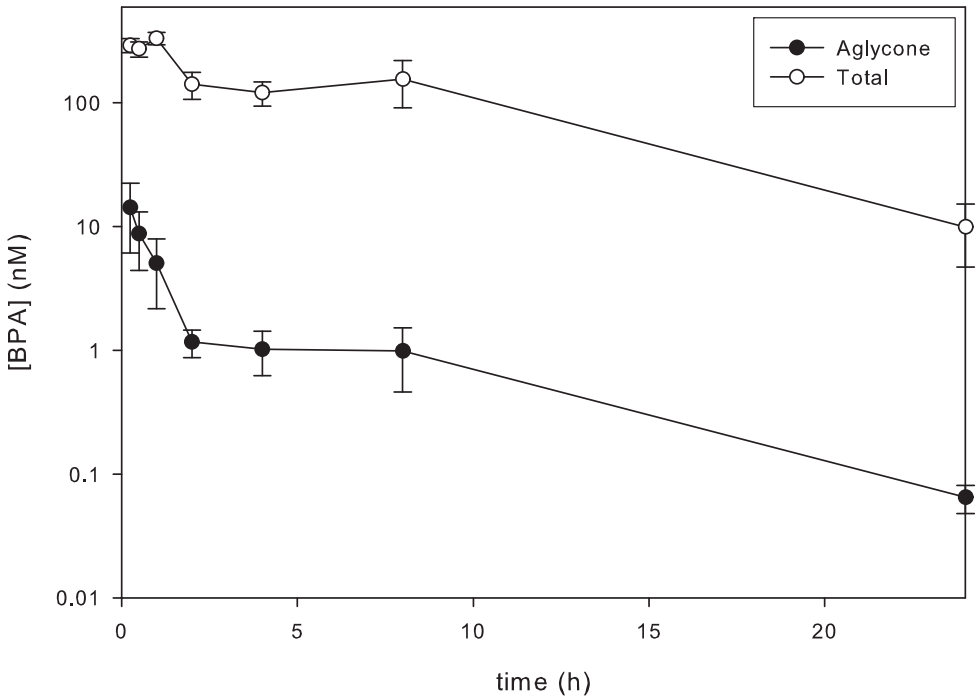

FIG. 1. Serum concentration-time profiles for total and aglycone d6-BPA in PND 4 Sprague Dawley rats (mean ± SD, $n = 4$ pups at each time point) treated by gavage with a single dose of 100 μg d6-BPA/kg bw in 0.3% carboxymethylcellulose in water.

interpreted as resulting from contamination by native BPA during the sample collection and storage. The background levels were consistently higher in PND 4 and PND 21 pups than in the PND 80 adults, presumably because of the different blood withdrawal procedures and the volumes of blood collected (cardiac puncture in young rats and tail vein withdrawal in adults). The evidence for sample contamination by native BPA despite evaluation of all blood handling materials prior to sample collection underscores the difficulty of preventing contamination of serum by native BPA because of numerous sources in laboratories (e.g., dust, plastic blood collection, and laboratory products; Twaddle *et al.*, 2010; Ye *et al.*, 2013). As a result, measurements of aglycone BPA were often foregone in favor of using the limited sample volumes available for analysis of total BPA in rats receiving the 2.5, 8, and 25 μg/kg bw/day doses. This strategy was deemed appropriate because extrapolation from d6-BPA pharmacokinetic studies (Fig. 1 and Doerge *et al.*, 2010a) showed the peak aglycone levels in serum (0.14nM per μg/kg bw dose unit in PND 4 rats) would be below those measured in the controls (Supplementary fig. 1). The levels of total BPA in naïve and vehicle control rats were consistently observed in the 1–10nM range (Figs. 2–4). Because the postexposure contamination of the blood samples by aglycone BPA made interpretation of total BPA levels difficult, it was deemed important to verify conjugated BPA levels directly as BPA-G. A high degree of concordance between the two methods was observed through paired sample comparisons of conjugated BPA (total − aglycone) versus BPA-G (e.g., in PND 21 controls, 105 ± 10%, $n = 16$; PND 80 controls, 100 ± 25%, $n = 13$). Small sample volumes precluded a similar comparative analysis for PND 4 serum. This concordance is also consistent with previous analyses using purified β-glucuronidase and sulfatase enzymes separately, which showed that BPA-G was the predominant BPA conjugate in rats (Doerge *et al.*, 2010a).

Total BPA exposures in naïve and vehicle control rats produced blood concentrations of BPA-G similar to those observed from dosing with the two lowest doses (2.5 and 8 μg/kg bw/day, Figs. 2–4). Although the low level of BPA contamination measured in the basal diet (<5 ppb) would contribute to total native BPA in serum, this was deemed unlikely to contribute significantly. Daily consumption of as much as 5 μg BPA/kg diet at a rate of approximately 15% of body weight, even as a bolus, would result in <1 μg/kg bw contribution associated with a predicted 5pM increment at peak serum concentration based on pharmacokinetics in previous studies in PND 21 and adult rats (0.4–0.7nM for 100 μg/kg bw given by bolus gavage; Doerge *et al.*, 2010a). Potential exposure to BPA from the vehicle, cage materials, bedding, and drinking water was also carefully evaluated and found to be minimal (Delclos *et al.*, 2014), and the additional source(s) of exposure has not been identified.

Analysis of Serum from BPA-Treated Rats

Increases in serum levels for both aglycone and total BPA above levels measured in controls were observed for all ages of rats tested above approximately 80 μg/kg bw (Figs. 2–4).

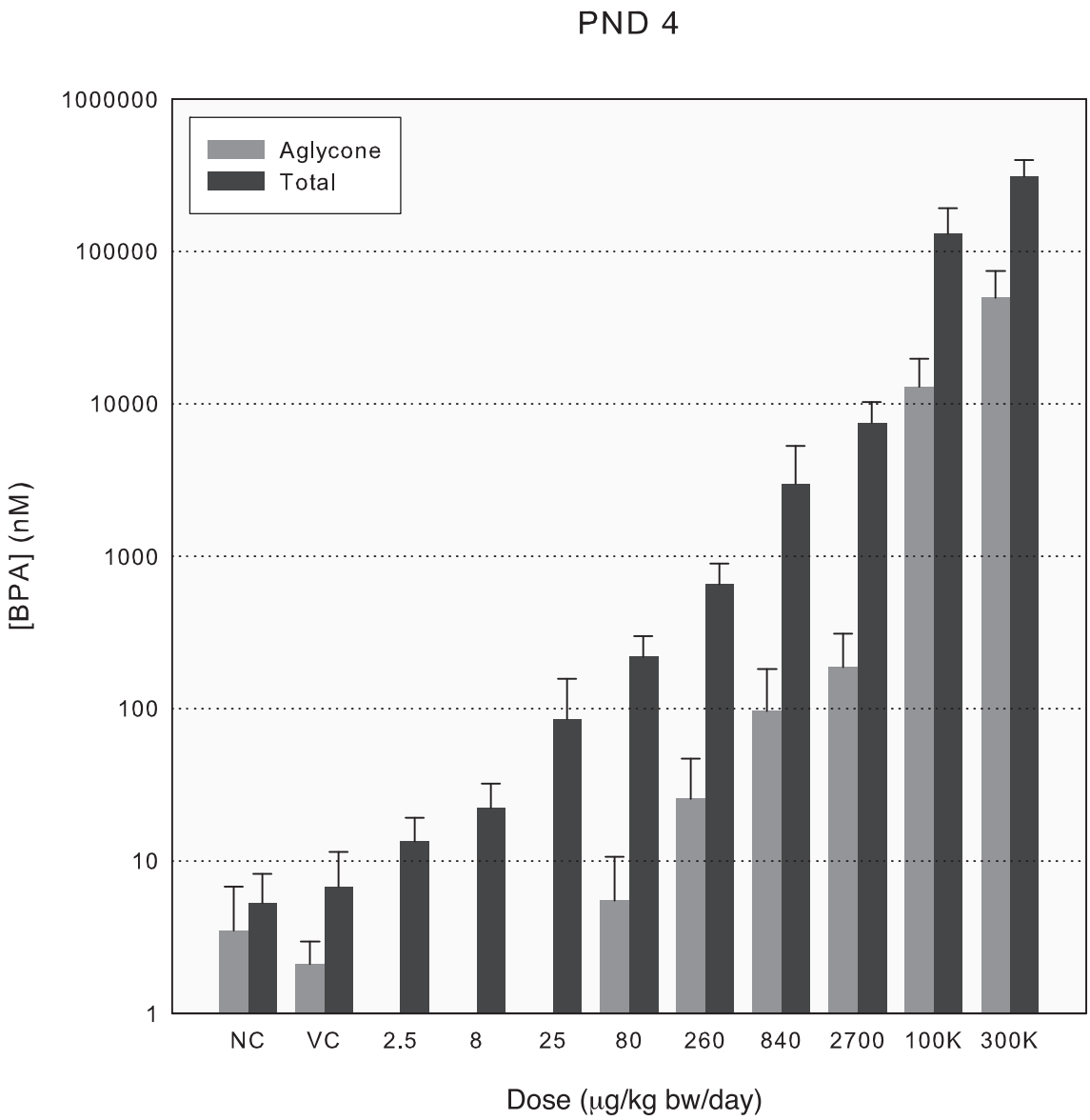

FIG. 2. Aglycone and total BPA serum concentrations at C_{max} across treatment groups for PND 4 rats. Values shown by treatment group are means of either total or aglycone BPA ± SD for dosed males and females, combined ($n \geq 8$). Aglycone BPA was not quantified in 2.5–25 μg/kg bw dose groups.

Although differences in levels of aglycone BPA observed in PND 21 and PND 80 rats were minimal, levels in PND 4 pups were consistently higher in accordance with the immaturity of phase II metabolism of BPA previously found in rodents, but not nonhuman primates (Doerge *et al.*, 2010a; Yang *et al.*, 2013). The fraction of total BPA present as aglycone was evaluated for doses above 80 μg/kg bw, where the impact of contamination was minimal, to evaluate further the possible impact of dose on phase II metabolism. The aglycone fraction was higher in PND 4 pups and appeared to diverge from the ∼4% range to >10% at the two highest doses (Supplementary fig. 1). No deviation from the 1–2% aglycone fraction was seen in PND 21 and PND 80 rats except for the highest dose of BPA in PND 21 rats.

The peak levels of aglycone and total d6-BPA determined under similar dosing conditions in the pharmacokinetic study for PND 4 pups (Fig. 1) and our previous evaluation of C_{max} for d6-BPA gavage on PND 21 and PND 90 (Doerge *et al.*, 2010a) made it possible to compare measured and simulated serum levels for all doses for newborn, weanling, and adult rats (Supplementary figs. 1–3).

Analysis of Serum from EE2-Treated Rats

Serum levels of EE2 in male and female PND 4, PND 21, and PND 80 rats were determined in controls, 0.5 and 5 μg EE2/kg bw-treated rats using identical sample collection to that described for the BPA-treated rats ($n = 5$–7 per sex per group).

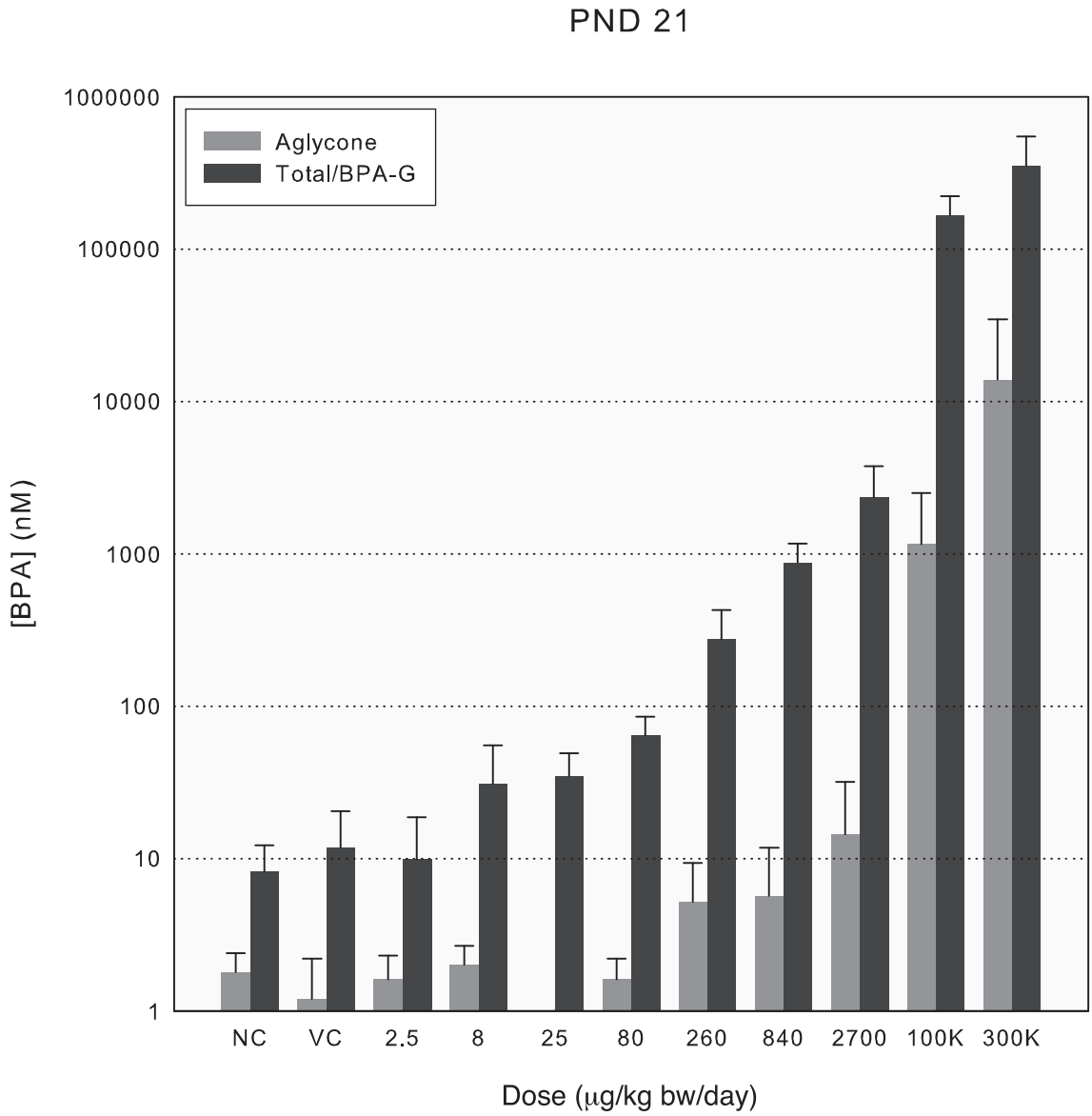

FIG. 3. Aglycone and total BPA/BPA-glucuronide serum concentrations at C_{max} across treatment groups for PND 21 rats. Values shown by treatment group are means of either total or aglycone BPA ± SD for dosed males and females, combined ($n \geq 8$), and BPA-glucuronide combined means ± SD for naïve and vehicle control groups (NC, $n = 8$ and VC, $n = 8$, respectively). Aglycone BPA was not quantified in the 25 μg/kg bw dose group.

Because there were no significant differences between levels in male and females (*t*-test), the values shown in Figure 5 are combined by treatment group. Serum levels of EE2 in vehicle and naïve control rats of all ages were consistently below the LOD (5pM or 1.5 pg/ml, Fig. 5). As seen with BPA, EE2 levels were far higher in PND 4 and decreased successively on PND 21 and PND 80. The effect of age on the degree of conjugation was evaluated in a subset of samples by comparing EE2 levels before and after total enzymatic deconjugation. On PND 4, 100% of EE2 was present unconjugated, on PND 21, 50% was unconjugated, and on PND 80, 10% was unconjugated (not shown).

Endogenous Steroid Hormone Levels

The derivatization method for E2 produced a level of analytical sensitivity that was useful for evaluating life-stage-dependent changes in serum levels in Sprague Dawley rats using the naïve and vehicle control groups. Levels of E2 in males and females were greater than LOD (1.5 pg/ml, 5pM) in all but newborn females and adult males (Fig. 6). E2 was measured in male pups at PND 4 and PND 21, but was undetectable in adult male rats at PND 80 and PND 180 (not shown). In females, E2 was <LOD on PND 4, and similar at PND 21 and PND 80. Enzymatic deconjugation studies showed that E2 was present exclusively in the unconjugated form.

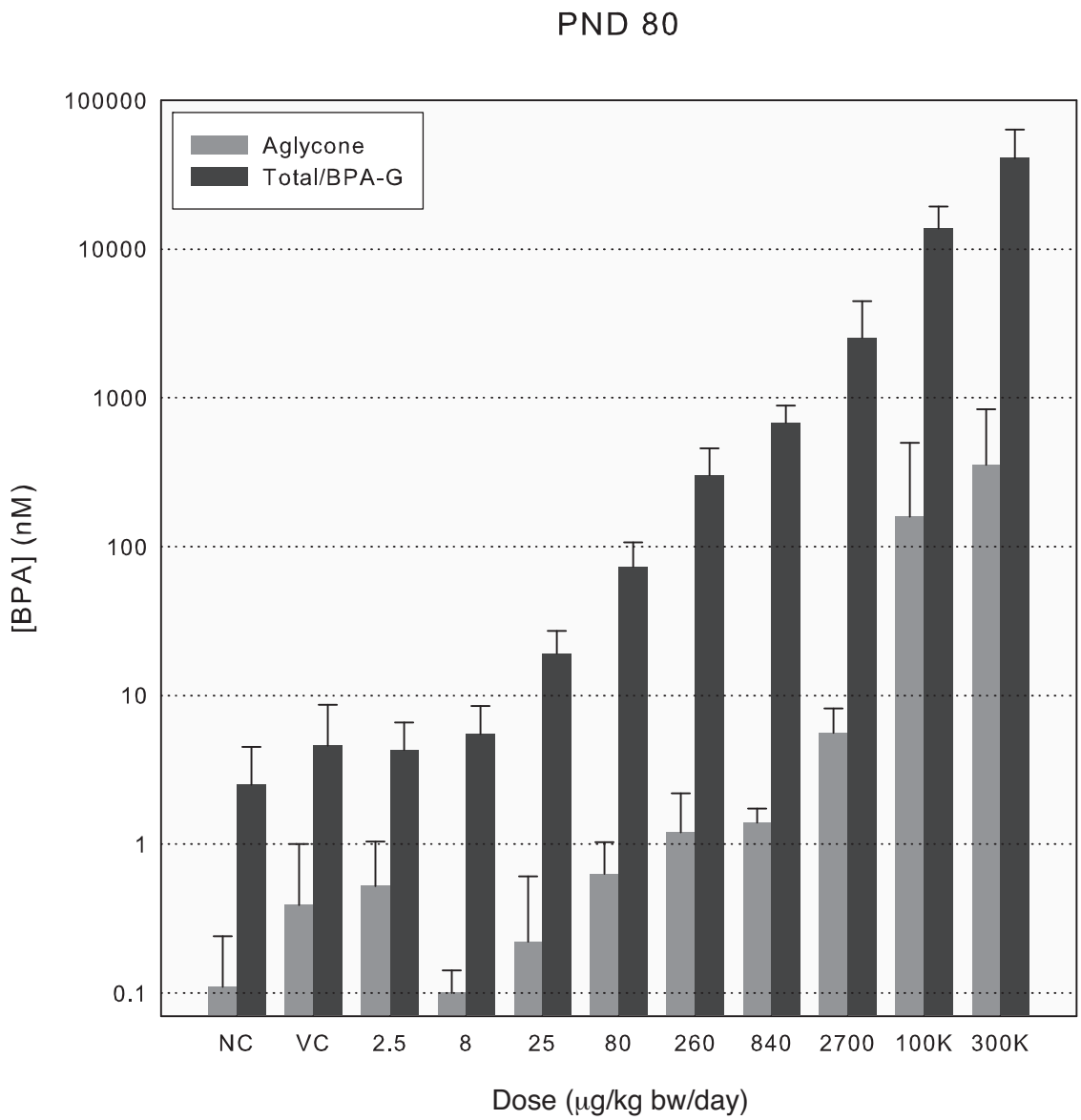

FIG. 4. Aglycone and total BPA/BPA-glucuronide serum concentrations at C_{max} across treatment groups for PND 80 rats. Values shown by treatment group are means of either total or aglycone BPA ± SD for dosed males and females, combined ($n \geq 8$), and BPA-glucuronide combined means ± SD for naïve and vehicle control groups (NC, $n = 17$ and VC, $n = 15$, respectively).

Testosterone levels were measured in rats at different ages (Supplementary fig. 4). Because insufficient serum volumes were available from PND 4 pups, serum was instead collected from pups culled at PND 1. Sufficient serum volumes were available from control groups of PND 21 and PND 80 rats in the BPA study. In addition, serum was collected from older untreated Sprague Dawley rats (PND 100 and PND 180) that were obtained as retired breeders from the NCTR colony. Testosterone levels were detectable in male rats as young as PND 1 and increased until PND 100, with lower levels observed in PND 180 rats. Testosterone levels in female rats were <LOD (0.05 ng/ml, 0.17nM) on PND 1 and increased to measurable levels on PND 21 and PND 80. Testosterone levels in female rats did not exceed those observed in PND 1 males at any age.

The effect of BPA dosing on PND 80 serum hormone levels is described in the companion paper (Delclos *et al.*, 2014). Briefly, mean serum estradiol as measured by radioimmunoassay in females was significantly increased by approximately 67% and 113% in the 100,000 and 300,000 μg BPA/kg bw/day groups, respectively. In males, there were no treatment-related differences for serum testosterone levels as measured by radioimmunoassay.

In order to critically test an estrogenic mode of action for BPA, internal dosimetry of aglycone BPA was compared where

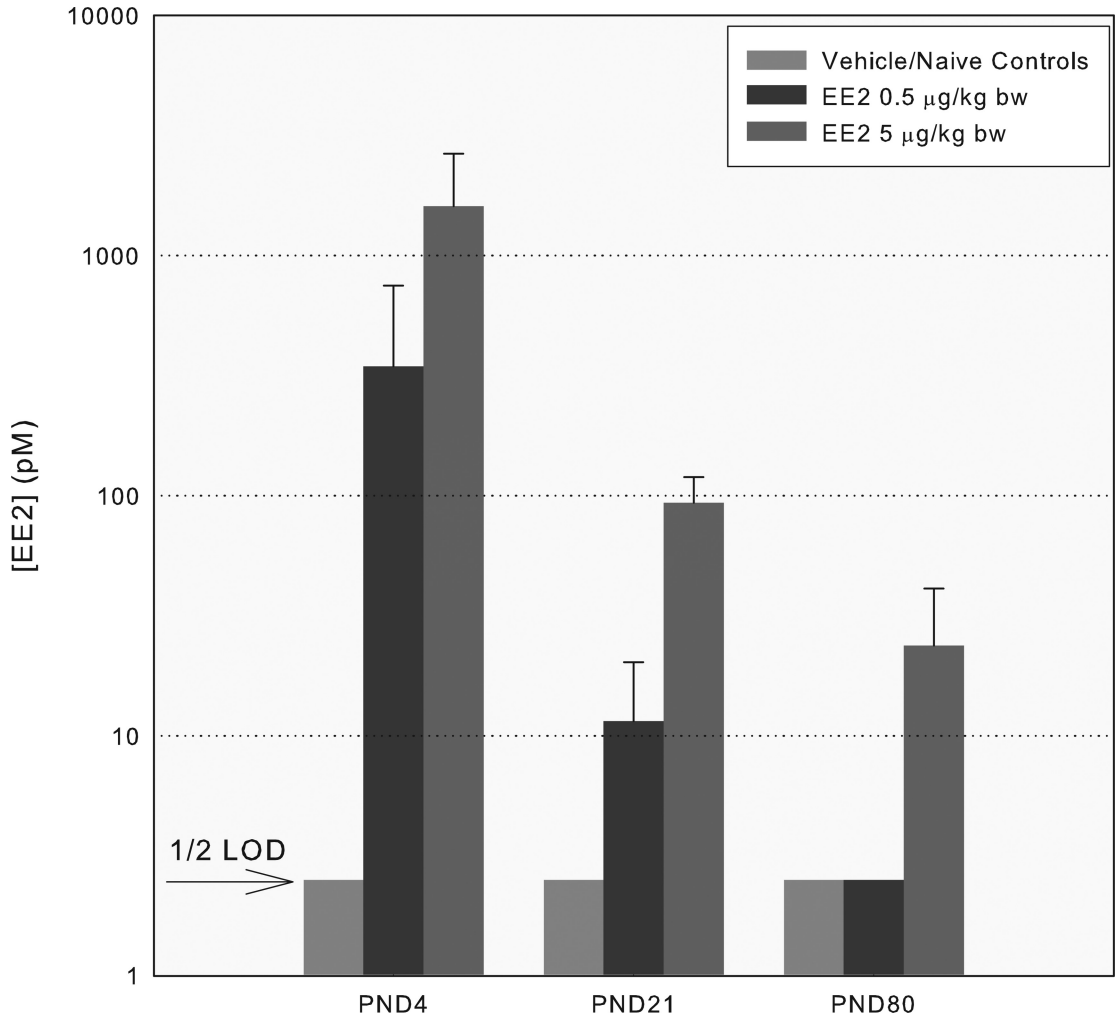

FIG. 5. EE2 C_{max} values following gavage treatment of Sprague Dawley rats at different ages. EE2 concentrations (unconjugated) were quantified using LC/MS/MS from serum collected from male and female rats approximately 30 min after gavage treatment (mean ± SD, $n = 5–7$/sex/group/age) of the designated ages (note: log ordinate scale; LOD 5pM, 1.5 pg/ml).

toxicological effects were observed in estrogen-responsive tissues and overlapped with those produced by the reference estrogen, EE2. As shown in the companion paper (Delclos *et al.*, 2014), only the two highest BPA doses met these criteria. E2-equivalent concentrations for binding to ERα were calculated by adjusting combined male and female group mean C_{max} values determined for the 100,000 and 300,000 μg/kg bw/day BPA dose groups (Figs. 2–4), group mean C_{max} values for both doses of EE2 (Fig. 5), and endogenous E2 levels in either male or female rats (Fig. 6) by using the respective relative ERα ligand binding affinity (1 for E2, 1.2 for EE2, and 0.0002 for BPA) where the K_D for E2 with ERα is 200pM (Gutendorf and Westendorf, 2001). Figure 7 shows the estrogenic internal exposures for each treatment group and life stage.

DISCUSSION

Internal Dosimetry as a Critical Design Element in the Interpretation of Toxicology Studies

A limitation in the design of most BPA toxicity studies, including previous guideline-compliant studies conducted in Sprague Dawley rats (Stump *et al.*, 2010; Tyl *et al.*, 2002), is the lack of evaluation of internal dosimetry, particularly in the "low dose region" (Teeguarden and Hanson-Drury, 2013). The comprehensive evaluation of tissue histopathology and reproductive and developmental endpoints in this subchronic BPA exposure study was matched by comprehensive evaluation of the levels of aglycone (i.e., unconjugated and receptor-active) and conjugated (i.e., inactive) BPA from daily oral administration throughout perinatal and adult life stages. The ages of rats evaluated were determined by sacrifice times chosen to include evaluation of development during early neonatal (PND 4), weaning

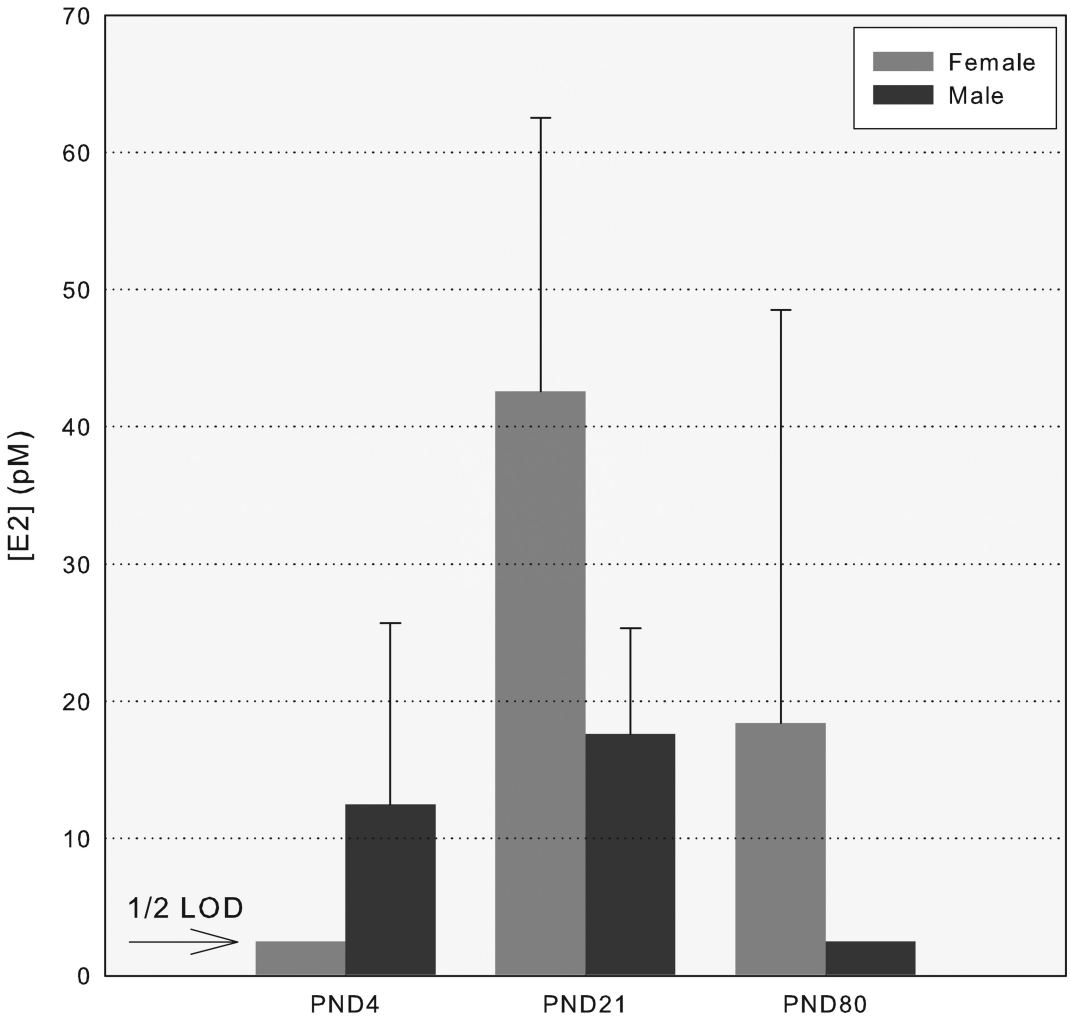

FIG. 6. E2 levels in male and female Sprague Dawley rats at different ages. Serum concentrations of E2 (unconjugated) were quantified using LC/MS/MS (LOD 5pM, 1.5 pg/ml) from naïve and vehicle control rats (mean ± SD, $n = 5$–10/sex/age).

(PND 21), and adult (PND 80) periods. These measurements in this rat model complement previous evaluations of pharmacokinetics of d6-BPA during fetal (GD 12–20), neonatal (PNDs 3–21), and adult (PND 90) life stages in the same rat strain (Doerge *et al.*, 2010a, 2011a), as well as in CD-1 mice (Doerge *et al.*, 2011b), nonhuman primates (Doerge *et al.*, 2010b; Patterson *et al.*, 2013), and PBPK modeling of the rat, monkey, and human data sets (Fisher *et al.*, 2011; Yang *et al.*, 2013). The pervasive postexposure contamination of serum by native aglycone BPA observed in vehicle and naïve control rats required specific evaluation of methodological blanks using commercial rat serum determined to contain undetectable levels of BPA. In addition, the wide range of BPA doses required complementary analytical strategies in order to quantify the resulting serum levels of aglycone and total BPA. Finally, the finding of the glucuronide metabolite of BPA indicated low level unintended exposure in vehicle and naïve control rats, and necessitated an independent method for directly quantifying native BPA-G. The use of two doses of the reference estrogen, EE2, required implementation of additional methodology, which conveniently permitted concurrent evaluation of the principal endogenous estrogen, E2. Testosterone levels were also determined at different life stages. A goal was to compare directly the dose-response for BPA and the reference estrogen, EE2, in the context of normal endogenous E2 levels in males and females at different ages. The overall goal of these measurements was to use internal dosimetry for BPA to interpret the dose-response for any histopathological or clinical chemistry endpoints affected (see Delclos *et al.*, 2014) and to test the hypothesis of estrogen receptor-mediated signaling disruption during critical developmental windows in rats.

Life-Stage Dependence of BPA Pharmacokinetics

The pharmacokinetics of d6-BPA in PND 4 rat pups were evaluated initially because different vehicle (0.3% CMC vs. 10% ethanol in water) and age (PND 4 vs. PND 3) were used than in the previous study of newborn rats, even though the

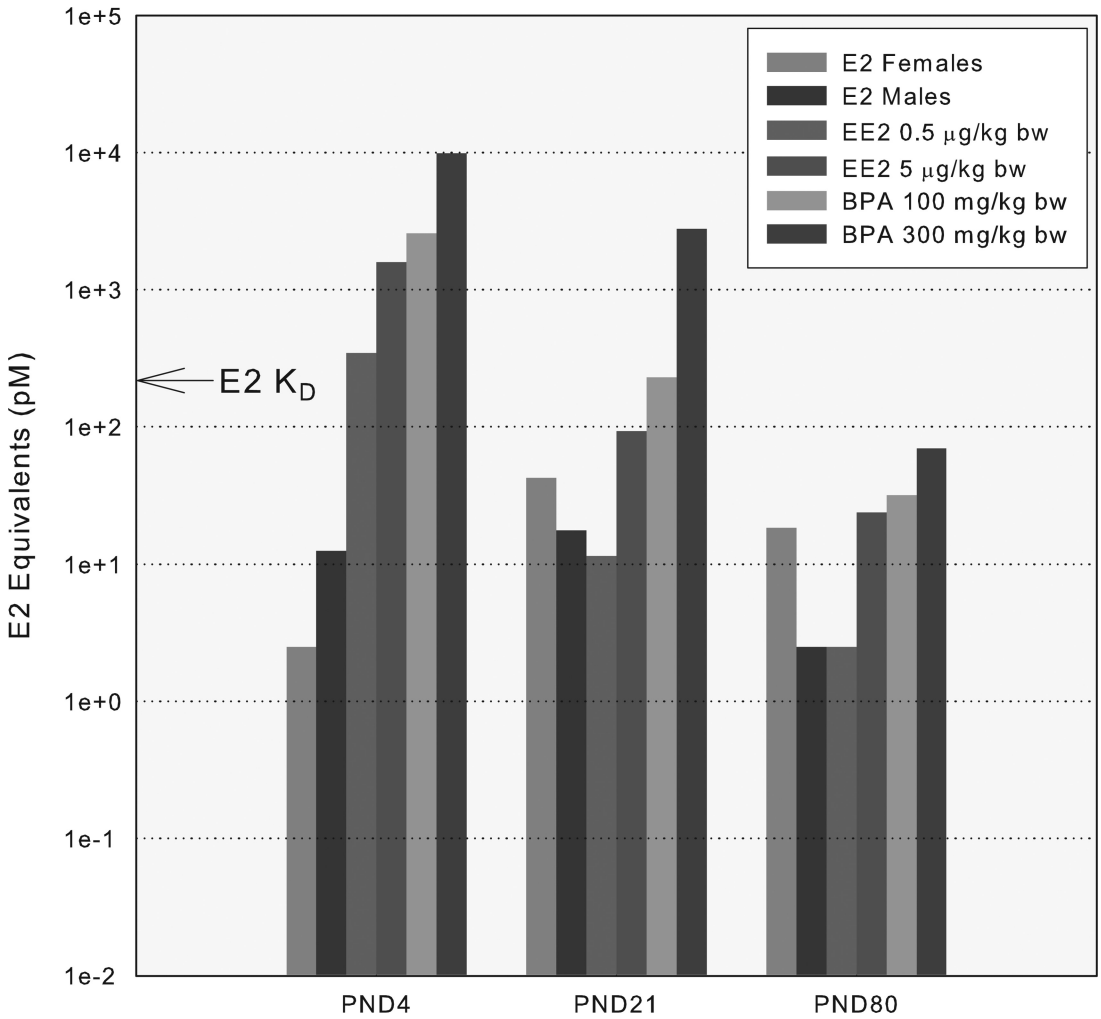

FIG. 7. Comparison of estrogen receptor α (ERα) binding-equivalent serum concentrations of endogenous E2 with EE2- and BPA-treated rats of different ages (see the Results section for description of the process used). The combined male + female C_{max} values for BPA in the 100,000 and 300,000 μg/kg bw treatment groups are shown along with combined C_{max} values for both doses of EE2 and the endogenous E2 values obtained in either male or female pups at various life stages. The K_D for E2 with ERα of 200pM is shown for reference.

same rat strain and dose were used (100 μg/kg bw; Doerge *et al.*, 2010a). The pharmacokinetic parameters for aglycone d6-BPA in PND 4 pups (C_{max}, AUC, and elimination half-time) were intermediate between those reported previously for PND 3 and PND 10, consistent with the age-dependent development of metabolic and excretory function in neonatal rats that combine to decrease internal exposures as maturation proceeds (Doerge *et al.*, 2010a). Because pharmacokinetics of BPA aglycone appeared to be linear with dose up to at least 840 μg/kg bw (Figs. 2–4), dose extrapolation of C_{max} from this controlled exposure was used to simulate levels in PND 4 rat serum from all doses of BPA tested (Supplementary fig. 1). Similarly, C_{max} values from Doerge *et al.* (2010a) were used to simulate BPA levels in PND 21 and PND 90 rats using the range of doses tested here in comparison with the measured values (Figs. 3 and 4 vs. Supplementary figs. 2 and 3).

The large numbers of rat pups being dosed daily during the main study made adherence to a single time point for collection of blood samples impossible, so a time window of 0.25–0.75 h was selected as a practical compromise to evaluate C_{max} for both aglycone and total BPA at the different ages. The mean postdose times at the three ages were in the range of 0.43–0.53 h (see the Experimental section for details). The serum concentrations of EE2 and E2 were also evaluated using the same postdosing time window and were not evaluated specifically for serum concentration-time profiles. The pharmacokinetics of orally administered EE2 in adult female Sprague Dawley rats have been previously reported (Twaddle *et al.*, 2003).

Figures 2–4 show the aglycone and total BPA (i.e., aglycone + conjugated forms) or BPA-G in the vehicle and naïve controls levels at C_{max} using a log scale. Aglycone levels were as much as 1000-fold lower than the corresponding total levels, depending

on age and dose. In PND 4, and to a more limited extent in PND 21 rats, plots of the aglycone fraction of total BPA showed an apparent discontinuity in the higher dose region (Supplementary fig. 5), which suggested that saturation of phase II metabolism in the gastrointestinal tract or liver could be occurring at the two highest doses of BPA. No such discontinuity was observed in PND 80 rats.

Methodological Limitations in the Conduct and Interpretation of BPA Dosimetry

The impact of serum contamination by BPA was evident in measurements of aglycone BPA in vehicle and naïve controls (Figs. 2–4). Measured and simulated levels of aglycone BPA levels in dosed-rat serum did not exceed the levels observed in controls until the 80 μg/kg dose (Figs. 2–4 and Supplementary figs. 1–3). The aglycone fractions were higher than those observed after gavage with doses uncompromised by contamination or saturation of metabolism (<4%, Supplementary fig. 5). These findings were consistent with a significant body of evidence supporting the propensity for contamination of blood by aglycone BPA during sample collection and processing (Markham *et al.*, 2010; Twaddle *et al.*, 2010; Ye *et al.* (2013). This problem was encountered in our measurements of aglycone BPA despite careful prescreening of sample collection apparatus before initiation of the experimentation (see the Experimental section). Because procedural blanks were determined daily and subtracted from each determination, we conclude that the contamination occurred during sample collection and/or storage (i.e., postexposure). It is for this reason that internal dosimetry was independently evaluated by using a "low dose" of isotopically labeled BPA in Figure 1 and previous pharmacokinetic studies, for which environmental contamination is not problematic (Doerge *et al.*, 2010a,b).

The serum concentrations of conjugated BPA, determined in dosed rats after complete enzymatic hydrolysis of conjugated metabolites and subtraction of aglycone, were similar to those predicted from single dose pharmacokinetic studies (Fig. 1 for PND 4, and Doerge *et al.*, 2010a for PND 21 and PND 90 rats). The notable exception was that the concentrations of BPA-G observed in vehicle and naïve control groups were similar to the levels of conjugated BPA observed in the 2.5 μg/kg bw/day groups at all ages tested. These findings are best interpreted as resulting from similar levels of aggregate exposure to BPA, which suggests some unidentified source(s) of BPA exposure in these rats. This finding may represent a technical limitation of using such a comprehensive dose range in toxicology studies despite checking all input media (i.e., diet, water, vehicle, cages, bedding), which were tested *a priori* for background levels of BPA and found to be negligible. Although the source of the unintended exposure leading to BPA-G in serum from vehicle and naïve control groups was not ultimately identified, this did not affect interpretation of the companion toxicology study (Delclos *et al.*, forthcoming) because no biologically significant effects of BPA were observed below the 100,000 μg/kg bw/day dose. It is similarly important that interpretation of other "low dose" effects from BPA studies could be affected, particularly in studies that do not include thorough evaluations of internal dosimetry and background contamination of input media. Indeed, some recent toxicity studies include dosing with isotopically labeled BPA, which obviates postexposure contamination problems; however, unless native BPA in untreated animals is also critically evaluated (i.e., BPA-G measurements), unintended exposure at low levels cannot be excluded. Other studies continue to dose with native BPA despite potential background problems. For example, a recent study on the carcinogenic potential of BPA (Acevedo *et al.*, 2013) reported detectable levels of native total BPA from untreated rats (range 0.1–0.9 ng/ml, 0.4–3.6nM) along with detectable aglycone BPA (range 0.05—0.3 ng/ml, 0.2–1.2nM). These observations, which could be consistent with either unintended internal exposure (total BPA) or postexposure sample contamination (aglycone BPA), were not considered by the authors as influencing the reported study outcomes. Similarly, the native BPA dosimetry reported by Prins *et al.* (2011) in PND 3 Sprague Dawley pups could be compromised by contamination by the following criteria: (1) the aglycone fraction of total BPA reported at C_{max} after oral administration was 25%, a value far higher than the 7% reported in an independent investigation of d6-BPA pharmacokinetics in Sprague Dawley pups (PND 3, Doerge *et al.*, 2010a) or 4% in PND 4 pups (Fig. 1); (2) the levels of native BPA in serum did not change between 0.5 and 2 h [the last time point reported by Prins *et al.* (2011)] as opposed to a 10-fold decrease during the same time period when d6-BPA was measured (Doerge *et al.*, 2010a; Fig. 1). However, there is always uncertainty inherent in comparing studies using quite different protocols (i.e., oil vs. aqueous vehicles, individual pup vs. pooled serum samples, different levels of analytical sensitivity and volumes analyzed). Although both these publications did report important measurements of aglycone and total BPA, the absence of a critical evaluation of the totality of pharmacokinetic evidence on BPA did not permit an adequate assessment of the potential impact of either postexposure sample contamination or unintentional exposure *in vivo*.

The contamination by native aglycone BPA observed in the current study is consistent with a substantial body of experimental evidence available in the literature that documents the pervasive influence of environmentally derived BPA on analysis of blood, urine, and tissue samples (Calafat *et al.*, 2013; Markham *et al.*, 2010; Patterson *et al.*, 2013; Teeguarden *et al.*, 2011; Twaddle *et al.*, 2010; Vandentorren *et al.*, 2011; Ye *et al.*, 2013). Indeed, analytical chemists responsible for nation-level BPA biomonitoring programs have repeatedly emphasized the unsuitability of blood as a matrix because of its particular propensity for contamination (Calafat *et al.*, 2008; Koch and Calafat, 2009; Koch *et al.*, 2012; Ye *et al.*, 2013). This analytical challenge is well documented for many other ubiquitous environmental chemicals, including phthalate diesters (Kessler *et al.*, 2001), polybrominated diphenyl ethers (PBDEs), poly-

chlorinated biphenyls (PCBs), triclosan, benzophenone-3, and parabens (Ye *et al.*, 2013). Nonetheless, a less extensive set of papers reporting aglycone BPA levels in human serum in the ng/ml range has been interpreted as reflecting either a large unknown source of exposure (Vandenberg *et al.*, 2010), release from depot tissues (Stahlhut *et al.*, 2009), or extensive dermal and sublingual exposure (Vandenberg *et al.*, 2013). A recent meta-analysis of BPA levels in human serum from 93 studies comprised of >30,000 subjects from 19 countries concluded that BPA aglycone levels in individuals exposed to upper bound aggregate daily doses (<1 μg/kg bw) are in the pM range, and orders of magnitude below levels detectable using current analytical techniques (Teeguarden *et al.*, 2013). This conclusion was based on comparison of blood concentration estimates made from urinary and blood biomonitoring measurements, controlled pharmacokinetic studies using stable isotope-labeled BPA in animals and humans, and simulations from PBPK modeling. A consistent theme from such investigations is that although thorough *a priori* evaluation of blood collection materials and field blanks is essential to minimize the likelihood of sample contamination, it is insufficient to preclude it. An emerging corollary is that pharmacokinetic and biomonitoring data from humans provide objective post hoc criteria to evaluate the plausibility of true exposures versus unintended contamination events. Specifically, (1) the systemic fraction of total BPA present as aglycone, which is <1% for oral exposures and ∼10% for parenteral; (2) concurrent measurements of total BPA concentrations in urine and blood samples (urine ∼40-fold greater than blood); and (3) evaluation of total estimated intake and its relationship with oral bolus C_{max} in blood for total BPA (∼20nM per μg/kg bw dose; Teeguarden *et al.*, 2011, 2013; Völkel *et al.*, 2002).

In light of this body of information regarding the impact of BPA contamination during sampling, it was not surprising that postexposure contamination of rat serum was observed in the current study despite extensive evaluation *a priori* of collection materials. However, the finding of BPA-G, a metabolite that cannot be derived from contamination, in vehicle and naïve control rat serum was unexpected. The source for this unintended internal exposure was never determined despite rigorous evaluation of diet, water, cages, bedding, vehicle, and careful attention to dose certification and delivery (see Delclos *et al.*, 2014) but a seemingly likely source is within the animal room itself. This possibility arises because a majority of BPA is excreted in feces in the aglycone form by the rat (Twaddle *et al.*, 2010). Therefore, the feces contained in cages of dosed rats represents a large reservoir for potential exchange to untreated controls housed alongside them. This hypothesis was evaluated by swabbing the inside and outside of cages from control, medium and high doses from a separate ongoing BPA study (see Experimental). The low ng levels found were insufficient to establish appreciable transmission through particulates. Although the low volatility of BPA would predict minimal exposure via inhalation, air sampling was not undertaken because of numerous plastic components in the air sample monitoring apparatus and the propensity to introduce background contamination during sample collection.

Life-Stage Dependence of Pharmacokinetics for the Reference Estrogen, EE2

Similar treatment of rats with EE2 showed evidence for dose-dependent and age-dependent changes in serum concentrations of EE2 (Fig. 5). These measurements show that the unconjugated EE2 levels and the unconjugated fraction were age-dependent, similar to BPA, presumably because of the development of phase II metabolic capabilities. Enzymatic hydrolysis procedures indicated that the aglycone fraction of EE2 was markedly higher than that seen for BPA in age-matched rats. This metabolic immaturity confers extra susceptibility to both EE2 and BPA exposures during the neonatal period because aberrant estrogenic signaling during this critical developmental window in rodents could produce irreversible effects that manifest in adulthood.

Methodological Strategy for Concurrent Evaluation of Endogenous E2 Levels Across Life Stages

There is a consistent body of evidence in the literature for overestimation of low pg/ml levels of E2 by antibody-based measurements, particularly in men (∼20 pg/ml), boys (<0.1 pg/ml), and postmenopausal women (<3 pg/ml) (Faupel-Badger *et al.*, 2010; Haisenleder *et al.*, 2011; Hsing *et al.*, 2007; Huhtaniemi *et al.*, 2012; Klein *et al.*, 1994). This discrepancy prompted the development of a sensitive LC/MS/MS-based method for small volumes of serum that is much less susceptible to the cross-reactivity problems that can affect the accuracy of RIA and ELISA with which to evaluate the rats in this study, particularly the males. Figure 6 shows the levels of E2 determined in naïve and vehicle control male and female rats at the same life stages evaluated for BPA and EE2. These data were not generated to evaluate comprehensively the complexity associated with all life-stage-specific and daily fluctuations in endogenous hormone levels, but to provide a direct comparison with contemporaneous measurements of BPA and EE2. Further, the reported values represent total serum aglycone E2 and do not incorporate the potentially modulating effect of serum protein binding on tissue availability. The higher levels of E2 observed in PND 4 males versus females was interpreted as incomplete development of ovarian function by this age in females as opposed to more complete development of testicular synthesis of testosterone (∼0.2 ng/ml or 1nM in male pups as young as PND 1, Supplementary fig. 4) that would be available for aromatization to E2. The consistent finding of undetectable E2 levels (<0.5 pg/ml) in adult male Sprague Dawley rats by LC-MS/MS is not consistent with reports in the literature of much higher values derived from antibody-based methods (e.g., 50, 90, and 10 pg/ml in 3-, 12-, and 20-month-old Sprague Dawley male rats, respectively; Wu *et al.*, 2009). This methodological discrepancy is critical to interpreting the reliability of ani-

mal models for hormonal carcinogenesis in which endogenous hormone levels in adult male rats (PND 90) are manipulated through subcutaneous silastic implants of E2 and testosterone to achieve E2 levels of 50–125 pg/ml (Lane *et al.*, 1997; Ofner *et al.*, 1992).

Relationship Between Estrogen Receptor-Based Dosimetry and Developmental Effects of BPA and EE2

It was important to evaluate contributions to the dose-response relationship between potential estrogenic effects reported in the companion BPA/EE2 toxicology study and internal dosimetry measurements reported here. This goal was accomplished by comparing the E2-equivalent molar concentrations of EE2 and BPA using relative ERα binding affinities. Receptor affinities have been reported for these three estrogens with several estrogen receptor types, including the classical nuclear receptors, ERα and ERβ (Gutendorf and Westendorf, 2001), and membrane-bound GPR30 (Thomas and Dong, 2006). Because well-recognized biological activities (e.g., uterine weight gain) are associated with its activation, ERα was chosen as an example, although the same relative affinity approach could be applied to any other receptor type. The ERα binding affinities have been reported using identical methodology for EE2 and BPA, relative to E2 = 1, as 1.2 and 0.0002, respectively (Gutendorf and Westendorf, 2001). Although the focus on relative binding affinity does not include other important factors that can serve to modulate overall potency (e.g., coactivator recruitment and receptor density), ligand binding affinity often correlates with cellular responses to estrogens (Jeyakumar *et al.*, 2011). As a point of reference, a representative value for the K_D for E2 binding to ERα is 200pM (Jeyakumar *et al.*, 2011). Accordingly, 10% receptor occupancy occurs at E2 concentration at approximately 20pM and 90% occupancy occurs at respective concentrations of 2000pM. The dosimetry comparison presented here is derived from measured levels of total serum ligands, but it is the free (unbound) tissue levels in equilibrium with receptors that determine toxicodynamic responses (Mendel, 1989). Although these ligands could be anticipated to interact differentially with rat serum binding proteins, the free fractions for serum albumin are similar for BPA (6%, Teeguarden *et al.*, 2005), EE2 (2%, Kuhnz *et al.*, 1990), and E2 (5%, Plowchalk and Teeguarden, 2002). Unraveling the complex relationships between ligand-protein binding kinetics and the dependence of tissue responses on either total or free ligand concentrations (Mendel, 1989) is beyond the scope of this paper; however, such an analysis could be made across life stages and between species using computation approaches previously described (Teeguarden and Barton, 2004).

Figure 7 shows the serum estrogenic dosimetry relationships in male and female rats at different ages for the two highest BPA doses (100,000 and 300,000 μg/kg bw) and both EE2 doses (0.5 and 5 μg/kg bw) compared with the contemporaneous endogenous levels of E2. When plotted on a log scale, it is clear that on PND 4, EE2 levels exceed those of endogenous E2 and the respective values are similar at PND 21 and PND 80. Therefore, based on internal dosimetry and receptor occupancy, ERα-mediated signaling, especially at the high dose of EE2, could be possible in rats of both sexes in which daily dosing started the day after birth. The two high doses of BPA produced internal estrogenic exposures in males and females greater than either endogenous E2 levels or exogenously administered EE2 at all ages examined.

The relevance of these ERα-dosimetry relationships during rodent development to predicting changes in estrogen-responsive tissues is supported by the results presented in the companion paper where significant treatment effects were observed in both sexes of rats at the 300,000 μg BPA/kg bw/day dose levels and only in females at the 100,000 μg/kg bw/day (see Delclos *et al.*, forthcoming). In females, the effects partially overlapped those of EE_2, in terms of a consistent pattern of changes in the PND 90 ovary (increased cystic follicles, depleted corpora lutea and antral follicles), PND 80 serum hormone levels (increased E2 and prolactin, decreased progesterone), and disruption of the estrous cycle. The degree of overlap was less prominent in males where both doses of EE_2 stimulated mammary gland hyperplasia at PND 21 and PND 90, whereas the high dose delayed the time of testicular descent and preputial separation, depressed male reproductive organ weights, and caudal epididymal sperm counts, and increased degeneration of the germinal epithelium. The highest dose of BPA (300,000 μg/kg bw/day) did not affect these endpoints, except for the delay in the time of testicular descent, and the 100,000 μg/kg bw/day dose had no treatment-related effects in males. Based on the internal dosimetry and ERα occupancies achieved discussed above, even more estrogenic effects might have been expected at the two highest doses of BPA.

CONCLUSIONS

This investigation is the first to evaluate thoroughly internal dosimetry for orally administered BPA throughout perinatal and adult life stages in rats over a range of doses spanning approximately 100- to 10,000,000-fold above median aggregate human daily intake, from which best estimates of steady state blood levels of aglycone BPA are in the low picomolar range (Teeguarden *et al.*, 2013). The concurrent evaluation of two doses of a reference estrogen, EE2, and endogenous levels of E2 throughout the life stages facilitates critical evaluation of estrogenic hypotheses for effects observed in histological and clinical chemistry endpoints evaluated in parallel. Only the two highest doses of BPA produced a similar pattern of internal dosimetry and ERα occupancy, compared with E2 and EE2, and a similar pattern of estrogenic effects *in vivo* as shown in the companion paper (Delclos *et al.*, 2014). Although alternative hypotheses for cellular targets of BPA have been promulgated, the data presented here for clearly adverse effects support an estrogenic mode of action *in vivo*. Despite care taken *a priori* to minimize

contamination of serum samples during collection and storage (i.e., postexposure), trace level measurements of aglycone BPA were clearly compromised at the lower doses. This finding adds to a large body of evidence suggesting that the propensity for sample contamination will continue to make measurements of native aglycone BPA in blood difficult to interpret. Evidence was also presented to suggest that some unidentified source of BPA in the animal rooms led to levels of BPA-G in vehicle and naïve control rats similar to those in the 2.5 μg BPA/kg bw/day dose despite extensive screening of possible contamination in input media (feed, water, cage material, bedding, room dust) and strict attention to dose certification and delivery. This finding could reflect a technical limitation in conducting studies of this complexity and suggests that it may be difficult to interpret studies reporting adverse effects from "low dose" BPA that do not similarly document and interpret internal dosimetry in the context of the totality of pharmacokinetic evidence for BPA.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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EXHIBIT 4

(Table 1 – Summary of studies cited by Peretz et al. to support purported adverse effects on early oogenesis and ovarian follicle formation)

Table 1. Epidemiological and experimental animal studies cited by Peretz et al. to support adverse effects on early oogenesis and ovarian follicle formation.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Lawson et al., 2011	BPA was from Sigma-Aldrich (purity not provided)	C57BL/6J inbred mice from pathogen-free colony - The Jackson Laboratory	n = 18 (based on 3 biological samples of 6 ovaries) 1 dose BPA	Timed matings followed by daily treatments of dams starting 11 day postcoitus (dpc) and sacrafice (groups of 18) at 12, 12.5, 13.5 & 14.5 dpc. Ovaries were pooled into 3 biological samples of 6 sets of ovaries	Oral - Eppendorf pipette. Daily 20 - 30 µL (placebo or BPA) tocopherol-stripped corn oil	Meiotic gene expression in fetal ovaries. Dosing spanned the anticipated start of fetal meiosis 12.5 - 13 dpc. Expression of 16 meiosis specific genes, 17 genes shared in meiotic & somatic cells and 17 housekeeping genes were tracked using Affymetrix chips.	20 ng/g (**no dose validation**). Some changes in gene expression were measured. Background BPA and phytoestrogen levels were not reported.	One way ANOVA to assess significance and fold-change in gene expression. Most changes were under 2-fold.	FDA Comments: Ovaries were pooled across litters. There was no direct linkage of subtile gene expression changes to biological response. The biological relevance of reported gene changes is not understood**.**
Rodriguez et al., 2010	BPA and DES were from Sigma-Aldrich (purity not provided)	Wistar rats	n ≥ 8 (but also stated as 5 males & 5 females when possible; no data is presented for males) 2 Doses BPA & 2 doses DES (positive control)	Liters from 10 timed-pregnant dams were adjusted to 10 pups (5 males and 5 females when possible) and cross-fostered to the 5 treatment groups to minimize the use of siblings. Pups were treated on PND 1, 3, 5, & 7. Pups were sacraficed on PND 8 and ovaries were fixed.	sc injection (placebo, BPA or DES) in corn oil	Primordial follicle population, follicle growth, expression of certain proteins, oocyte survival, incidence of multioocyte follicles, Erβ positive follicles, AR expression in granulosal cells, PR expression in ovarian/follicular cells.	20 mg/kg and 0.05 mg/kg (no dose validation). At 20 mg/kg, primordial follicle population ↓, growing follicles ↑, p27 expression in granulosal cells & oocytes of primordial & growing follicles↑, ERβ expression & proliferation in recruited follicles, ERα positive folicles↑. No significant effects at the low dose. No changes were observed on oocyte survival, ERβ positive follicles, incidence of multi-oocyte follicles (MOFs), AR, expression p27 expression, or apoptosis. Potential phytoestrogen exposure was not evaluated.	Kruskal-Wallis analysis of overall significance, Dunn post hoc test to compare each group to th control. Fisher exact probablility test to evaluate proportions.	FDA Comments: The biological significance and/or function of the molecules evaluated to follicle development and physiology have not been clearly demonstrated.
Rivera et al., 2011	BPA (99% purity from Aldrich) and DES (Sigma)	Lambs - Corriedale ewes (age 2 - 4) mated to Hampshire Down rams.	n=6 1 dose BPA & 1 dose DES (positive control)	Female lambs born in Aug. and Sept. from single delivery (no twins) and pasture raised. Lambs treated daily on PND's 1 (day of birth) - 14. Ovaries were removed on PND 30.	Treatment was sc (nape of neck). Corn oil vehicle.	Ovary weight measured. Follicle #'s from each ovary, % follicles at each stage, & MOF's measured (microscopy/counting). ERα, ERβ, AR, Ki67, & p27 ovarian expression (immunostaining/microscopy), & estradiol & testosterone serum levels measured (RIA). Ki67 positive cells used for proliferative index (anti-Ki67 rabbit polycolonal antibody).	50 µg/kg BPA (**no validation**). 5 µg/kg DES. BPA had ovarian weight ↓, growing follicles (transitional & primary) ↑, incidence of MOF's ↑, proliferation of large preantral cells & small antral follicles and p27 expression ↑, # small antral atretic follicles ↑. No effects on expression of ERα, ERβ, AR, or serum estradiol or testosterone. Phytoestrogen content of pasture was not evaluated.	Kruskal-Wallis for overall significance. Dunn post hoc to compare each group to the control.	FDA Comments: The sc dosing has limited relevance. There was a single dose so no dose response. The observed effects are not clearly associated with adverse effects and the significance for adult sheep needs to be determined.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Hunt et al., 2012	Deuterated BPA (CDN Isotopes; purity not reported)	Rhesus macaques (*Macaca mulata*) Females aged 6 - 13 yr naturally mated. Female fetus identified by sonography (confirmed at tissue collection). Early treatment fetuses evaluated after cesarean at GD 100. Late treatment fetuses evaluated at term.	**1 dose level** (given at 2 timings and 2 exposure routes) n = 5 treated early oral n = 6 treated early implant n = 5 treated late oral n = 6 treated late implant	Pregnant rhesus females with female fetuses were treated GD 50 - 100 by oral or implant routes & female fetuses removed by cesarean at GD 100 or at term and fetal ovaries were studied.	Oral doses were given in small pieces of fruit. Implant doses were given via Silastic tubing capsules and the vehicle in the slow release capsules was not reported.	Meiotic chromosome behavior was evaluated in pachytene cells by immunofluorescence staining. MLH1 foci in pachytene cells were counted to assess recombination, synaptic defects were identified by SYCP3 staining and centromere associations were identified with CREST antiserum. Follicle formation was evaluated in ovaries by embedding in parafin, sectioning, & staining with hematoxylin & eosin. Follicles were counted by blinded observers.	BPA dose: 400 µg/kg and blood levels were measured. Serum BPA levels around the time of the last dose were as follows: Oral doses at GD 50 - 100 0.51 ± 0.20 ng/mL. Oral doses at GD 100 - term 0.31 ± 0.13 ng/mL Implant doses at GD 50 - 100 0.45 ± 0.23 ng/mL. Implant doses at GD 100 - term 0.90 ± 0.13 ng/mL. BPA treatment via Silastic implant at GD 50 - 100 had MLH1 foci ↑ but overlap (50.4 ± 7.0 for 33 cells from BPA treated and 42.2 ± 5.9 from 70 cells for control; technical difficulties occurred in the oral treatment group). No changes were observed in synaptic defects. Centromere associations for implant dosing ↑. Oral dosing follicles with 2 - >5 oocytes ↑ and >5 oocytes both BPA treatments ↑.	Statistical methods may not have been reported.	FDA Comments: **Intradermal implant and continuous exposure is of questionable relevance.** Control values for number of oocytes per follicle were notably different between the controls for the single exposure group and the implant group. The increase in MLH1 foci for implant doses is based on comparison to only 2 control animals. Extrapolation from rhesus to humans is confounded by differences between species (metabolism, immunoreactivity, seasonal breeding pattern & associated hormonal control of ovulation). Tests were run in two different rhesus breeding seasons.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Zhang et al., 2012	BPA was from Kefeng, Shanghai, China (no purity provided)	CD-1 mice (Vital River, Beijing, China)	3 BPA doses. Number of mice per treatment group not stated. 7 - 8 pups per treatment group were evaluated for oocytes per cyst and follicles per region, but the number of litters is not clear.	Pregnant dams were treated daily from GD 12.5 - 18.5; on PND 3, 5, & 7 ovaries from pups were collected and fixed (neutral buffered formalin). In addition, groups of control and high dose dams were sacrificed on GD 15.5, 17.5, and 19.5 to collect developing fetal oocytes.	Oral- Eppendorf pipette. Vehicle 0.1% DMSO.	Immunostaining and microscopy were used to count oocytes, primordial, primary and secondary follicles. Meiosis prophase I assay was used on developing fetal oocytes. Oocytes from 13.5, 15.5, and 17.5 dpc had bisulfite-treated DNP amplified by PCR for the Stra8 gene. Oocyte RNA from 17.5 dpc was extracted and evaluated by quantitative PCR using primers for Dmc1, Rec8, Scp3, Stra8, ERα, ERβ, and β-actin.	0.02 mg/kg BPA 0.04 mg/kg BPA 0.08 mg/kg BPA **(**no dose validation). At PND 3 high dose BPA pups had percentage of oocytes in cyst ↑, percentage in primary follicle ↓ and # oocytes/section ↑ - but then # oocytes/section at PND 7 ↓. No differences were observed in the number of oocytes per section at PND 5, or oocyte distribution at PND 5 and 7. BPA did not affect litter size or offspring ratio. At GD 15.5, 17.5, and 19.5 the percentage of oocytes at different phases of meiosis differed between high dose BPA and control fetal ovaries suggesting a delay in meiotic progression to prophase I. At GD 17.5 Stra8 expression at high dose BPA ↓. **Background BPA and phytoestrogen levels were not reported.**	Differences between treatments and controls used ANOVA and differences were calculated with Tukey's test; it is not clear whether the litter or the pup was considered the experimental unit.	TSG Comment: The biological relevance of reported effects including possible delay in meiosis is not understood. Effects in litter size or offspring ratio were not observed.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Veiga-Lopez et al., 2013	BPA (≥99%, Aldrich Chemical)	Suffolk ewes (2 - 3 years old). Breeder rams were from animal providers.	n = 5 at GD 65 and n =5 at GD 90 1 BPA dose	Ewes were treated GD 30 - GD 90. One ovary was removed from dam fetuses on GD 65 and GD 90 and used for gene expression studies.	Treatment was daily by sc injection. The vehicle was corn oil.	Quantitative RT-PCR was used to evaluate gene expression.	BPA dose: 50 mg/kg. Age dependent changes in ovarian mRNA for steroidogenic enzymes, hormone receptors, growth factors and insulin-related biomarkers did not differ between control and treated at either GD except at GD 65 treated Cyp 19 ↑ and SRD5A1 ↑. miRNA's ↓ due to BPA at both GD's but miRNA regulators (endoribonuclease enzymes Drosha & Dicer) were not affected. BPA levels were 0.43 ± 0.09 ng/mL in control and 2.62 ± 0.52 ng/mL in BPA-treated fetuses based on umbilical arterial samples at GD 90 (n=3-4/group).	2-way ANOVA was used for significance, Bonferroni post hoc test was used to evaluate differences between treatments. An independent t test was used to evaluate differences in BPA concentrations.	FDA Comments: High phytoestrogen hay (alfalfa) was fed to the pregnant ewes (phytoestrogen level not analyzed). Despite down regulation of miRNA's, no changes occurred in gene expression of miRNA regulators, ovarian steriodogenic enzymes, receptors, or growth factors except transient upregulation of Cyp 19 and SrD5A1 on GD 65. The biological significance of these transient changes remain to be determined.

EXHIBIT 5

(Table 2 – Summary of studies cited by Peretz et al. to support purported alteration of female steroidogenesis)

Table 2. Epidemiological and experimental animal studies cited by Peretz et al. to support alteration of female steroidogenesis.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Bloom, 2011a	Not applicable	Women of childbearing age.	Subset of study with 58 female patients; 37 male partners: measure-ments from 186 embryos, 27 couples.	This epidemiological study evaluated the relationship between BPA serum concentrations in couples and the quality of embryos produced through in vivo fertilization (IVF).	Not applicable	Serum BPA measured on the day of oocyte and sperm collection by HPLC with Coularray detection; LOD = 0.3 ng/mL. Embryo quality was indicated by cell number (ECN) and fragmentation score (EFS).	There was no association between maternal BPA level and ECN or EFS.	Models were created for ECN and EFS, including age, race, paternal age and race.	FDA Comments: Major limitations in the study included the small size and the limited ability to control for known confounders of ECN and EFS.
Mok-Lin, 2010	Not applicable	Women between 18 and 45 years of age	84 women: 61 had 1 IVF cycle; 18 had 2 cycles; 5 had 3 cycles; for a total of 112 IVF cycles	The epidemiological study is described as prospective cohort in design, but is cross-sectional in nature based on the timing of the collection of the urinary BPA samples in relation to the outcome measures.	Not applicable	Urinary BPA concentrations were measured in 203 urine samples collected during 112 IVF cycles. Fifteen samples had BPA concentrations below the LOD	In a mixed effect regression model accounting for multiple IVF cycles, and adjusting for age, BMI, and day 3 FSH level, there was an average decrease of 213 pg/ml in peak serum estradiol levels for each log unit increase in urine volume-adjusted BPA.	Age, body mass index (BMI), and day 3 FSH, all factors known to be associated with decreased ovarian response during IVF, were adjusted for in the statistical analyses.	FDA Comments: Findings in IVF patients may not relate to those not using IVF; the small sample size limited power for analysis by infertility type; there was no indication whether the known diurnal variation in urine BPA was accounted for; not known if all materials used in sample aliquatting was BPA-free; authors indicated that results were preliminary.
Ehrlich, 2012a	Not applicable	Female IVF patients age 18 to 45 years	Total n = 167	Epidemiological prospective study to determine if there was a correlation between BPA urine concentration and egg implantation failure following IVF treatment.	Not applicable.	Spot urine samples measured at CDC by solid phase extraction HPLC isotope dilution tandem mass spectrometry. Implantation failure was defined as a serum β-hCG level < 6 mIU/mL typically measured 17 days after egg retrieval (12 to 14 days after uterine transfer).	42% of the IVF cycles resulted in implantation failure (IF). The odds of IF increased linearly with increasing quartiles of urinary BPA concentrations; however, correlation was statistically insignificant after adjusting for age, day of embryo transfer and IVF protocol (p-trend=0.06 corrected for age and day of transfer; trend = 0.12 corrected for age, day of transfer and IVF protocol).	The findings don't provide a convincing statistical correlation between urinary BPA concentration and the failure of implantation in women undergoing IVF.	FDA Comment: There was no statistical correlation between urinary BPA concentration and implantation failure in IVF; infertility factors and health status of patients and partners plus previous IVF failure may confound analysis; it was not not stated if the embryologist that selected the embryos for implantation was blinded to groups.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Kandaraki, 2011	Not applicable	Women < 40 years old.	71 PCOS cases; 100 age and BMI-matched controls	The goal of this epidemiological study was to measure serum BPA levels and explore a possible link between BPA and polycystic ovary syndrome (PCOS); reported as cross-sectional, described as case-control.	Not applicable	PCOS components measured directly in blood samples included: insulin, total testosterone, SHGB, androstenedione, LH, and FSH.	Mean BPA levels (ng/mL) were found to be significantly higher in PCOS cases compared to BMI-matched controls.	Multiple linear regression.	FDA Comments: BPA measurements in blood are uncertain due to ELISA method detecting non-specific binding; BPA levels may be unreliable due to possible contamination, human subject variability; BPA blood levels taken at one point in time; causal association between BPA levels and PCOS risk based on temporality can't be assessed due to study design.
Ehrlich, 2013	Not applicable	Caucasian women (88%), average age, 35 years of age.	Follicular fluid samples from 61 women	This epidemiologicical study was designed to investigate a possible relationship between urinary BPA concentrations and CYP19A1 gene expression in granulosa cells collected from women enrolled in a larger study.	Not applicable	RNA was extracted from follicular samples and quantitative PCR assays were used to measure CYP19 gene expression; serum FSH levels were measured on cycle day 3 and peak serum estrodiol was measured 1.5 days prior to oocyte retrieval from each participant.	No association was observed between urinary BPA levels (quartiles) and CYP19 gene expression levels	Simple statistics were used to characterize participants; mixed-effect statistical models were used to test for associations between BPA concentrations and CYP19A1 gene expression	FDA Comments: "The authors stated that both the adjusted and unadjusted models “suggested” a non-monotonic dose response between urinary BPA and parameter estimates for log CYP19 gene expression. However, the data did not support that statement, since none of the parameter estimates in either model was statistically significant, nor was any test for trend."
Galloway, 2010	Not applicable	Men and women maximum age 75.	720 total, a subset of a larger study of 1268 individuals.	The study was intended to clarify the association between serum and urine BPA levels and estradiol, testosterone and SHBG.	Not applicable	Urine and serum levels of BPA were measured, plus serum testosterone, sex hormone binding globulin (SHBG) and estradiol.	Only a few minr associations were identified; in men, higher BPA excretion was associated with higher testosterone levels, and in premenopausal women, higher BPA excretion was associated with higher SHBG.	20 separate multivariable linear regression models were developed to evaluate the association between BPA and measured parameters in pre- and post-menopausal women, adjusting for confounding factors	FDA Comments: This study does not clarify the relationship between BPA and testosterone, and does not provide evidence of new risk information.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Fernandez, 2010		Female Sprague-Dawley rats, neonatal.	7/group	Non-GLP; the goal was to evaluate the effects of BPA on sex hormone levels in serum, ovarian morphology, ovulation, and fertility.	Subcutaneous exposure, daily PND1-10; castor oil vehicle.	At 4 months of age, sex hormones were measured and ovaries were evaluated by histopathology.	BPA doses were approximate: 0; 0.25-0.62 mg/kg (low); 2.5-6.2 mg/kg (mid); and 25-62.5 mg/kg (high); Ovarian weight, but not body weight, was lower in the mid and high dose groups. In the high dose group there were a large number of ovarian cysts, fewer corpora lutea, and more atretic follicles. The high dose group did not ovulate and had no pups; mid dose group had fewer pups, and low dose animals showed no effect.		FDA Comments: Study design issues include use of an in-house SD rat, housing pups in different dose groups in the same cage, and approximate dosing; that aside, the data do show a dose-response and so the study may be useful for hazard identification.
Tan, 2013		Female ICR-mice, pre-natal.	3-5/dose	Non-GLP study to determine if BPA is a multi-targeted endocrine disrupter.	Exposure by gavage in a vehicle of ethanol/corn oil (1:9, v/v) from GD 13-16	Mice were euthanized on GD 17; serum testosterone, 17 beta estradiol (E2) and corticotropin-releasing hormone (CRH) were measured as was expression of *crh* , protein kinase C isoforms and CREB.	BPA doses were: 0, 2, 20, or 200 mg/kg/day; serum levels of T, E2, and CRH were reported to increase in groups exposed to 20 or 200 mg/kg/day BPA, and placental expression of *crh* mRNA increased in the 200 mg/kg/day group.	Data were analyzed by ANOVA followed by Bonferroni's multiple comparison test if significant differences were idetntified.	FDA Comments: sstudy limitations include: small sample size--3-5 animals/group; potential contamination in caging, water, food not evaluated, standard devications smaller than expected for endpoints measured.
Kobayashi, 2012	BPA purity, >99.6%	Sprague Dawley rats	10/ dose/ sex	Non-GLP; Pre-and post-natal dietary BPA exposure to investigate the effects of low-dose BPA exposure on reproductive development in F1 male and female rat offspring.	Dietary exposure from GD6 through PND 21	Reproductive outcomes measured included: dam weight gain, gestation length, sex ratio, and live births per litter. F1 offspring were necropsied at 5 weeks (n=10/group/sex) or 3 months (n=10/group/sex); Blood was collected for steroid hormone analysis; reproductive organs were removed and weighed.	BPA doses: 0, 0.017, 0.17, and 1.7 mg/kg/day; No effects on the reproductive outcomes for the F0 dams or F1 pups. There were no BPA related changes in body weight or reproductive organ weights in F1 male and female rats except a reduction in epididymis weight in the 3-month old F1 males in the high dose group.		FDA Comments: Authors stated the toxicological significance of the decrease weight of the epididymous was unknown; there was no discussion of potential environmental contamination, it is not clear how offspring were selected from litters for measured parameters.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Mendoza-Rodriguez, 2011		Pregnant Wistar rats	5/dose	Non-GLP; female offspring were monitored for estrous cyclicity beginning at 3 months of age for 4 weeks, then sacrificed at estrus.	Exposure via drinking water in glass bottles from GD6 to PND 21	The study evaluated the effects of pre-and post-natal BPA exposure on uterine epithelium proliferation.	A single dose of BPA was administered, approximately 1.2 mg/kg/day.		FDA Comments: This study has no utility for hazard ID due to study design and methodological issues; lack of description of contamination control, offspring treatment (culling/cross-fostering), use of an inappropriate test unit (pups instead of litter), small sample size, and the use of only a single dose level.
Rivera et al., 2011	BPA (99% purity from Aldrich) and DES (Sigma)	Lambs - Corriedale ewes (age 2 - 4) mated to Hampshire Down rams.	n=6 1 dose BPA & 1 dose DES (positive control)	Female lambs born in Aug. and Sept. from single delivery (no twins) and pasture raised. Lambs treated daily on PND's 1 (day of birth) - 14. Ovaries were removed on PND 30.	Treatment was sc (nape of neck). Corn oil vehicle.	Ovary weight measured. Follicle #'s from each ovary, % follicles at each stage, & MOF's measured (microscopy/counting). ERα, ERβ, AR, Ki67, & p27 ovarian expression (immunostaining/ microscopy), & estradiol & testosterone serum levels measured (RIA). Ki67 positive cells used for proliferative index (anti-Ki67 rabbit polycolonal antibody).	50 µg/kg BPA (no validation). 5 µg/kg DES. BPA had ovarian weight ↓, growing follicles (transitional & primary) ↑, incidence of MOF's ↑, proliferation of large preantral cells & small antral follicles and p27 expression ↑, # small antral atretic follicles ↑. No effects on expression of ERα, ERβ, AR, or serum estradiol or testosterone. Phytoestrogen content of pasture was not evaluated.	Kruskal-Wallis for overall significance. Dunn post hoc to compare each group to the control.	FDA Comments: The sc dosing has limited relevance. There was a single dose so no dose response. The observed effects are not clearly associated with adverse effects and the significance for adult sheep needs to be determined.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Varayoud, 2011		Newborn Wistar-derived rats were dosed on PND 1, 3, 5, and 7 and tested on PND 80	Approx. 10-13/ dose group	Non-GLP; to evaluate if early postnatal exposure to BPA has an effect on female reproductive performance, uterine homeobox A10 (Hoxa 10) and Hoxa10-target gene expression, ovarian steroid levels or uterine estrogen receptor alpha and progesterone receptor expression.	Subcutaneous injection exposure on PND 1, 3, 5, and 7, in a corn oil vehicle (charcoal stripped).	Pregnancy rates, corpora lutea, implantation and resorption sites, gene expression, ovarian steroid levels, estrogen and progesterone receptor expression during pre-implantation period	BPA doses, 0, 0.05 mg/kg or 20 mg/kg ; Implantation sites were significantly reduced for the BPA 20 me/kg/day group.		FDA Comments: This study is generally mechanistic, evaluating possible modes of BPA action rather than endpoints of potential toxicity; a limitation of the study is that a dose of 20 mg/kg/day BPA makes the uterine environment non-normal.
Lee SG, 2013	Not reported.	Adult female Sprague-Dawley rats, 8 weeks of age	n= 30/ dose, but sample sizes n=3 to 5 / analysis	The study was intended to determine if long-term exposure to BPA disrupts 17β-estradiol (E2) production by granulosa cells of the ovary.	Exposure via gavage daily for 90 days; vehicle 0.5% DMSO in corn oil.	Serum testosterone (T), E2, FSH, and LH levels were measured by ELISA.	BPA doses were 0.001 or 0.1 mg/kg bw, estradiol benzoate (EB); 0.001 mg/kg bw; or vehicle;	Data were analyzed using ANOVA and Duncan's post hoc test.	FDA Coments: The BPA dose range is too small to detect a dose-response effect, the sample sizes were too small to detect a significant effect,
Berger, 2010	BPA purity 97%, Sigma -Aldrich	CF-1 female mice, 3-5 months of age	Between 4 and 9 subjects /dose	Non-GLP; gestational exposure GD 1-4; sacrifice and uterine removal on GD 6 for histomorphology, immunohisto-chemical staining and Western blot analysis	Subcutaneous injection in peanut oil vehicle	Uterine implantation sites, uterine luminal area, luminal epithelial cell height, estrogen and progesterone receptor density	BPA doses: 0, 100, 200, and 300 mg/kg/day	Dose was first treated as a categorical condition within a one-way ANOVA, it was also related to each quantitative measure through linear regression, with the associated r2 value tested via an F-test.	TSG Comments: This is a mechanistic study not relevant to hazard ID or listing based on sacrifice of P0 females and offspring on GD6, and use of high doses of BPA.

EXHIBIT 6

(Table 3 – Summary of studies cited by Peretz et al. to support purported reduction in oocyte quality)

Table 3. Epidemiological and experimental animal studies cited by Peretz et al. to support reduction in oocyte quality.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Fujimoto, 2011	Not Applicable	Infertile female patients and male partners undergoing IVF treatment.	58 females, 37 male partners	Epidemiological cross-sectional design to investigate the relationship between serum BPA and oocyte maturation and fertility outcomes in women undergoing IVF.	Not applicable	Unconjugated BPA concentrations were measured in blood samples by HPLC.	Median fasting blood BPA levels for women were 2.53 ng/ml (86.4% > LOD) and non-fasting blood BPA levels for men 0.34 ng/ml (51.6% > LOD); there was no association between BPA and oocyte maturation when all cases were considered; an association was seen for the nine Asian women in the intracytoplasmic sperm injection (ICSI) group (as distinguished from the conventional insemination group).		FDA Comments: There was no association between BPA and oocyte maturation for all cases; an association was seen only for 9 Asian women; study findings may not be generalizable to the population of normal women; the source study for subjects in this study is an investigation that collected data on lead, cadmium, and mercury exposures.
Mok-Lin, 2010	Not applicable	Women between 18 and 45 years of age	84 women: 61 had 1 IVF cycle; 18 had 2 cycles; 5 had 3 cycles; for a total of 112 IVF cycles.	The epidemiological study is described as prospective cohort in design, but is cross-sectional in nature based on the timing of the collection of the urinary BPA samples in relation to the outcome measures.	Not applicable	Urinary BPA concentrations were measured in 203 urine samples collected during 112 IVF cycles. Fifteen samples had BPA concentrations below the LOD.	In a mixed effect regression model accounting for multiple IVF cycles, and adjusting for age, BMI, and day 3 FSH level, there was an average decrease of 213 pg/ml in peak serum estradiol levels for each log unit increase in urine volume-adjusted BPA.	Age, body mass index (BMI), and day 3 FSH, all factors known to be associated with decreased ovarian response during IVF, were adjusted for in the statistical analyses.	FDA comments: Findings in IVF patients may not relate to those not using IVF; the small sample size limited power for analysis by infertility type; there was no indication whether the known diurnal variation in urine BPA was accounted for; not known if all materials used in sample aliquatting was BPA-free; authors indicated that results were preliminary.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Ehrlich, 2012a	Not applicable	Female IVF patients age 18 to 45 years of age.	Total n = 167	Epidemiological prospective study to determine if there was a correlation between BPA urine concentration and egg implantation failure following IVF treatment.	Not applicable.	Spot urine samples measured at CDC by solid phase extraction HPLC isotope dilution tandem mass spectrometry. Implantation failure was defined as a serum β-hCG level < 6 mIU/mL typically measured 17 days after egg retrieval (12 to 14 days after uterine transfer).	42% of the IVF cycles resulted in implantation failure (IF). The odds of IF increased linearly with increasing quartiles of urinary BPA concentrations; however, correlation was statistically insignificant after adjusting for age, day of embryo transfer and IVF protocol (p-trend=0.06 corrected for age and day of transfer; trend = 0.12 corrected for age, day of transfer and IVF protocol.).	The findings don't provide a convincing statistical correlation between urinary BPA concentration and the failure of implantation in women undergoing IVF.	FDA Comments: There was no statistical correlation between urinary BPA concentration and implantation failure in IVF; infertility factors and health status of patients and partners plus previous IVF failure may confound analysis; it was not not stated if the embryologist that selected the embryos for implantation was blinded to groups.
Chao, 2012	Not described	Female CD-1 mice, dosed on PND 7-14	Number/ dose not stated; 110 mice in one assay, 73 mice in the second assay.	Non-GLP study designed to evaluate potential effects of BPA on several genes to determine if alterations in gene expression influence oocyte development.	Exposure by "hypodermical injection" daily PND 7-14, vehicle was physiological saline with 0.1% DMSO.	On PND 15, the methylation status of the maternally imprinted genes, Igf2r and Peg3 and the paternally imprinted gene, H19, were evaluated in oocytes; on PND 21 the numbers and types of follicles, and the oocyte spindle assembly were evaluated, and on PND 15 and 21 the diameter of all oocytes were measured to determine if BPA altered follicular development.	BPA doses: 0, 20, or 40 µg/kg/day; BPA decreased the methylation of the maternally imprinted Igf2r and Peg3 genes but had no effect on the paternally imprinted H19 gene in both experiments; a decrease in the number of primordial follicles was identified with later increases in primary, secondary, and antral follicles, but not in the total number of follicles; an increase in oocytes with abnormalities in the spindle apparatus during meiosis I was noted.	Differences between groups were analyzed by t-tests or by one-way ANOVA followed by Tukey's tests.	FDA Comments: There was a lack of clarity in the description of the experimental design and methods; the protocol did not describe how female pups were grouped with the dams or describe how many female pups were assigned to treatment groups, selected for measurements, or pooled for the molecular studies; only 2 BPA-treated groups were used so dose response could not be assessed; route of exposure was not clearly described, no information was presented on housing conditions, diet, or source and purity of BPA.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Muhlhauser, 2009	Not specified.	21-day old female offspring of mated C57BL/6J mice.	Number/ dose not stated	Non-GLP study conducted to evaluate the effect of diet on the results of BPA studies of the periovulatory oocyte.	Breeding mice were placed on 1 of 2 rodent diets at least 1 wk prior to mating: 1) a low phytoestrogen diet; or 2) a high-phytoestrogen-containing soy-based diet.	Female (F1) mice received daily oral doses of 0, 20, 40, 100, 200, or 500 µg/kg body weight BPA in a corn oil vehicle for 7 days preceding oocyte collection.	Oocytes of treated females were collected, and meiotic spindle and chromosome characteristics compared between control and BPA-treated mice; authors identified a significant diet-related variation in both the frequency of abnormalities in oocytes from untreated females and in the response to BPA.	Statistical comparisons were based on contingency table analyses or Fisher exact tests with Bonferroni corrections used for multiple comparison procedures; it is not clear how litter effects were taken into account.	TSG Comments: The study study concludes that exogenous estrogens are a serious confounding variable in BPA studies; which is useful information but does not support a listing determination.

EXHIBIT 7

(Table 4 – Summary of studies cited by Peretz et al. to support purported impairment of uterine endometrial cellular proliferation)

Table 4. Epidemiological and experimental animal studies cited by Peretz et al. to support impairment of uterine endometrial cellular proliferation.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Cobellis, 2009	Not applicable	Fertile women, age 21-42.	n=58 with endo-metriosis, n=11 controls	Epidemiological; reported as case-control but lacked elements of case-control design	Not applicable	Lack of BPA detection in any of the control sera suggests the possibility of control selection bias, measurement error, or misclassification bias; small sample size became smaller due to many BPA samples below the LOQ	BPA was detected in serum samples of 30 cases, but only 15 samples were above the LOQ (range: 0.79-7.12 ng/mL; mean: 2.91±1.74 ng/mL).		FDA Comments: This study was unable to adequately address the goal to assess the association between endometriosis and BPA levels. Issues with "case control' design: no case or control definitions; no study period given; exclusion and inclusion criteria not fully detailed; refusals/nonparticipants not addressed
Buck Louis, 2013	Not applicable	Menstruating women aged 18-44	Operative cohort: 495 women; Population cohort; 131 women	Epidemiological matched cohort design intended to explore the relation between BPA and endometriosis.	Not applicable	Surgically visualized disease was used to define endometriosis in the operative cohort and pelvic magnetic resonance imaging visualized endometriosis for the population cohort. Total BPA concentrations were quantified using HPLC coupled with mass spectrophotometry.	A positive finding was only observed in the population cohort in which the sample size for endometriosis compared to the cases without endometriosis is small (n=14 vs. n=113)	Statistical significance was evaluated using the Student t test or Wilcoxon nonparametric test for continuous data. Logistic regression was used to estimate the odds ratio (OR) for an endometriosis diagnosis for BPA by cohort. Age, body mass index , and urinary creatinine were potential confounders.	FDA Comments: There were no significant correlations between BPA and endometriosis in the operative or population cohort in the unadjusted or adjusted (age, BMI, creatinine) models; there was uncertainty in the endometriosis diagnosis by MRI.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Signorile, 2010		Pregnant Balb/c mice	Groups of 6 pregnant rats/dose	Non-GLP; female rats evaluated at 3 months of age for effects from perinatal exposure	BPA dosed by subcutaneous injection from GD1 to PND 7 in a vehicle of 2% EtOH in saline	Histology of pelvic organs	BPA doses: 0, 100, or 1000 µg BPA/kg/day; a significant increase in cystic ovaries was found in BPA-treated rats (10% in controls vs 45% in BPA 100 and 50% in BPA 1000), as was an increase in endometriosis in adipose tissue; there was no dose-response for the presence of free BPA in liver between the 100 or 1000 ug/kg doses	Statistical analysis did not account for litter effects.	FDA Comments: This study did not meet criteria for hazard ID (or listing under Prop 65) because the statistical analysis did not address the litter effects adequately; in addition, an isolated mouse colony was used and the diet was unusual. Cystic ovaries have been reported in other mouse strains and rats exposed to BPA.
Hiyama, 2011	BPA Source: Sigma-Aldrich	ICR mice 8 weeks old were mated	Not clearly presented	Non-GLP; study intended to evaluate the effects of BPA exposure on the reproductive organ across generations.	Subcutaneous injection daily GD 12-GD 16; sesame oil vehicle	Endpoints included: morphology of uterus and ovary, and the methylation pattern of HOXA10 gene using the F2 generation not exposed to BPA.	BPA doses were: 0, 100, 200, 500, and 1, 000 mg/ kg/ day, GD 12-16.	Statistical analysis was performed with Student's t-test after the check homoscedasticity with F test.	TSG Coments: These doses are too high to be useful for hazard ID or support for Prop 65 listing; methodological limitations include: treatment of litter vs pup as experimental unit not addressed, subject number in F1 generation not clear, relationship between gene changes and possible adverse effects not clear.
Berger, 2010	BPA purity 97%, Sigma -Aldrich	CF-1 female mice, 3-5 months of age	Between 4 and 9 subjects /dose	Non-GLP; gestational exposure GD 1-4; sacrifice and uterine removal on GD 6 for histomorphology, immunohistochemical staining and Western blot analysis	Subcutaneous injection in peanut oil vehicle	Uterine implantation sites, uterine luminal area, luminal epithelial cell height, estrogen and progesterone receptor density	100, 200, and 300 mg/kg/day	Dose was first treated as a categorical condition within a one-way ANOVA, it was also related to each quantitative measure through linear regression, with the associated r2 value tested via an F-test.	TSG Comments: This is a mechanistic study not relevant to hazard ID or listing based on sacraficce of P0 females and offspring on GD6, and use of high doses of BPA.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Bosquaizzo, 2010		Female Wistar rats		Non-GLP; the goal was to determine if neonatal exposure to BPA can alter expression of vascular endothelial growth factor (VEGF) and proliferation of endothelial cells in female rats on PND 80 after ovariectomy (OVX) and hormone treatment	Subcutaneous injection of BPA on PNDs 1, 3, 5, and 7in corn oil vehicle.	VEGF expression and endothelial cell proliferation were assessed.	BPA doses were: 0, 0.05 or 20 mg/kg; decreased VEGF expression and endothelial cell production were observed in uterine tissue after hormonal treatment in OVX rats exposed to the low dose of BPA, and no effects were seen at the high dose (20 mg/kg).		FDA Comments: This study is not useful for hazard ID (or listing), only as supporting study: vascular endothelial growth factor (VEGF) expression and endothelial cell proliferation may be important for an understanding of the BPA MOA for reproductive effects, but by themselves do not constitute an "adverse" effect from a toxicological perspective.
Bromer, 2010	Not specified	CD-1 mice, gestational	Not specified	The study goal was to determine whether effects seen following in utero ip exposure of dams to BPA are mediated through aberrant methylation of the Hoxa 10 gene.	IP injection in sesame oil vehicle daily on GD 9-16	Female offspring were euthanized 2 or 6 weeks after birth and uteri were evaluated by histological, immuno-histochemical and genomic analyses.	The authors indicate that: "Bisphenol-A exposure in utero leads to epigenetic alterations in the developmental programming of uterine estrogen response."	Fisher's exact test was used to compare DNA methylation frequencies of the Hoxa10 gene between BPA-treated and control mice. Student's t test compared the mean number of methylated sites per region.	FDA Comments: "Since this study dealt with a mechanistic endpoint, it should not be used for either hazard ID or risk assessment, but may be useful as a supporting study."
Mendoza-Rodriguez, 2011		Pregnant Wistar rats	5/dose	Non-GLP; female offspring were monitored for estrous cyclicity beginning at 3 months of age for 4 weeks, then sacrificed at estrus.	Exposure via drinking water in glass bottles from GD6 to PND 21	The study evaluated the effects of pre-and post-natal BPA exposure on uterine epithelium proliferation.	A single dose of BPA was administered, approximately 1.2 mg/kg/day.		FDA Comments: This study has no utility for hazard ID due to study design and methodological issues; lack of description of contamination control, offspring treatment (culling/cross-fostering), use of an inappropriate test unit (pups instead of litter), small sample size, and the use of only a single dose level.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Aldad, 2011	Not provided	Female African green monkeys of unstated reproductive age	3/group	Not GLP; Monkeys surgically oophorectomized, treated w/ BPA alone or with beta estradiol benzoate (EB).	Dosed w/ BPA via minipumps and EB via silastic implants; cholesterol vehicle.	The effect of BPA on EB-induced endometrial progesterone receptor (PR) expression in oophorectomized female monkeys was evaluated.	Immunohistochemistry and analysis of endometrial PRs were performed; EB-induced PR expression in the endometrial glands and stroma significantly compared to vehicle control. BPA had no effect alone, but repressed 38% and 50% of glandular and stromal expression, respectively.		FDA Comments: Only a single dose level of BPA was used; methods described are inadequate to determine the validity of the system; dosing was by implanted Alzet minipumps (BPA) and subcutaneous silastic implants (EB), which are not relevant to human exposures, systemic levels of BPA were not provided; and potential sources of BPA contamination were not characterized.

EXHIBIT 8

(Table 5 – Summary of studies cited by Peretz et al. to support purported decrease in uterine receptivity)

Table 5. Epidemiological and experimental animal studies cited by Peretz et al. to support a decrease in uterine receptivity.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Ehrlich, 2012b	Not applicable	Cohort of 174 women age 18-45 undergoing IVF	25% of the women (n=44) were assayed >2 IVF cycles	Epidemiological / Observational: To evaluate the association between urinary BPA and ovarian response and early stage reproductive outcomes.	Not applicable	Combined free and conjugated BPA was measured in addition to peak serum estradiol, oocyte maturation, fertilization, embryo quality, cleavage rate.	After adjustment for age and other potential confounders such as smoking, there was a significant inverse relationship between urinary BPA concentrations and the number of oocytes, number of fertilized oocytes, and estradiol concentrations.	Trend test analysis based on quartiles of measured serum BPA.	FDA Comments: Study limitations include: variability in BPA measurements, the small study sample size, data from women undergoing IVF treatment may not be generalizable to fertile, normally cycling women, evaluation does not consider potential contributions from the male partner.
Xiao, 2011		C57BL6 mice exposed from GD5 through GD 3.5	BPA Doses for 4 different assays: 1) 0 (n=14), 0.025 (n=7), 0.5 (n=7), 10 (n=10), 40 (n=9), and 100 (n=9) mg /kg bw/day; 2) 0 (n=8) and 100 mg BPA/kg bw/day (n=5); 3) 0 (n=5) or 100 mg/kg bw/day (n=4); 4) 0 or 100 mg/kg bw/day (n=4-7).	To investigate the effects of preimplantation exposure to BPA on preimplantation embryo development, embryo transport, and uterine receptivity	BPA was administered by subcutaneous injection om a sesame oil vehicle daily from GD0.5 through GD 3.5	Assay 1) number and position of implantation sites; Assay 2) embryo presence and stage of development; Assay 3) uterine receptivity to blastocysts; Assay 4) gestation length, litter size, postnatal survival rate.	Authors concluded that high doses of BPA (40 or 100 mg/kg/day) by the sc route have adverse effects on processes critical for embryo implantation.	Data were analyzed by one-way ANOVA with Dunnett's t test or X2 with Fischer's exact test.	FDA Comments: Study is mechanistic in nature and not useful for hazard ID (or consideration for Prop 65 listing); limitations incllude: high doses used for most assays, low and unequal subject numbers for dose groups, administration of rodent chow that contains phytoestrogens and assays with only one or two treatment groups.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Berger, 2010	BPA purity 97%, Sigma -Aldrich	CF-1 female mice, 3-5 months of age	Between 4 and 9 subjects /dose	Non-GLP; gestational exposure GD 1-4; sacrifice and uterine removal on GD 6 for histomorphology, immunohistochemical staining and Western blot analysis	Subcutaneous injection in peanut oil vehicle	Uterine implantation sites, uterine luminal area, luminal epithelial cell height, estrogen and progesterone receptor density	100, 200, and 300 mg/kg/day	Dose was first treated as a categorical condition within a one-way ANOVA, it was also related to each quantitative measure through linear regression, with the associated r2 value tested via an F-test.	TSG Comments: This is a mechanistic study not relevant to hazard ID or listing based on sacrifice of P0 females and offspring on GD6, and use of high doses of BPA.
Varayoud, 2011		Newborn Wistar-derived rats were dosed on PND 1, 3, 5, and 7 and tested on PND 80	Approx. 10-13/ dose group	Non-GLP; to evaluate if early postnatal exposure to BPA has an effect on female reproductive performance, uterine homeobox A10 (Hoxa 10) and Hoxa10-target gene expression, ovarian steroid levels or uterine estrogen receptor alpha and progesterone receptor expression.	Subcutaneous injection exposure on PND 1, 3, 5, and 7, in a corn oil vehicle (charcoal stripped).	Pregnancy rates, corpora lutea, implantation and resorption sites, gene expression, ovarian steroid levels, estrogen and progesterone receptor expression during pre-implantation period	BPA doses, 0, 0.05 mg/kg or 20 mg/kg ; Implantation sites were significantly reduced for the BPA 20 mg/kg/day group.		FDA Comments: This study is generally mechanistic, evaluating possible modes of BPA action rather than endpoints of potential toxicity; a limitation of the study is that a dose of 20 mg/kg/day BPA makes the uterine environment non-normal.
Salain 2009a	BPA purity 99.8%	Male Holtzman rats exposed PND 1 through 5, assessed PND 75		The study evaluated the effect of neonatal exposure to BPA on adult male rat sperm parameters and fertility.	BPA dosing was a daily subcutaneous injection from PND 1 through PND 5;	Serum hormone levels (LH, FSH, T, and E) and testis histopathology were examined in the control, 400 and 1600 µg/kg/day groups	BPA doses: approximately 200, 400, 800 and 1,600 µg/kg/day; Females mated with male rats exposed to doses of 400 ug/kg/day and higher showed significant loss in post-/pre-implantation survivability and a decrease in litter size.	The fact that 1 treated male was mated to two untreated females was not taken into account in the statistical analysis. A nested ANOVA (within the treated male) would have been appropriate.	TSG and FDA Comments: This study is not useful for evaluation of potential female reproductive toxicity; it isn't clear whether neonates were cross-fostered to limit litter effects;

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Salain, 2009b	Not reported	Pregnant Holtzman rats exposed GD 12 through PND 21; F1 males were tested on PND 75		The study examined the effects of perinatal exposure of male rats to BPA on fertility parameters in the F1, F2, and F3 generations	P0 female rats were dosed with BPA by gavage daily at 0, 1.2 and 2.4 µg/kg bw from GD 12 until PND 21	Endpoints in untreated females bred with F1 male rats exposed to BPA neonatally include: post-implantation loss, and litter size; endpoints in F1 males include hormone levels and fertility measurements.	The authors report a significant increase in post-implantation loss, a decrease in litter size, and a decrease in male sperm count and motility in F1, F2 and F3 males; The authors conclude that perinatal exposure to BPA affects the male germ line leading to impairments in the fertility of F1 male offspring and their subsequent F2 and F3 generations.	Only the P0 dam was dosed and thus, was the experimental unit; this was not taken into account in the statistical analysis.	TSG and FDA Comments: This study is not useful for evaluation of potential female reproductive toxicity because male rats were studied; In-house bred rats were fed an in-house prepared diet, consequently replication of the results may be problematic; the fact that the F0 female was not considered the experimental unit is a critical flaw in the statistical analysis.
Tiwari & Vanage, 2013	BPA purity ~ 99% Sigma Chemical Co, US	Male Holtzman rats, presumably in-house bred, 8 weeks of age.	7 males/ dose, each bred w/ 2 untreated females	Non-GLP; study goals to evaluate the effect of BPA on germ cell development during spermatogenesis and assess possible genotoxicity in male germ cells	Oral exposure daily for 6 consecutive days in 8-week old male rats; sesame oil vehicle	In untreated females paired with treated males: mating index, gestation index, number of corpora lutea, number of implantations, number of live fetuses, pre- and post-implantation loss; the Comet assay assesed damage to sperm DNA, and other sperm parameters were measured in treated males.	BPA doses: 0, 10 µg/kg bw and 5 mg/kg bw	Statistical analyses included Bartlett's test for homogeneity, ANOVA with Dunnett's post-hoc t-test; non-parametric tests such as the Kruskal-Wallis test were used for count data.	FDA Comments: Multiple methodological issues were identified, plus the study evaluated effect of BPA on spermatogenesis in male, it was not designed to show an impact on female reproductive toxicity.
Hiyama, 2011	BPA Source: Sigma-Aldrich	ICR mice 8 weeks old were mated	Not clearly presented	Non-GLP; study intended to evaluate the effects of BPA exposure on the reproductive organ across generations.	Subcutaneous injection daily GD 12-GD 16; sesame oil vehicle.	Endpoints included: morphology of uterus and ovary, and the methylation pattern of HOXA10 gene using the F2 generation not exposed to BPA.	BPA doses were: 0, 100, 200, 500, and 1, 000 mg/ kg/ day, GD 12-16.	Statistical analysis was performed with Student's t-test after the check homoscedasticity with F test.	TSG Comments: These doses are too high to be useful for hazard ID or support for Prop 65 listing; methodological limitations include: treatment of litter vs pup as experimental unit not addressed, subject number in F1 generation not clear, relationship between gene changes and possible adverse effects not clear.

EXHIBIT 9

(Table 6 – Summary of studies cited by Peretz et al. to support purported increased implantation failure in several strains and species)

Table 6. Epidemiological and experimental animal studies cited by Peretz et al. to support increased implantation failure in several strains and species.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Cabaton, 2011	BPA purity was assessed; study materials tested for estrogenicity	CD-1 mouse; age prenatal through 32 weeks.	18-21/dose	Non-GLP; One F1 female/litter was evaluated after pre- plus post-natal BPA exposure for reproduction effects using a continual forced breeding model.	Pre- and post-natal exposure via osmotic minipump implanted in dam, GD6 through PND 16; Vehicle, 50% dimethyl sulfoxide (DMSO)	Cumulative number of pregnancies and pups were counted in addition to time to first litter, deliveries/day, sex of pups	BPA doses: 0, 25, 250, and 25,000 ng/kg/day; DES reference estrogenic control: 10 ng/kg/day; cumulative number of pups was significantly decreased relative to control in the 25 and 25,000 ng/kg/day dose groups, but not in the 250 ng BPA/kg/day group.		FDA Comments: DMSO is not an appropriate solvent as it may alter the effects of the BPA; the dose response is unusual given a response in the low and high dose groups but not the mid-dose group, and forced breeding alters maternal hormones and may alter sensitivity to BPA through mechanisms not relevant to humans.
Kobayashi, 2010 (Kyoto)	Dietary BPA dose levels not confirmed.	C57BL/6J mice	12 males, 12 females of the F1 and F2 generations	Non-GLP; 2-generation exposure to evaluate reproduction and development.	Dietary exposure, F0 dams beginning GD6 ending at F2 pups, 15 weeks of age.	Body weight, food consumption, F0 gestation length, offspring body length, tail length, anogenital distance (AGD), ratio of AGD to cube root of body weight, organ weights, sperm count, sperm motility and progressivity	BPA doses: 0, 0.05, 0.5, and 5 mg/kg/day; Authors conclude there were no treatment-related effects.	litter as the unit of analysis	FDA Comments: A few statistically significant effects on body weight (increased), weights of epididymides (increased), and sperm motility (decreased) observed and dismissed by the authors as relatively small in magnitude and not consistent with age or across generations.
Kobayashi, 2012	BPA purity, >99.6%	Sprague Dawley rats	10/ dose/ sex	Non-GLP; Pre-and post-natal dietary BPA exposure to investigate the effects of low-dose BPA exposure on reproductive development in F1 male and female rat offspring.	Dietary exposure from GD6 through PND 21	Reproductive outcomes measured included: dam weight gain, gestation length, sex ratio, and live births per litter. F1 offspring were necropsied at 5 weeks or 3 months; Blood was collected for steroid hormone analysis; reproductive organs were removed and weighed.	BPA doses: 0, 0.017, 0.17, and 1.7 mg/kg/day; No effects on the reproductive outcomes for the F0 dams or F1 pups. There were no BPA related changes in body weight or reproductive organ weights in F1 male and female rats except a reduction in epididymis weight in the 3-month old F1 males in the high dose group.		FDA Comments: Authors stated the toxicological significance of the decrease weight of the epididymous was unknown; there was no discussion of potential environmental contamination, it is not clear how offspring were selected from litters for measured parameters.

Publication	Chemical Source (purity)	Species & Strain (sex & age)	#/Dose	Study Design (guideline, GLP?, test conditions)	Exposure Route (frequency, vehicle)	Endpoints Assessed (parents or offspring, methods)	Doses & Observations (analytical validation)	Statistics (methods)	Comments
Salian, 2009a	BPA purity 99.8%	Male Holtzman rats exposed PND 1 through 5, assessed PND 75		The study evaluated the effect of neonatal exposure to BPA on adult male rat sperm parameters and fertility.	BPA dosing was a daily subcutaneous injection from PND 1 through PND 5;	Serum hormone levels (LH, FSH, T, and E) and testis histopathology were examined in the control, 400 and 1600 µg/kg/day groups	BPA doses: approximately 200, 400, 800 and 1,600 µg/kg/day; Females mated with male rats exposed to doses of 400 ug/kg/day and higher showed significant loss in post-/pre-implantation survivability and a decrease in litter size.	The fact that 1 treated male was mated to two untreated females was not taken into account in the statistical analysis. A nested ANOVA (within the treated male) would have been appropriate.	TSG and FDA Comments: This study is not useful for evaluation of potential female reproductive toxicity; it isn't clear whether neonates were cross-fostered to limit litter effects;
Salian, 2009b	Not reported	Pregnant Holtzman rats exposed GD 12 through PND 21; F1 males were tested on PND 75		The study examined the effects of perinatal exposure of male rats to BPA on fertility parameters in the F1, F2, and F3 generations	P0 female rats were dosed with BPA by gavage daily at 0, 1.2 and 2.4 µg/kg bw from GD 12 until PND 21	Endpoints in untreated females bred with F1 male rats exposed to BPA neonatally include: post-implantation loss, and litter size; endpoints in F1 males include hormone levels and fertility measurements.	The authors report a significant increase in post-implantation loss, a decrease in litter size, and a decrease in male sperm count and motility in F1, F2 and F3 males; The authors conclude that perinatal exposure to BPA affects the male germ line leading to impairments in the fertility of F1 male offspring and their subsequent F2 and F3 generations.	Only the P0 dam was dosed and thus, was the experimental unit; this was not taken into account in the statistical analysis.	TSG and FDA Comments: This study is not useful for evaluation of potential female reproductive toxicity because male rats were studied; In-house bred rats were fed an in-house prepared diet, consequently replication of the results may be problematic; the fact that the F0 female was not considered the experimental unit is a critical flaw in the statistical analysis.