May 23, 2008

Dr. Barbara S. Shane
Executive Secretary for the NTP BSC
NTP Office of Liaison, Policy and Review
NIEHS
P.O. Box 12233
MD A3-01
Research Triangle Park, NC 27709

Sent as e-mail attachment to: shane@niehs.nih.gov

Re: Comments on the draft NTP Brief on bisphenol A

Dear Dr. Shane:

I am pleased to provide these comments on behalf of the Polycarbonate/BPA Global Group in regard to the draft NTP Brief bisphenol A. The Polycarbonate/BPA Global Group represents the leading global manufacturers of bisphenol A and polycarbonate plastic, who for many years have supported and conducted scientific research to understand whether bisphenol A has the potential to cause health or environmental effects and to support scientifically sound public policy.

Please do not hesitate to contact me if I can be of further assistance to clarify any comments or if additional information is needed. I can be reached at (703) 741-5588 or by e-mail at steve_hentges@americanchemistry.com.

Regards,

Steven G. Hentges, Ph.D.
Executive Director
Polycarbonate/BPA Global Group

Attachment

Comments of the Polycarbonate/BPA Global Group
on the Draft NTP Brief on Bisphenol A

May 23, 2008

1) **The NTP Draft Brief fails to comply with the Data Quality Act when it relies on inadequate studies for its conclusions**

   a. The Data Quality Act and NIH's Information Quality Guidelines require that the NTP Brief be based on studies conducted in accordance with sound and objective scientific practices that can be substantially reproduced and on data collected by accepted methods

   b. NTP did not apply rigorous scientific standards to ensure that studies upon which it relies meet the requirements of the Data Quality Act

2) **Under the Data Quality Act, NTP cannot rely on studies that use non-oral routes of exposure because they lack "utility"**

   a. The use of the subcutaneous route of administration for *adults* is inappropriate for human health risk assessment due to route dependency in the metabolism and pharmacokinetics of bisphenol A

   b. The use of the subcutaneous route of administration in *neonates* is inappropriate for human health risk assessment due to route dependency in the metabolism and pharmacokinetics of bisphenol A

3) **NTP improperly relied on numerous studies that do not meet the requirements of the Data Quality Act to reach the conclusions of "some concern for adverse effects" from low doses of bisphenol A on the mammary gland, prostate gland, earlier age for puberty in females, and neural and behavioral effects**

   a. Mammary gland

   b. Prostate gland

   c. Earlier age for puberty in females

   d. Neural and behavioral effects

The following review of the NTP process and the large body of data available on bisphenol A supports the conclusions of the CERHR expert panel and the other panels of experts that indicate a lack of concern for bisphenol A low dose effects based on the extensive weight of evidence. Our comments focus primarily on NTP's failure to rigorously evaluate each study upon which it relied in accordance with the requirements of the Data Quality Act.

1) The draft NTP Brief fails to comply with the Data Quality Act when it relies on inadequate studies for its conclusions

a. The Data Quality Act and NIH's Information Quality Guidelines require that the NTP Brief be based on studies conducted in accordance with sound and objective scientific practices that can be substantially reproduced and on data collected by accepted methods

The Data Quality Act, 44 U.S.C. Sec. 3516 et seq., required federal agencies to adopt information quality guidelines that ensure and maximize the quality, objectivity, utility, and integrity of information distributed by the agency. The Department of Health & Human Services (HHS) Guidelines for Ensuring and Maximizing the Quality, Objectivity, Utility, and Integrity of Information Disseminated to the Public include provisions applicable to the National Institutes of Health ("NIH"), of which the National Toxicology Program (NTP) is a part. With regard to "influential scientific information" the Guidelines require that:

> "influential" scientific, financial, or statistical information in official Government documents **must be based on studies that can be substantially reproduced** if the original or supporting data were to be independently reanalyzed using the same methods.… **NIH is committed to applying rigorous scientific standards to ensure the accuracy, reliability, and reproducibility of research results.**
>
> ***
>
> NIH makes every effort to ensure that … scientific conclusions are based on: (1) **The best available science and supporting studies, particularly peer-reviewed studies, conducted in accordance with sound and objective scientific practices**; and (2) **data collected by accepted methods or best available methods** (if the reliability of the method and the nature of the decision justifies use of the data)."

United States Department of Health & Human Services Guidelines for Ensuring the Quality of Information Disseminated to the Public, National Institutes of Health, Sec. VII (NIH Guidelines) (emphasis added).

It is clear that the NTP Brief on bisphenol A is "influential" scientific information covered by the requirements of the Data Quality Act and the NIH Guidelines. According to the NIH Guidelines, *"Influential"* means that the *"NIH can reasonably determine that dissemination of the information will have or does have a clear and substantial impact on important public policies or important private sector decisions, or will have important consequences for specific health practices, technologies, substances, products, or firms."* It can hardly be disputed that the Brief will have a clear and substantial affect on private sector decisions, and on substances, products, and firms. The stated goal of the Brief "is to provide the public, as well as government health, regulatory, and research agencies, with the NTP's conclusions regarding the potential for the chemical to adversely affect human reproductive health or children's development." Furthermore, the NTP Brief on bisphenol A is an official government document. (NIH Guidelines indicate that scientific research papers, journal articles, and similar authoritative materials (unless they contain a disclaimer), other official reports, and consensus panel reports are all among the NIH information that is covered by the NIH Guidelines.) Therefore, it is clear that the preparation of the NTP Brief is subject to the NIH Guidelines.

As the NTP Brief is subject to the requirements of the Data Quality Act, it must be based on *"studies that can be substantially reproduced",* and the scientific conclusions therein must be based on the *"best available science . . . conducted in accordance with sound and objective scientific practices" and "data collected by accepted methods or best available methods".* (see NIH Guidelines)

b. NTP did not apply rigorous scientific standards to ensure that studies upon which it relies meet the requirements of the Data Quality Act

NTP must apply appropriate data quality evaluations to data upon which it has based its conclusions. In its preparation of the draft Brief, NTP admittedly "did not establish strict criteria for determining which studies from the bisphenol A literature to consider for the evaluation." (see draft NTP Brief, page 10) The failure to properly evaluate the quality and utility of studies relied upon in the draft NTP Brief has resulted not only in inconsistent evaluation of the scientific quality and validity of studies, but also in conclusions based on numerous studies that have not:

- followed accepted methods (for example, OECD or other regulatory guideline studies);
- been generally accepted by the scientific community as to their validity or relevance for human health risk assessment; or
- been reproduced by other investigators.

Reliance on studies of questionable scientific value in reaching the conclusions of the draft Brief undercuts its fundamental purpose "to provide timely, unbiased, *scientifically sound* evaluations of the potential for adverse effects on reproduction or development resulting from human exposures to substances in the environment," and violates the principles of the Data Quality Act. (see draft NTP Brief, page 2)

In preparing the draft NTP Brief, a weight of evidence approach should be employed to systematically review and critically analyze the studies; that weight of evidence approach should include the following analytical elements:[1]

Elements Focused on Internal Validity

- Rigor – Studies should be evaluated for their proper conduct and analysis. Greater weight should be given to better-conducted studies. Some studies may have been performed so poorly that their results should be substantially or entirely discounted.

- Power – The statistical power of an experimental design should be examined for its ability to detect effects of a given magnitude. Studies of higher statistical power should be given higher weight over studies of lower statistical power that are otherwise comparable.

- Corroboration – The replication of findings among similar studies and the observation of similar effects under relevant conditions may increase the confidence that the findings represent a real effect in experimental animals as replication is a fundamental principle of the scientific process. Conversely, lack of corroboration across many studies with similar experimental conditions is grounds to doubt the validity of an experimental result reported in one or a few studies. In a multi-generational study, an exposure-related effect should appear across generations.

Elements Focused on External Validity

- Universality – The degree to which a finding (i.e., either the presence or absence of an effect) is consistently reproduced in validated study designs and test systems increases the confidence that it is valid and may apply to humans. In contrast, if an effect is restricted to a certain species, strain, or route of administration, the ability to generalize the response to other species or routes becomes more questionable.

[1] Gray, G. M., Baskin, S. I., Charnley, G., et al. 2001. The Annapolis accords on the use of toxicology in risk assessment and decision-making: An Annapolis Center workshop report. Toxicological Methods. 11(3):225-231.

- Proximity – When clearly established effects have been shown in a species with greater similarity to humans or at a dose level similar to that expected in humans, such results weigh more heavily than those in dissimilar species, by inappropriate routes, or at markedly different dose levels. In this regard, the significant route dependency and species dependency in the metabolism and pharmacokinetics of bisphenol A must be taken into account. In humans, the oral route of exposure is most relevant. By this route in humans, bisphenol A is subject to virtually complete pre-systemic clearance by first-pass metabolism to a primary metabolite (bisphenol A-glucuronide) that is then rapidly eliminated in urine. The primary metabolite has no known biological activity and, in particular, has been shown to have no estrogenic activity.

- Relevance – From what is known about the underlying biological basis for a toxic response in animals, it may be possible to judge (based on knowledge of animal and human physiology) whether similar metabolism, mechanisms of damage and their repair, and molecular targets of action should be expected to operate in humans. Confidence in applicability to humans can increase or decrease accordingly.

- Cohesion – The extent to which all of the data are consistent and are subject to a single, biologically plausible explanation increases weight compared to a situation where inconsistencies require ad hoc explanations and exceptions to general patterns. A common hypothesis is that bisphenol A may act via an estrogenic mode of action at low doses to cause adverse effects. The weight of evidence analysis should therefore examine the data to determine if there is a replicable pattern of estrogenic effects within and across the many studies. Lack of a consistent pattern of effects significantly reduces the biological plausibility of the hypothesis that bisphenol A acts via an estrogenic mechanism to cause reproductive and developmental effects at low doses.

2) Under the Data Quality Act, NTP cannot rely on studies that use non-oral routes of exposure because they lack "utility"

The closer that a study comes to replicating the human situation, the greater utility it has in evaluating potential effects on humans and the greater weight it should be given in a weight of evidence analysis. The vast majority of bisphenol A exposure is oral - through the diet (~99%). (see draft NTP Brief, pages 4 and 33) By the relevant oral route of exposure in humans, the bioavailability of bisphenol A is extremely low and there is no indication of biologically active metabolites, in particular estrogenic metabolites. Subcutaneous or parenteral injections result in blood levels of biologically active parent bisphenol A that are much higher than those seen after oral exposure. As a result, the CERHR expert panel report concluded that injection studies:

> *"Would produce irrelevantly high internal does of active parent compound, and would tend to produce "false positive" effects from the point of view of the human oral situation. Thus, the Panel viewed those otherwise adequate studies which injected bisphenol A as providing "supplemental" information (i.e. of limited utility), unless they also analyzed the levels of parent compound and metabolites after the injection."* (see NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of bisphenol A, November 26, 2007, page 122)

NTP too recognized that:

> *"Because the majority of exposure to bisphenol A occurs through the diet, laboratory studies that use the oral route of administration are considered the most useful to assess potential effects in humans. . . . [and that] none of the reproductive and developmental toxicity studies that treated animals by non-oral routes of administration determined the circulating levels of free bisphenol A or its metabolites.* ***As a result, studies that treat laboratory animals using non-oral routes of administration have often been considered of no or limited relevance for estimating potential risk to humans.***" (see draft NTP Brief, pages 11-12)

Yet, NTP failed to follow its own cautions or the Data Quality Guidelines when it relied on non-oral routes of administration studies for some of its conclusions. In preparing the draft NTP Brief, animal studies that use non-oral routes of exposure should be viewed as of limited or no utility for NTP's conclusions.

a. The use of the subcutaneous route of administration for *adults* is inappropriate for human health risk assessment due to route dependency in the metabolism and pharmacokinetics of bisphenol A

The draft NTP Brief's reliance on non-oral route of exposure studies is particularly unjustified since NTP itself recognized the significant scientific shortcomings of non-oral rodent studies involving adult animals. Specifically, the draft NTP Brief (page 13) states: "Studies in adult animals, including pregnant dams, that administered bisphenol A by subcutaneous injection or by a subcutaneous mini-pump were considered informative for identifying biological effects of bisphenol A but not for quantitatively comparing exposures in laboratory animals and humans."

Nevertheless, NTP partially based its conclusion that there is "some concern for adverse effects" on development in fetuses, infants, and children on such studies. In particular, NTP's conclusion that there is "limited evidence of adverse effects" on the mammary gland from low doses of bisphenol A is based on two studies[2,3] in which adult dams are dosed by subcutaneous osmotic pumps. The findings reported in these studies have not been replicated or corroborated in studies using the oral route of exposure, which is most relevant for humans. Consequently, reliance on these studies to support a conclusion of some concern for effects on the mammary gland does not meet the requirements of the Data Quality Act.

With respect to potential effects of low doses of bisphenol A on the mammary gland, a more appropriate conclusion is "insufficient evidence for a conclusion" (in Figure 2b) and "insufficient hazard and/or exposure data" (in Figure 3).

<u>Basis for not relying on studies that use non-oral routes of administration in adult rodents</u>

As stated in the draft NTP Brief (page 11): "There is scientific consensus that doses of bisphenol A administered orally and subcutaneously cannot be directly compared in adult laboratory animals because the rate of metabolism of bisphenol A differs following oral and non-oral administration." Consequently, since humans are exposed to bisphenol A by the oral route of exposure, laboratory animal studies using non-oral routes of exposure are of limited relevance for risk assessment.

It is well known that bisphenol A is efficiently biotransformed to bisphenol A-glucuronide in the gut wall[4] and the liver[5] after oral administration. In contrast,

---

[2] Durando, M., Kass, L., Piva, J., Sonnenschein, C., Soto, A. M., Luque, E. H., and Munoz-de-Toro, M. 2006. Prenatal bisphenol exposure induces preneoplastic lesions in the mammary gland of Wistar rats. Environmental Health Perspectives. 115(1):80-86. (Note: page numbers incorrectly cited in the draft NTP Brief)

[3] Murray, T. J., Maffini, M. V., Ucci, A. A., Sonnenschein, C., and Soto, A. M. 2007. Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol A exposure. Reproductive Toxicology. 23(3):383-390. (Note: page numbers incorrectly cited in the draft NTP Brief)

[4] Inoue, H., Yuki, G., Yokota, H., and Kato, S. 2003. Bisphenol A glucuronidation and absorption in rat intestine. Drug Metabolism and Disposition. 31(1):140-144.

parenteral routes of administration, which bypass this efficient first-pass metabolism, have been shown to result in higher bioavailability of free bisphenol A and route-dependent metabolism, producing a potentially different toxicity profile.[6]

Most notably, a study on the metabolism and pharmacokinetics of bisphenol A in rats investigated a single dose of 10 or 100 mg/kg ^{14}C-labelled bisphenol A administered by oral gavage, intraperitoneal or subcutaneous injection.[6] Unchanged bisphenol A and radioactivity were determined in blood and plasma samples. Radioactivity was determined in urine and fecal samples. At 7 days post-dosing, animals were sacrificed and selected tissues (brain, liver, kidneys, peri-renal fat, gonads, uterus, and skin) and remaining carcass were analyzed for radioactivity. Examination of metabolic products was also conducted by HPLC on selected urine and fecal samples. The authors concluded:

- "The relative bioavailability of BPA was highly route-dependent. Estimation of BPA AUC for sc administration resulted in values from 7-fold (high dose females) to 245-fold (high does males) greater than for oral administration"

- "Oral administration resulted in Cmax values that were from 1 to 2 orders of magnitude lower than those found following ip or sc administration"

- Three unidentified metabolites not seen following oral doses were observed following ip or sc exposure.

- "Both ip and sc administration resulted in larger fractions of plasma radioactivity comprised of unchanged parent compound. Unchanged parent comprised 27-51% and 65-76% of total plasma radioactivity following ip and sc administration, respectively, whereas oral administration resulted in only 2-8% of total plasma radioactivity comprised of unchanged parent compound"

A metabolism study in mice[7] reported that no *qualitative* differences between metabolites following subcutaneous administration and oral administration were observed (although the data were not shown). However, no information on *quantitative* differences in the metabolites formed as a function of the route of administration was provided. The

---

[5] Inoue, H., Yokota, H., Makino, T., Yuasa, A., and Kato, S. 2001. Bisphenol A glucuronide, a major metabolite in rat bile after perfusion. Drug Metabolism and Disposition. 29(8):1084-1087.

[6] Pottenger, L.H., Domoradzki, J.Y., Markham, D.A., Hansen, S.C., Cagen, S.Z., and Waechter, J.M. 2000. The relative bioavailability and metabolism of bisphenol A in rats is dependent upon the route of administration. Toxicological Sciences. 54:3-18.

[7] Zalko, D., Soto, A.M., Dolo, L., Dorio, C., Rathahao, E., Debrauwer, L., Faure, R., and Cravedi, J.P. 2003. Biotransformations of bisphenol A in a mammalian model: Answers and new questions raised by low-dose metabolic fate studies in pregnant CD1 mice. Environmental Health Perspectives. 111(3):309-319.

quantity of phase I metabolites, in particular, is important because these metabolites may have significant bioactivity that could contribute to the production of adverse effects.[8]

As expected, based on differences in metabolism and pharmacokinetics, a significant difference in the toxicity of bisphenol A when administered by the oral and subcutaneous routes of exposure has been demonstrated experimentally in an immature rat uterotrophic assay.[9] Female rats were given daily subcutaneous injections of 0, 8, 40 or 160 mg/kg bisphenol A, or daily oral (gavage) doses of 0, 40, 160 or 800 mg/kg bisphenol A, on postnatal days 18-20. Animals were sacrificed 24 hours after the final dose and uteri removed and weighed. A repeat study using the same experimental protocol was conducted. Statistically significant increases in the relative wet and dry uterine weights were seen at 160 mg/kg and above after oral dosing and at 8 mg/kg (lowest dose investigated) and above for subcutaneous dosing. This >20 fold higher sensitivity after subcutaneous dosing is consistent with the much lower bioavailability of bisphenol A following oral administration.

The use of laboratory animal studies using non-oral routes of administration for either hazard identification or risk assessment is also tenuous based on the known pharmacokinetics and metabolism in humans and non-human primates following oral administration. In two human studies[10,11] and a monkey study[12], free bisphenol A was below the limit of detection in all urine and blood samples after oral administration. These data demonstrate that laboratory animal studies using non-oral routes of administration, which result in much higher bioavailability of free bisphenol A, are not directly applicable to human risk assessment

Furthermore, the data from the recent paper of Taylor et al. (2008) (if valid; see comments below) is not relevant for studies dosing adults as only neonates were dosed.[13] Further discussion on this study is provided in the following section in regard to its utility for studies on neonates.

Based on these considerations, it is clear that the use of non-oral routes of administration in rodents is not the best available method to assess effects of bisphenol A for use in a

---

[8] Yoshihara, S., Mizutare, T., Makishima, M., Suzuki, N., Fujimoto, N., Igarashi, K., and Ohta, S. 2004. Potent estrogenic metabolites of bisphenol A and bisphenol B formed by rat liver S9 fraction: Their structures and estrogenic potency. Toxicological Sciences. 78(3):50-59.

[9] Yamasaki, K., Sawaki, M., and Takatsuki, M. 2000. Immature rat uterotrophic assay of bisphenol A. Environmental Health Perspectives. 108(12):1147-1150.

[10] Völkel, W., Bittner, N., and Dekant, W. 2005. Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by HPLC-MS/MS. Drug Metabolism and Disposition. 33(11):1748-1757.

[11] Völkel, W., Colnot, T., Csanady, G.A., Filser, J.G., and Dekant, W. 2002. Metabolism and kinetics of bisphenol A in humans at low doses following oral administration. Chemical Research in Toxicology. 15(10):1281-1287.

[12] Kurebayashi, H., Harada, R., Steward, R.K., Numata, H., and Ohno, Y. 2002. Disposition of a low dose of bisphenol A in male and female cynomolgus monkeys. Toxicological Sciences. 68:32-42.

[13] Taylor, J. A., Welshon, W. V., and vom Saal, F. S. 2008. No effect of route of exposure (oral; subcutaneous injection) on plasma bisphenol A throughout 24 hr after administration in neonatal female mice. Reproductive Toxicology. 25(2):169-176.

human health risk assessment. Consequently, data from such studies cannot be relied upon under the Data Quality Act.

b. The use of the subcutaneous route of administration in *neonates* is inappropriate for human health risk assessment due to route dependency in the metabolism and pharmacokinetics of bisphenol A

The draft NTP Brief (page 12) states: "neonatal rats do have the ability to metabolize bisphenol A as indicated by the presence of bisphenol A glucuronide in the blood and the inability to detect the free form within the measurement sensitivity of the assay by 12 to 24-hours after treatment in females and males respectively" and "Neonatal rats appear to be able to more efficiently metabolize bisphenol A when given at lower dose levels than at higher dose levels." Taken together, these comments indicate that NTP understands that the question is not whether neonates have the capability to metabolize bisphenol A (they do), but rather whether neonates have the capacity to metabolize very low doses that are relevant for human exposures.

This understanding is based partly on a study, discussed further below, that found that in 4-day old male and female rats treated with 1 mg/kg of bisphenol A, 98-100% of the administered dose was detected in plasma as bisphenol A-glucuronide.[14] Nevertheless, NTP then stated (page 13) that "data from studies where bisphenol A was given by subcutaneous injection were considered as useful in the NTP evaluation as oral administration when treatment occurred during infancy." This position is based on the results of one flawed study that reported no differences in the blood concentration of free bisphenol A as a function of route of administration (oral versus subcutaneous injection) in 3-day old female mice following treatment with either 0.035 or 0.395 mg/kg of bisphenol A.[13]

As a result of this position, NTP partially based its conclusion that there is "some concern for adverse effect" on development in fetuses, infants and children on a study involving subcutaneous injection of bisphenol A into neonates. In particular, NTP's conclusion that there is "limited evidence of adverse effects" on the prostate gland from low doses of bisphenol A is based on one study in which neonatal rats were administered bisphenol A by subcutaneous injection.[15] The findings reported in this study have not been replicated or corroborated in studies using the oral route of exposure and, consequently, reliance on this study to support a conclusion of some concern for effects on the prostate gland does not meet the requirements of the Data Quality Act.

---

[14] Domoradzki, J. Y., Thornton, C. M., Pottenger, L. H., Hansen, S. C., Card, T. L., Markham, D. A., Dryzga, M. D., Shiotsuka, R. N., and Waechter, J. M. 2004. Age and dose dependency of the pharmacokinetics and metabolism of bisphenol A in neonatal Sprague-Dawley rats following oral administration. Toxicological Sciences. 77(2):230-242.

[15] Ho, S.-M., Tang, W.-Y., Belmonte de Frausto, J., and Prins, G. S. 2006. Developmental exposure to estradiol and bisphenol A increases susceptibility to prostate carcinogenesis and epigenetically regulates phosphodiesterase type 4 variant 4. Cancer Research. 66(11):5624-5632.

With respect to potential effects of low doses of bisphenol A on the prostate gland, a more appropriate conclusion is "insufficient evidence for a conclusion" (in Figure 2b) and "insufficient hazard and/or exposure data" (in Figure 3).

Basis for not relying on studies that use non-oral routes of administration in neonatal rodents

As discussed in our earlier comments to the final CERHR report, the far-reaching conclusions presented by Taylor et al.[13] are not supported by the experimental data. The study was conducted to test the hypothesis that the known rapid metabolism of bisphenol A to the corresponding glucuronide in adults would not occur in neonates due to lower UDP-glucuronosyltransferase activity at early stages of development. However, due to several significant flaws in the design and execution of this study, that hypothesis is not tested in this study at all and, as a result, the author's conclusions are tenuous if not completely unfounded. The significant flaws include:

- Conjugated metabolites are neither directly not indirectly measured

  The procedure to extract plasma (with tert-butyl methyl ether) is reported to extract bisphenol A but not water soluble conjugates. Consequently, since the extraction procedure excludes conjugated metabolites entirely from the analysis, the study provides no direct or indirect measure of whether or to what extent bisphenol A was metabolized to the glucuronide or to any other metabolite. In particular, if sulfotransferase is expressed in the neonatal mouse, as would be expected, the analysis is also incapable of measuring the extent of sulfonation of bisphenol A to the corresponding sulfate. The study is thus incapable of testing the hypothesis that conjugative metabolism of bisphenol A in neonates does not occur.

  As noted above, non-oral routes of exposure can produce a different profile of metabolites compared to oral exposure and the extent to which additional metabolites formed with non-oral routes of exposure contribute to toxicity observed in non-oral studies is not known. An assessment of the presence or significance of additional metabolites formed by non-oral exposure in neonates is precluded in this study since the extraction and analytical procedure does not measure any metabolites.

- No mass balance was conducted to determine the fate of the administered dose

  Based on data presented in the paper, only a very small fraction of the dose administered was recovered in the HPLC fractions reported to be bisphenol A. At C_{max}, approximately 0.04% of the administered radioactivity was recovered in these fractions. No information is provided to account for the remaining 99.96% of the administered dose. In particular, no information is provided to determine

whether or how much of the administered bisphenol A has been metabolized or to what metabolite(s). The conclusions of the study are thus based on analysis of only a tiny fraction of the dose administered.

- Bisphenol A itself is not positively identified in the analytical procedure

  A further limitation is that quantitation of parent bisphenol A in plasma was based only on scintillation counting of HPLC fractions. The fractions were collected over a very broad peak with a base peak width of over three minutes that elutes very close to the solvent front. No structural confirmation was conducted (e.g., mass spectrometry) to confirm that the radiolabeled peak consists partly or entirely of bisphenol A.

  Without positive identification of structure, it cannot be certain whether the fractions contain bisphenol A, an impurity originating with the ^{3}H-bisphenol A sample,[16] or other substances resulting from radiolabel exchange.[17] At a minimum, the authors should have analyzed $^{3}H_2O$ via the stated HPLC method, to verify that the purported bisphenol A peak was not tritiated water arising from metabolic/exchange loss of the radiolabel.

  In addition to the lack of positive structural identification, the chromatographic procedure has essentially no capability of separating individual components that may be co-eluting over the three minute retention window assigned to bisphenol A.[18]

  To the extent that the detected peak represents bisphenol A, no analytical controls (e.g., spike and recovery experiments with authentic bisphenol A-glucuronide and bisphenol A-sulfate conjugates) were included to be sure that the bisphenol A was truly present in plasma rather than forming adventitiously by hydrolysis of small amounts of conjugated metabolites during the extraction and analytical procedure.

---

[16] According to the supplier, ^{3}H-bisphenol A is provided as an ethanol solution with purity ≥97%. Some or all of the peak reported to be bisphenol A, at only 0.04% of the administered dose, could easily be an impurity.

[17] Radiolabel exchange of tritium from metabolism or during processing/analysis has been reported in other cases. For example, see Marcus, S. N. and Heaton, K. W. 1988. Evidence for loss of tritium from 3β-tritiated deoxycholic acid during enterohepatic cycling in man. Lipids. 23(6):629-630, and Raeside, J. I., Christie, H. L., Renaud, R. L., Waelchli, R. O., and Betteridge, K. J. 2004. Estrogen metabolism in the equine conceptus and endometrium during early pregnancy in relation to estrogen concentrations in the yolk-sac fluid. Biology of Reproduction. 71:1120-1127.

[18] Given the HPLC column (4.6 X 100 mm) void volume of 1.16 mL, it requires 1.4 minutes for an unretained component to elute from the column at the stated flow rate of 0.82 mL/min. A standard column efficiency calculation [$N = 16\ (t_r / W_b)^2$] yields a value of 36 which is much lower than what is typical for this column (a one-minute wide peak yields N = 324).

As discussed in Section 2a above, the seminal and most instructive study on the route-dependency of metabolism and pharmacokinetics of bisphenol A is Pottenger et al. (2000).[6] This study showed a clear route-dependency in the bioavailability of bisphenol A and its metabolites between subcutaneous and oral administration with subcutaneous administration resulting in much greater bioavailability, as well as unique metabolites not observed following oral administration.

Similarly, in neonatal rats orally administered 1 or 10 mg/kg bisphenol A at postnatal days 4, 7, or 21, bisphenol A was metabolized to bisphenol A-glucuronide at all three ages, although an age dependency in the number and concentration of plasma metabolites was observed, consistent with the ontogeny of UDP-glucuronosyltransferases.[14] This study demonstrated nearly complete glucuronidation of a 1 mg/kg dose of bisphenol A, even in postnatal day 4 rats (98-100% of administered bisphenol A was detected in plasma as bisphenol A-glucuronide). As the dose of 1 mg/kg was clearly below the level where available UDP-glucuronosyltransferase activity was saturated, the much lower oral doses of bisphenol A that are typical of environmental exposures would be within the range of linear pharmacokinetics and would be efficiently conjugated.

These data indicate that there is sufficient capacity from early in neonatal life to efficiently metabolize bisphenol A to the non-estrogenic bisphenol A-glucuronide after oral exposure to low doses. At higher doses of 10 mg/kg and above, glucuronidation of bisphenol A may be saturated.

Consistent with these data, increased sensitivity of offspring (neonates) to bisphenol A was not observed in guideline multigeneration studies in both rats and mice in which dams and offspring were exposed to dietary bisphenol A.[19,20,21] Developmental effects were observed only at high doses exceeding the maximum tolerated dose in the dams.

Under the Data Quality Act. NTP must rely on the best available science, conducted by accepted scientific methods. As demonstrated by the discussion above, the use of non-oral methods of administration in neonates (and reliance on the Taylor study) does not meet this standard.

---

[19] Tyl, R.W., Myers, C.B., Marr, M.C., Thomas, B.F., Keimowitz, A.R., Brine, D.R., Veselica, M.M., Fail, P.A., Chang, T.Y., Seely, J.C., Joiner, R.L., Butala, J.H., Dimond, S.S., Cagen, S.Z., Shiotsuka, R.N., Stropp, G.D., and Waechter, J.M. 2002. Three-generation reproductive toxicity study of dietary bisphenol A in CD Sprague-Dawley rats. Toxicological Sciences. 68:121-146.

[20] Ema, M., Fujii, S., Furukawa, M., Kiguchi, M., Ikka, T., and Harazono, A. 2001. Rat two-generation reproductive toxicity study of bisphenol A. Reproductive Toxicology. 15:505-523.

[21] Tyl, R. W., Myers, C. B., Marr, M. C., Sloan, C. S., Castillo, N. P., Veselica, M. M., Seely, J. C., Dimond, S. S., Van Miller, J. P., Shiotsuka, R. N., Beyer, D., Hentges, S. G., and Waechter, J. M. 2008. Two-generation reproductive toxicity study of dietary bisphenol A (BPA) in CD-1® (Swiss) mice. Toxicological Sciences. In Press.

3) NTP improperly relied on numerous studies that do not meet the requirements of the Data Quality Act to reach the conclusions of "some concern for adverse effects" from low doses of bisphenol A on the mammary gland, prostate gland, earlier age for puberty in females, and neural and behavioral effects

a. Mammary gland

Two studies[2,3] were cited in the draft NTP Brief to support the conclusion that there is "limited evidence of adverse effects" on the mammary gland from low doses of bisphenol A, which provides part of the basis for the conclusion of "some concern for adverse effects" on development in fetuses, infants and children. Neither of these studies satisfies the requirements of the Data Quality Act.

Referring to the study by Durando et al. (2007),[2] the draft NTP Brief (page 21) noted "the CERHR Expert Panel on Bisphenol A considered this study critically flawed", but is was nevertheless relied upon by NTP. This discrepancy between the conclusions of the CERHR Expert Panel on Bisphenol A and this draft NTP Brief is a direct consequence of not having established strict criteria for acceptance of studies for consideration in the draft NTP Brief and is one of the key short-comings of this draft NTP Brief.

The NTP Brief cites the following significant methodological error in the Durando et al. study: "*The study by Durando et al. (44) implied that 99.9% DMSO was used in the mini-pump ["Pumps are designed to deliver 25 BPA (Sigma-Aldrich de Argentina S.A., Buenos Aires, Argentina) or only DMSO (99.9% molecular biology grade, Sigma-Aldrich de Argentina S.A.)"]. The manufacturer of the mini-pump does not recommend use of DMSO concentrations greater than 50% because it can degrade the pump reservoir material and potentially result in tissue inflammation and edema. For this reason, the CERHR Expert Panel on Bisphenol A considered this study critically flawed (2). The NTP concurs that use of a high concentration of DMSO is a technical short-coming, but is not convinced that this factor could account for the observed results. The NTP also considered the possibility that potential pump degradation could result in variations in administered dose, but concluded that the study was still useful to consider in the context of other findings.*" It is not feasible to reach NTP's conclusion on the validity of this study without substantial additional information. Indeed, the misuse of critical dosing equipment is adequate to invalidate the study. Therefore, NTP's reliance on this study does not satisfy NIH's commitment "to applying rigorous scientific standards to ensure the accuracy, reliability, and reproducibility of research results" under the Data Quality Act and NIH Guidelines.

With respect to Murray et al. (2007),[3] the CERHR Expert Panel found that this study was inadequate due to small sample size, route of administration, and lack of clarity on statistical analysis. This study reported that the mean values of % hyperplastic ducts were found to be significantly elevated for all doses, but with no apparent dose dependence (consistently in the range of 25% on PND50) despite a 400-fold difference in

range of estimated dose administered. Even more confounding is the consistent decrease in degree of response to bisphenol A of this same endpoint over time; i.e., the animals subsequently sacrificed on PND95 showed a statistically significant increase only in the lowest dose group (2.5 µg/kg bw/day) and even for this group the mean % hyperplastic ducts had decreased to approximately 13%. This information would lead to the conclusion that either this lesion is reversible or that it is not a reproducible phenomenon.

Equally perplexing is the inconsistency between the Murray et al. and Durando et al. data from the same laboratory. Durando et al. reported a statistically significant increase in % hyperplastic ducts in animals treated with 25 µg/kg bw/day bisphenol A and examined on PND110 and PND180. In marked contrast, Murray et al. showed no significant increase in % hyperplastic ducts in animals treated with 25 µg/kg bw/day and sacrificed on PND95, a sacrifice time period which closely approximates that of the PND110 group. This inconsistency in findings for the same endpoint for two studies from the same laboratory, in and of itself, should lead to a requirement for replication by another group of investigators before this information is considered relevant as a potential human health concern. This apparent discrepancy casts substantial doubt on the reproducibility of this endpoint as a bisphenol A-related effect for any of the doses tested by Murray et al.

The studies by Murray et al. and Durando et al. were from the same laboratory and used an osmotic pump to deliver bisphenol A to the pregnant dams. The critical data not provided in these publications to allow for assessment of their relevance and applicability to human health risk assessment is the metabolism and kinetics of free bisphenol A in blood when bisphenol A is delivered via an osmotic pump compared to comparable doses delivered via the oral route, and whether there were any route-dependent differences in metabolites. Based on the studies of Pottenger et al., (2000),[6] it is likely that dosing by osmotic pump also results in the extrahepatic metabolism of bisphenol A as described for subcutaneous administration.

The draft NTP Brief develops a line of logic attempting to relate hyperplastic ducts, carcinoma in situ and exposure to low doses of bisphenol A leading to a predisposition to development of cancer. However, Murray et al. clearly state "Whether the incidence of CIS might be increased in the bisphenol A-exposed animals at later time points remains to be determined." Thus, the implications made in the draft NTP Brief of extending the generalization of hyperplastic ducts leading to tumor formation or metastatic lesions is not supported.

The study by Moral et al. (2008)[22] offers additional insight on the reported findings by Murray et al. and Durando et al. Moral administered bisphenol A to dams during the gestation period using two of the same doses used by Murray et al. (25 µg/kg bw/day and

[22] Moral, R., Wang, R., Russo, I. H., Lamartiniere, C. A., Pereira, J., and Russo, J. 2008. Effect of prenatal exposure to the endocrine disruptor bisphenol A on mammary gland morphology and gene expression signature. Journal of Endocrinology. 196(1):101-112.

250 µg/kg bw/day). However, the major difference was that Moral et al. used oral gavage, a more relevant route of administration for determination of potential human health effects. The endpoints that allow for comparison of these two studies are those related to morphologic analysis of mammary glands and measures of cellular proliferation. Assessing whether bisphenol A administration was related to any morphologic findings, Moral found no change attributable to bisphenol A as a function of dose (25 or 250 µg/kg bw/day) or time (animals sacrificed on PND 21, 35, 50 and 100) for terminal end buds and number of alveolar buds when compared to controls. The number of terminal ducts was statistically elevated only for animals gavaged with 250 µg/kg bw/day and sacrificed on PND 21 and PND 100 but not during the intervening times (PND 35 or PND 50). The number of lobules type 1 was found to be elevated only on PND 35 and again only for animals dosed with 250 µg/kg bw/day. Taken together, these findings put into question the reproducibility and ultimate relevance of reported increases in % hyperplastic ducts at 25 and 250 µg/kg bw/day on PND 50 and PND 95 as reported by Murray et al. The second finding by Moral et al. was that the bisphenol A exposed groups did not show a major influence of bisphenol A on cell proliferation index. This finding stands in direct contrast to the reported effects of bisphenol A significantly increasing cell proliferative activity on PND 50 and PND 95 by Murray et al.

With but a single exception, the other studies cited in the draft NTP Brief on mammary gland effects involved administration of bisphenol A via a mini-pump**.** As discussed above, the use of such studies is inappropriate and not the best available method.

The recent study by Moral et al. using the oral route of exposure and having direct points of comparison (dose and time of sacrifice) for the critical endpoints enumerated by the draft NTP Brief adds considerable reason to question whether there is sufficient experimental evidence to reach the level of certitude to conclude there is "limited evidence of adverse effects" on the mammary gland.

As noted by NTP, the current literature is not sufficient to establish the reproducibility of the studies relied upon by NTP to reach a conclusion of "some concern" for effects on the mammary gland. With respect to potential effects of low doses of bisphenol A on the mammary gland, a more appropriate conclusion is "insufficient evidence for a conclusion" (in Figure 2b) and "insufficient hazard and/or exposure data" (in Figure 3).

b. Prostate gland

Two studies[15,23] were cited in the draft NTP Brief to support the conclusion that there is "limited evidence of adverse effects" on the prostate gland from low doses of bisphenol A, which provides part of the basis for the conclusion of "some concern for adverse effects" on development in fetuses, infants and children. Neither of these studies satisfies the requirements of the Data Quality Act.

With regard to Timms,[23] NTP stated (page 23), "These effects were observed in fetal mice and it is unclear if they persist into adulthood or relate to a clear adverse health outcome." Furthermore, NTP noted (page 23), "It is important to note that other studies have not reported severe consequences of urinary tract constriction in adult animals exposed during development that might be predicted based on the finding by Timms *et al*. including bladder stones, hydronephrosis, hydroureter, or other indications of kidney toxicity." As the results of this study have not been replicated or corroborated, or even relate to a clear adverse health outcome, this study is not adequate under the Data Quality Act to support a conclusion of "some concern" for prostate effects from exposure to low doses of bisphenol A.

In the other study relied on by NTP, Ho et al. (2006)[15] investigated the effect of short-term neonatal exposure to bisphenol A on susceptibility of Sprague Dawley rats to prostate cancer. On PND 1, 3, and 5 male pups were subcutaneously injected with bisphenol A at 0.1µg/pup (0.010 mg/kg bw), or estradiol benzoate (EB). At PND 90, half of the rats from each treatment group were implanted with silastic capsules containing 17β-estradiol (E) and testosterone (T) and the other half were implanted with empty capsules; the capsules were left in place for 16 weeks when rats were sacrificed, prostates were removed, and histopathological evaluations were conducted on each lobe. Immunohistochemistry techniques were used to measure proliferation. Apoptosis was measured using the TUNEL technique. In addition, PCR techniques were used to study methylation pattern and expression changes in prostate cell signaling proteins on PND 10, 90 (before adult E+T treatment), and 200.

In animals that did not receive E+T in adulthood, bisphenol A exposure had no effects on dorsal prostate weight, histopathology alterations, proliferation index, or apoptotic index. Overall, this study showed that short-term neonatal exposure of male rats to 10 µg/kg bw bisphenol A by subcutaneous administration had no effect on the prostate gland later on in life (at 6-7 months of age). The observation of hypomethylation of the cell signaling gene, PDE4, in the bisphenol A-treated animals, in itself does not represent an adverse effect.

[23] Timms, B. G., Howdeshell, K. L., Barton, L., Bradley, S., Richter, C. A., and vom Saal, F. S. 2005. Estrogenic chemicals in plastic and oral contraceptives disrupt development of the fetal mouse prostate and urethra. Proceedings of the National Academy of Sciences. 102(19):7014-7019.

In animals treated with E+T in adulthood, bisphenol A exposure resulted in a statistically significant increased incidence and severity of PIN. In the bisphenol A/E+T group compared to the E+T group, the proliferation index was increased and the apoptosis index was decreased in regions where PIN was observed.

Although the study authors claim that PIN is a precancerous lesion leading to prostate cancer, as the animals were sacrificed at 6-7 months of age, this could not be verified and, hence, the toxicological significance of PIN in animals remains unknown. Due to the following experimental limitations it is difficult to establish whether the increased incidence of E+T-induced prostate lesions was a real, bisphenol A-related effect.

- The data reported by the authors were obtained in a single experiment with a low number of animals per group (6 to 10 males)
- The authors used only a single bisphenol A dose group and consequently no information on dose response is available
- No information was provided on the background variation of PIN in this strain of rats and on the experimental variation of E+T-induced PIN
- PIN was investigated at a single time point and no information on progression is available
- No information is given how new-born pups were assigned to experimental groups (i.e., it is not indicated if all doses were represented within litter rearing units)
- The authors used subcutaneous injection to dose offspring (see discussion on limitations of subcutaneous dosing in section 2b above)

NTP's reliance on this study is presumably supported by their interpretation of the validity of the Taylor et al. (2008) study.[13] As detailed in section 2b above, the Taylor et al. study has serious methodological flaws that include among others: conjugated metabolites were neither directly nor indirectly measured, no mass balance was conducted to determine the fate of the administered dose, and bisphenol A itself was not positively identified in the analytical procedure. Therefore, the Taylor et al. study can not be used to support the study by Ho et al., which uses a non-validated method to evaluate endpoints, specifically prostate gland lesions, that have unknown etiology and consequence.

The CERHR expert panel evaluated the Ho et al. paper as adequate and of limited utility. Concerning the prostate gland, the CERHR expert panel overall concluded "the Expert Panel has minimal concern" and indicated additional data are needed to understand the biology of PIN in animal models and its relationship to prostate cancer.

In addition to the CERHR expert panel's appropriate conclusion on the uncertainty of the adversity of PIN lesions, it should also be noted that PIN lesions were not observed in prostates from a large number of control, estradiol-treated or bisphenol A-treated adult males in mouse and rat multigeneration studies of Tyl et al.[19,21,24] Lesions of this type would have been observed in these studies had they been present.

The draft NTP Brief also indicated that a recent study,[25] not previously evaluated by the CERHR expert panel, is consistent with Ho et al.: "The findings of Ho *et al.* (*43*) are consistent with a recent report of increased expression of cytokeratin 10 (CK10), a cell-marker associated with squamous differentiation, in adult male offspring of pregnant mice orally treated with 0.020 mg/kg bw/day bisphenol A during gestation (*181*)."

This recent publication by Ogura et al (2007)[25] investigated in vitro and in vivo differentiation of mouse prostate by immunohistochemistry; anti-cytokeratin 14 (CK14) antibodies were used to identify basal epithelial cells and anticytokeratin 10 (CK10) antibodies to identify keratinization of the epithelium. In the experiment mentioned in the draft NTP Brief, the authors dosed pregnant female BALB/c mice by gavage with 20 µg/kg/day bisphenol A (or 0.2 µg/kg/day DES) from GD 13–18 and prostates of male offspring were investigated at 12 weeks of age. The authors reported that prostatic ducts in DES and bisphenol A groups were morphologically indistinguishable from the control group and added "However, in both the DES and BPA treated-group, CK14 positive basal epithelial cells appeared to co-express CK10 in prenatally drug-treated mice aged to 12 weeks." Due to the following limitations, no conclusion should be drawn from this study, and the paper should be ranked inadequate for the evaluation process.

- The number of dams used in the experiment is very low (N=3) and no information is given on the number of male offspring investigated
- Data are reported in a single figure, and the statement "CK14 positive basal epithelial cells *appeared to* co-express CK10" can not be judged without additional information
- No information is given regarding whether the histopathological samples were blinded during investigation of these "subtle molecular changes"
- No information is provided concerning historical data or the biological variability of CK14 or CK10 expression

[24] Tyl, R. W., Myers, C. B., Marr, M. C., Sloan, C. S., Castillo, N. P., Veselica, M. M., Seely, J. C., Dimond, S. S., Van Miller, J. P., Shiotsuka, R. S., Stropp, G. D., Waechter, J. M., and Hentges, S. G. 2008. Two-generation reproductive toxicity evaluation of dietary 17ß-estradiol (E2; CAS No. 50-28-2) in CD-1® (Swiss) mice. Toxicological Sciences. 102(2):392-412.

[25] Ogura, Y., Ishii, K., Kanda, H., Kanai, M., Arima, K., Wang, Y., and Sugimura, Y. 2007. Bisphenol A induces permanent squamous change in mouse prostatic epithelium. Differentiation. 75(8):745-756.

– Additionally, the observations are difficult to interpret because only a single dose of bisphenol A was investigated and histopathology was performed only at a single time-point

With respect to potential effects of low doses of bisphenol A on the prostate gland, a more appropriate conclusion is "insufficient evidence for a conclusion" (in Figure 2b) and "insufficient hazard and/or exposure data" (in Figure 3).

c. Earlier age for puberty in females

Two studies[26,27] were cited in the draft NTP Brief to support the conclusion that there is "limited evidence of adverse effects" on early onset of puberty in female rats from low doses of bisphenol A, which provides part of the basis for the conclusion of "some concern for adverse effects" on development in fetuses, infants and children. Neither of these studies satisfies the requirements of the Data Quality Act.

Howdeshell et al. (1999)[26] reported no change in the age to vaginal patency or first estrus but only in the interval between these two endpoints. This is a derived variable, not a directly measured endpoint, which is not standard in toxicology and should not be interpreted as a sensitive indicator of onset of puberty. In addition, Howdeshell et al. only reported an effect on puberty in females with an inter-uterine position of "0M", that is, a female positioned between two other females in utero, and not in animals with any other inter-uterine position.

NTP also stated: "These findings are supported by another study that noted an early onset of puberty in female ICR/Jcl mice whose mothers were treated with 0.02 mg/kg bw/day bisphenol A during gestation by subcutaneous injection (Honma et al., 2002)." The CERHR expert panel deemed this study[28] "of limited utility due to statistical questions about body weight and AGD and subcutaneous route of exposure." As noted in sections 2a and 2b above, NTP inappropriately relied on the Taylor et al. (2008) study[13] to conclude that the Honma et al. study provided valid support for conclusions on potential concerns for bisphenol A. In addition to the flaws in the Taylor methodology discussed in section 2b, the use of Taylor to support subcutaneous dosing of adult mice is inappropriate (see section 2a).

The studies cited by NTP to support an accelerated onset of puberty in females are not consistent with the large majority of the literature. There were no effects on puberty in

[26] Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G., and Saal, F. S. 1999. Exposure to bisphenol A advances puberty. Nature. 401:763-764.
[27] Ryan, B. C. and Vandenbergh, J. G. 2006. Developmental exposure to environmental estrogens alters anxiety and spatial memory in female mice. Hormones and Behavior. 50(1):85-93.
[28] Honma, S., Suzuki, A., Buchanan, D.L., Katsu, Y., Watanabe, H., and Iguchi, T. 2002. Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. Reproductive Toxicology. 16:117-122.

females reported by Tyl et al. (2002),[19] Tinwell et al. (2002),[29] Murray et al. (2007),[3] Ema et al. (2001),[20] Kubo et al. (2003),[30] Rubin et al. (2001),[31] Yoshida et al. (2004),[32] Ashby et al. (1999),[33] Markey et al. (2003),[34] and Tyl et al. (2008).[21]

In addition, a very recent paper[35] attempted to correlate environmental exposures to the onset of puberty in a cross sectional study of 192 9-year old girls. Although higher urinary phytoestrogen levels, when considered jointly with body mass index, were reported to delay the onset of puberty as judged by breast development, urinary bisphenol A levels were not associated with any of the studied endpoints of early onset of puberty. The results of this study are consistent with the experimental animal studies showing no bisphenol A-related early onset of puberty in females.

Vaginal opening (VO) time does correlate with onset of puberty in mice and should be considered as a reliable indicator of puberty.

The draft NTP Brief states that the age at first estrus is the most accurate indicator of puberty in rodents and that this occurs at the same time as vaginal opening in rats, but not mice. Data from a mouse two-generation study[24] using 17β-estradiol (conducted in accordance with international guidelines and thus data that is acceptable under the Data Quality Act) clearly show the mouse to be a sensitive model for assessing onset of puberty using days to vaginal opening. Vaginal opening time was accelerated 6.8 days at 0.15 ppm estradiol in the diet (30 µg /kg/day) and 7.3 days at 0.5 ppm estradiol in the diet (100 µg /kg/day). In addition, a variety of estrogenic agents have been shown to accelerate the occurrence of vaginal patency in mice, including the synthetic estrogen, diethylstilbestrol (Burroughs et al., 1985[36]; Honma et al., 2002[28]), the plant estrogen, coumestrol (Burroughs et al., 1985[36]), and the pesticide, methoxychlor (Walters et al,

[29] Tinwell, H., Haseman, J., Lefevre, P.A., Wallis, N., and Asbhy, J. 2002. Normal sexual development of two strains of rat exposed in utero to low doses of bisphenol A. Toxicological Sciences. 68:339-348.
[30] Kubo, K., Arai, O., Omura, M., Watanabe, R., Ogata, R., and Aou, S. 2003. Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. Neuroscience Research. 45(3):345-356.
[31] Rubin, B.S., Murray, M.K., Damassa, D.A., King, J.C., and Soto, A.M. 2001. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environmental Health Perspectives. 109(7): 675-680.
[32] Yoshida, M., Shimomoto, T., Katashima, S., Watanabe, G., Taya, K., and Maekawa, A. 2004. Maternal exposure to low doses of bisphenol A has no effects on development of female reproductive tract and uterine carcinogenesis in Donryu rats. Journal of Reproduction and Development. 50(3):349-260.
[33] Ashby, J., Tinwell, H., and Haseman, J. 1999. Lack of effects for low dose levels of bisphenol A and diethylstilbestrol on the prostate gland of CF1 mice exposed in utero. Regulatory Toxicology and Pharmacology. 30:156-166.
[34] Markey, C.M., Coombs, M.A., Sonnenschein, C., and Soto, A.M. 2003. Mammalian development in a changing environment: exposure to endocrine disruptors reveals the developmental plasticity of steroid-hormone target organs. Evolution and Development. 5(1):67-75.
[35] Wolff, M. S., Britton, J. A., Boguski, L., Hochman, S., Maloney, N., Serra, N., Liu, Z., Berkowitz, G., Larson, S., and Forman, J. 2008. Environmental exposures and puberty in inner-city girls. Environmental Research. In Press.
[36] Burroughs, C. D., Bern, H. A., and Stokstad, E. L. 1985. Prolonged vaginal cornification and other changes in mice treated neonatally with coumestrol, a plant estrogen. Journal of Toxicology and Environmental Health. 15(1):51-61.

1993[37]; Eroschenko, 1991[38]). As the ages of vaginal patency and first estrus can be affected by cohabitation with adult males (Vandenbergh, 1967[39]) or by exposure to bedding that has housed males (Vandenbergh, 1969[40]), it is important that studies measuring these endpoints in mice be properly controlled in regard to either cohabitation and/or the exact proximity of caging to males. Studies that do not indicate that this variable was very carefully and properly controlled should be viewed as suspect. For example, Howdeshell et al.(1999)[26] indicates only that females were housed "near" males but did not specify that the distance from males between treated and control animals was carefully controlled.

In a low dose bisphenol A study using the mouse model and same experimental design as the estradiol two-generation study, no effects on vaginal opening time were reported.[21] This study also used a concurrent positive control (0.5 ppm estradiol), which showed an accelerated age to vaginal opening consistent with the previous estradiol study. The multi-generation study of dietary estradiol was large in size (n= 25 litters/group), followed a globally harmonized guideline, and was conducted under Good Laboratory Practices. The current globally harmonized guidelines for assessing reproduction and development (OECD 416 and US OPPTS 870.3800) both recommend the use of vaginal opening time as a standard measure of puberty in females.

<u>The rat and mouse models are both sensitive for assessing onset of puberty.</u>

Onset of puberty using the day of vaginal opening was accelerated in both the rat and mouse models following administration of the potent estrogen, estradiol. Biegel et al. (1998)[41] and Tyl et al. (2006)[42] reported accelerated vaginal opening time from dietary estradiol in rat one-generation and two-generation studies, respectively. Tyl et al. (2008) reported accelerated vaginal opening time from dietary estradiol in mouse one-generation[43] and two-generation studies.[24]

---

[37] Walters, L. M., Rourke, A. W., and Eroschenko, V. P. 1993. Purified methoxychlor stimulates the reproductive tract in immature female mice. Reproductive Toxicology. 7(6):599-606.
[38] Eroschenko, V. P. 1991. Ultrastructure of vagina and uterus in young mice after methoxychlor exposure. Reproductive Toxicology. 5(5):427-435.
[39] Vandenbergh, J. G. 1967. Effect of the presence of a male on the sexual maturation of female mice. Endocrinology. 81(2):345-349.
[40] Vandenbergh, J. G. 1969. Male odor accelerates female sexual maturation in mice. Endocrinology. 84(3):658-660.
[41] Biegel, L. B., Cook, J. C., Hurtt, M. E., and O'Connor, J. C. 1998. Effects of 17 β-estradiol on serum hormone concentrations and estrous cycle in female Crl:CD BR rats: effects on parental and first generation rats. Toxicological Sciences. 44:143-154.
[42] Tyl, R. W., Myers, C. B., Marr, M. C., Castillo, N. P., Seely, J. C., Sloan, C. S., Veselica, M. M., Joiner, R. L., Van Miller, J. P., and Simon, G. S. 2006. Three-generation evaluation of dietary para-nonylphenol in CD (SD) rats. Toxicological Sciences. 92(1):295-310.
[43] Tyl, R. W., Myers, C. B., Marr, M. C., Castillo, N. P., Veselica, M. M., Joiner, R. L., Dimond, S. S., Van Miller, J. P., Stropp, G. D., Waechter, J. M., and Hentges, S. G. 2008. One-generation reproductive toxicity study of dietary 17β-estradiol (E2; CAS No. 50-28-2) in CD-1® (Swiss) mice. Reproductive Toxicology. 25(2):144-160.

Acquisition of puberty in both rats and mice consists of three stages in sequence: vaginal patency, first estrus and start of estrous cycling (EC). In the rat, the three stages occur in rapid succession, with first estrus typically at or immediately after vaginal patency (which occurs on pnd 30-34) and EC following soon after. In mice, vaginal patency (which occurs earlier, on or about pnd 26-28) is first, with first estrus occurring typically 1-3 weeks later, and EC after that. But all three stages are sensitive to the estrogenic environment (i.e., provide information on the hormonal status of the female), all have meaning and all three are useful indicators of puberty. It is anticipated that all three track together in both species; if vaginal patency is accelerated (as it is with estradiol), then the others will also exhibit acceleration. Note that only vaginal patency is currently specified in the US EPA OPPTS Guideline 870.3800 (1989) and the OECD Test Guideline No. 416 (2001).

Based on the analysis set forth above, using data that meets the requirements of the Data Quality Act, a more appropriate conclusion regarding the potential for low doses of bisphenol A to cause early onset of puberty in females is "insufficient evidence for a conclusion" (in Figure 2b) and "insufficient hazard and/or exposure data" (in Figure 3).

d. Neural and behavioral effects

We agree with the conclusions in the draft NTP Brief that state:

> "The NTP also concurs that additional research is needed to more fully assess the functional, long-term impacts of exposures to bisphenol A on the developing brain and behavior. Overall, the current literature provides a collection of findings that cannot yet be easily interpreted for biological or experimental consistency or for relevance to human health. Part of the interpretive difficulty lies in reconciling findings of different studies that use different experimental designs and different specific behavioral tests to measure the same dimension of behavior."

A review of developmental neurotoxicity testing datasets reveals several inadequate statistical approaches, including issues of Type I error control, power considerations, and ignoring gender, time, and litter allocation as factors in the analyses. The experimental design often consists of multiple measures for each animal, as well as repeated testing across time. Other complications include the use of littermates for the same or different tests, and the need to account for the influence of such genetic and maternal factors. The type of data collected varies with the behavioral test, including continuous, ordinal, and binary data. Appropriate statistical analyses of the data collected in developmental neurotoxicity studies is an important issue (see CERHR expert panel report). Studies that fail to do so do not satisfy the requirements of the Data Quality Act.

The influence of litter must be taken into account in the allocation of test animals as well as the statistical analyses. Since developmental neurotoxicity studies in general include numerous experimental procedures conducted on the dam and offspring at several ages, it is not unusual to have various significance tests if each was analyzed separately (Holson et al., 2007).

Hence, investigation of neural and behavioral endpoints using accepted methods and the now-established international test guidelines would bring a robust approach to inform the scientific and regulatory community on the potential of bisphenol A to produce neural and behavioral effects.

4. Figure 2b should clarify that the finding regarding reproductive toxicity relates only to high dose effects

Figure 2b indicates "Reproductive toxicity"; "Some evidence of adverse effects." It should be clarified that this statement relates only to "high" dose scenarios.