Response to the Final Draft of the NTP-CERHR report on the Reproductive and Developmental Toxicity of Bisphenol A

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Table of Contents

General Statement

This is the third set of comments submitted by our labs regarding the NTP/CERHR review of Bisphenol A. In our first set of comments, we highlighted factual inaccuracies and misunderstandings of our own publications in a point-by-point fashion. Many of these issues were not addressed in the second public draft of the document. In our second set of comments, we outlined seven major points we considered critical errors in the revised draft, namely: 1) The criteria established by the panel were arbitrary; 2) The assessment criteria were not set a priori; 3) These criteria were used inconsistently to weigh the evidence from each study to determine adequacy; 4) In many instances the data are misrepresented and/or misunderstood by the panel; 5) There is a lack of understanding about the proper use and importance of both positive and negative controls; 6) There is evidence of bias in the assessment of studies based on the source of funding; and 7) Many sections in this report illustrate a disregard for the nature of science. We concluded our second set of comments with a point-by-point review of many studies cited by the CERHR panel. In the current version of the NTP/CERHR review, we note that many of our comments and criticisms were used to correct errors in the draft; however some of the issues we raised were not addressed.

We stand by the seven major issues we highlighted previously in our June 2007 correspondence to this panel. **Many of these concerns are major and fundamental to the review process; they cannot be fixed by mere editing or word-swapping.** However, because we spent a considerable amount of time addressing each of these issues in June 2007, we will not belabor every one here. Instead, we will highlight several points that still require clarification or remain incorrect in the current draft.

Finally, many of the issues outlined in the following pages are repeatedly brought up by researchers in the BPA field – and by their critics. This is likely due to the striking differences in the backgrounds of researchers of BPA and other endocrine disruptors. Researchers with training in toxicology may approach scientific issues from a different angle than developmental biologists, endocrinologists, cell biologists, molecular biologists, and epidemiologists, among others. While these different perspectives can often lead to enriching collaborations between researchers in these varied fields, the belief by some toxicologists that there is no mechanism to explain low dose responses or non-monotonic dose response curves compels these scientists to reject a great majority of BPA research. **This simply is unwarranted.** Non-monotonic dose-response curves are a common phenomenon in Endocrinology, and the mechanisms underlying some responses exhibiting non-monotonicity have been described. Since Rachel Carson first investigated environmental toxicants that were disturbing the development of wildlife and again in the 16 years since the first Wingspread Conference, **low dose effects, the importance of understanding "critical periods", and the appearance of non-monotonic dose response curves following treatment with endocrine disrupting chemicals (EDCs) have been shown time and time again**. Ignoring these critical issues with regards to the current BPA literature will send this field back several decades.

We urge the panel to write a consensus statement that **all** panelists are comfortable signing, to ensure that the panel's findings are driven and accepted by the full committee of experts.

Summary of Findings

Route of exposure

The panel has chosen to remove or de-emphasize studies that did not use an oral route of exposure, stating that they are "not relevant to human exposures". There are several flaws in this reasoning:

1) While human exposure to BPA is thought to be primarily via the oral route, there is evidence that humans are exposed to BPA via other routes. As cited in the report, several studies detected BPA in air and dust samples, both indoor and outdoor (1-3). These studies indicate that **BPA exposure through air and dust is likely**. BPA has been detected in landfill leachates (4-6) and in treated leachates (6). BPA has also been detected in sewage treatment works effluents, rivers, creeks and drinking water (7-10). In rivers, the concentrations ranged from 500pg/L to over 100ng/L. BPA levels in drinking water ranged from 300pg/L to 2 ng/L. Thus, **BPA exposure through water used for both drinking and bathing is likely**.

BPA is used in many consumer products including digital media (CDs & DVDs), electrical and electronic equipment, automobiles, eyeglass lenses, sports safety equipment, bicycle helmets, etc. Exposures from these products are unknown. To date, studies have estimated BPA intake either from dietary sources alone (11) or from environmental contaminations (water, air and soil) and dietary sources (12). However, **no study has yet measured actual human exposures from all sources**.

2) In order for a method of dosing to be truly relevant to human exposures, it must generate a **constant** low-level concentration of BPA in blood [see (13) for a review of studies that have **consistently** demonstrated detectable concentrations of BPA in human blood samples]. Several methods of dosing that were considered "relevant" and acceptable by the panel required large bolus doses of BPA to be administered. These methods include oral gavage, drinking of BPA from a pipette, and eating of BPA-tainted cookies. None of these methods is perfect. In particular, gavage administration raises concerns because of associated stress, which results in altered hormone levels. Moreover, the current measurements of BPA in human populations would argue against exposure of humans to a single bolus of BPA per day.

Dosing animals via feed or in water are preferable methods because they allow for an oral exposure and they do not rely on a single bolus application; however, they are unreliable. The actual exposure level is only approximate because: i) in group housing, the amount consumed by each animal is unknown; ii) water often leaks from bottles, which may lead to inaccurate calculations; and iii) several studies cited by the panel indicate that animals exposed to higher doses of BPA actually decrease their intake by consuming less BPA-containing food or water. Data from studies that used these methods (gavage, pipette drinking, cookies, BPA-contaminated feed/water) are valuable and important. Many of these studies have demonstrated that exposure to BPA, even in a single dose, causes significant changes in various endpoints. However, **if the panel prefers to focus only on studies that are relevant for human exposures, the flaws in these exposure paradigms must be acknowledged as well**.

3) As developmental biologists, we are most concerned with fetal and neonatal exposure to BPA. An extensive literature is available suggesting that low-level exposure to other estrogens (including estradiol and diethylstilbestrol) during the perinatal period leads to altered development of estrogen-target organs. Unfortunately, **the panel fails to make the distinction between adult and fetal exposure in their review.** A fetus does not

eat; it is exposed to BPA through its mother's blood, and studies in humans indicate that low levels of BPA are regularly detected in blood. Therefore, **for studies of fetal/neonatal exposures, any route of exposure that allows BPA to circulate in the maternal blood is closely "replicating the human condition"** as the panel desires.

Our studies have used subcutaneous osmotic pumps to deliver extremely low (ng/kg) doses of BPA to pregnant dams. Even if 100% of this dose remained unconjugated and active, the circulating levels found in the dams' blood- i.e. the levels to which fetuses are exposed- would be significantly lower than the exposures considered "relevant" by the panel following oral exposure to BPA in the mg/kg range.

4) Because of the multiple sources of BPA and the consistent finding of BPA in human samples, human exposure is thought to be chronic and low-level. However, so far, no pharmacokinetic study of chronic, low dose BPA has been performed.

The panel has concluded that oral dosing is the only relevant mode of exposure under the incorrect assumption that all BPA delivered orally is inactivated via first-pass metabolism. There are several flaws with this assumption: i) the metabolic profile of BPA metabolites found in urine and feces of animals treated with 25 micrograms/kg of BPA is qualitatively similar at 24 hours **regardless of the route of administration** (14). A recent study indicates that neonatal rodents have limited ability to conjugate BPA to an inactive form, regardless of the mode of administration (subcutaneous vs. oral), again arguing against the removal of studies using subcutaneous exposure paradigms (15). Unfortunately, at this time there is no single study that monitors the levels of the parent compound after the administration of chronic low doses of BPA orally or subcutaneously, although chronic low dose exposure is the expected human condition; ii) **high dose studies are difficult to interpret** due to the low bioavailability of oral BPA in these conditions [16% for 10 mg/kg and 5.6% at 100 mg/kg-see (16)]. A study by Upmeier et al (16) clearly noted that **the plasma concentrations of parent compound after oral administration are far from negligible**. For example, while iv administration of 10mg/kg resulted in very high levels that declined very fast (700 ng/ml at 1h and 100 ng/ml at 2h), oral administration of the same dose resulted in a maintained level of 20-30 ng/ml for 8 hours, with a second peak at 6 hours due to enterohepatic recirculation. Hence, **the much-praised and constantly cited notion of rapid first pass and complete inactivation remains undocumented.** Again, the recently published study by vom Saal's group also illustrates that neonates have limited ability to metabolize BPA, regardless of the mode of exposure (15); iii) conjugation reactions are reversible and in the case of natural estrogens, the **conjugates can be hydrolyzed in target organs** releasing the active parent compound (17;18); iv) it is likely that BPA undergoes enterohepatic recycling, as was shown in several studies. In one of these (performed in a GLP lab), a significant amount of the parent compound was detected in feces (94%), bile (1%) and digestive tract (26%) 24 hours after subcutaneous administration of tritiated BPA, suggesting that the conjugates have been hydrolyzed by the intestinal bacteria and have been secreted directly into the intestine. Hence, **to assume that all the conjugated metabolites have been irreversibly inactivated is not warranted**. Due to all these facts, it is arbitrary to disregard, "downgrade" or discount data from studies that used a subcutaneous route of exposure, particularly when the overwhelming majority of truly low level exposure (ng/kg range) and a representative number of low dose studies (microgram/kg range) have used this route.

5) The panel's reasoning for challenging the use of DMSO has changed from draft to draft, suggesting that the panel members are searching for justifiable reasons for excluding a number of studies, many of which originated in our labs. In the original draft,

the panel did not state particular problems with the use of DMSO, and many of our studies were praised for utilizing extremely low doses via osmotic pump and for the difficult endpoints and organs we examined.

In the draft released in April 2007, studies using DMSO were criticized however, because “DMSO has significant biological activities of its own.” The panel cited Santos et al. 2003 (19) as evidence for this statement, even though the Santos et al paper was a commentary article reviewing the problematic effects of DMSO exposure (including systemic side effects such as nausea and vomiting, diarrhea, hemolysis, anaphylactic reactions, renal failure, etc.) that were observed when DMSO was used as a cryoprotectant for cells or transplants, including autologous bone marrow transplants, that were then directly infused into the bloodstream of patients. A more recent study of autologous stem cell transplants reported evidence of acute DMSO-induced systemic toxicity in individuals that received intravenous infusions of stem cells suspended in DMSO (20). In one case the patient received a 200 ml infusion and in another, a patient received a 500 ml infusion. In both cases the cells were suspended in 10% DMSO and administered directly into the bloodstream of highly compromised patients. Clearly, DMSO is not inert and can have effects on its own- some desirable and others not desirable. There are many studies in the literature that have documented the effects of DMSO administration and they appear to be related to the level of exposure, the i.v. route of administration, and the high flow delivery rate (21). The Alzet pumps used for subcutaneous administration of BPA release at the rate of 0.25 µl/hour. Because the vehicle is 50% DMSO, **approximately 0.125 µl of DMSO is released per hour.** These levels of exposure are **far below the levels routinely reported to have significant systemic effects when infused intravenously** and they are also below the dose of DMSO vehicle used in most reported studies.

In the most recent CERHR draft, it is stated that DMSO “has significant biological activities of its own (*315*), and the experience of the Panel is that DMSO can help move solutes into cells. Increasing the DMSO concentration can produce a greater solute effect, even when holding that solute concentration stable. The real impact of this for *in vivo* injections is uncertain…” Yet the report also states that “this effect is likely to be small at the dosing volumes administered in the studies considered here”. In fact, the very low levels of DMSO suggest that this is a non-issue. Again, even if 100% of the administered BPA were to be “helped into cells” by the 50% DMSO vehicle used, the levels of exposure would be within the range- if not significantly lower- than in studies where high doses were administered orally.

Additionally, in a footnote, the panel quoted an Alzet spokesperson about the use of DMSO in osmotic pumps. The manufacturer’s instructions specify the use of up to 50% DMSO. Significantly higher concentrations of DMSO can apparently lead to degradation of the pump reservoir, which would be expected to result in more rapid release of BPA and DMSO then intended and could lead to tissue inflammation and edema. As stated in our papers, 50% DMSO was the diluent used in our studies, a concentration considered acceptable by the pump’s manufacturer. In the unlikely- worse case scenario -that the osmotic pump degraded, exposing the dams to the complete contents of the chamber, based on the average weight of a dam and the volume of the pump, dams would have received a bolus dose of approximately 100ng BPA in a solution of 80-100 microliters- a high exposure that is still within the levels of other studies deemed “acceptable” by the panel. Finally, in our experience with thousands of osmotic pumps, we have never observed inflammation or edema as would be expected with pump failure as described above and would require treatment and/or euthanasia of the dams under our IACUC approved protocol.

Crucial issues to understand the literature and history of EDCs

The panel's ability to assess significant effects of BPA appears to have been colored by a lack of understanding of non-monotonic dose response (NMDR) curves. In the field of endocrinology, NMDR curves have been observed following exposure to numerous hormones (endogenous and exogenous) and in various hormone-related endpoints. **Endocrinologists are familiar with the vast amount of literature describing the mechanisms behind these phenomena.** NMDR curves are generated by the integration of two or more monotonic dose response curves that are occurring through different pathways and affecting a common end point with opposing effects (22). For instance, *in vitro* studies have shown that low doses of androgens can mediate a proliferative response in androgen-target cells while at a higher dose, they inhibit cell proliferation (23;24). When the end point is cell number, the resulting curve has the shape of an inverted U. These two pathways are induced independently of each other; they can be segregated, generating two differently behaving cell types, i.e. one that shows a monotonic proliferative response, (the cell number increases as the androgen dose increases) and another that shows a monotonic inhibitory response, (the cell number decreases as the hormone concentration increases) (22). Studies have determined that the biochemical events underlying these effects are distinct (25).

Non-monotonic dose response curves have also been studied, identified and accepted in the field of toxicology! Calabrese and Baldwin describe the importance of experimental design to detect the presence of a NMDR curve. In particular, studies of dose response curves must use a wide range of doses, including doses below the established LOAEL (26;27). A meta-analysis of 20,285 toxicology studies conducted between 1962 and 1998 found that only 1% of the published studies met the criteria set *a priori* to determine if a study was designed to detect a non-monotonic dose response curve (26). Of these studies, almost 40% satisfied the requirements for a NMDR curve, supporting the idea that **the occurrence of non-monotonic responses is non-random and may even be more common than monotonic dose response curves** (27;28).

Low-dose effects have also been challenged, with suggestions that there is no mechanism to explain how effects that are not seen at high doses can possibly occur at doses several fold lower. Again, an understanding of NMDR curves is the first such explanation of these phenomena. Several other mechanistic explanations have been offered, including non-genomic signaling through membrane-bound forms of estrogen receptor (29) and additivity with other estrogens present (30;31).

Finally, since before the time of Rachel Carson, developmental biologists and endocrinologists have demonstrated that small doses of chemicals, including hormones, can have drastically different effects on the developing organism compared to the adult. **The panel has repeatedly failed to distinguish between fetal and adult exposures.** There are many examples in the literature of how adults can be exposed to chemicals without any obvious deleterious effects, but exposure of animals (including humans) during early development (morphogenesis or organogenesis) to the same dose can have significant consequences (32). This has been demonstrated with exposure to radiation, vitamin A, thalidomide, and diethylstilbestrol, among others. The abundant number of studies examining differences in morphology, physiology and behavior in rodents due to extremely slight differences in hormone exposure due to intrauterine position are further evidence that low doses of hormones during fetal life can significantly impact the individual's development (33).

Continued use of arbitrary criteria

In our response to the April 2007 draft (submitted in June 2007), we highlighted several criteria used by the expert panel to disqualify or de-emphasize studies that we considered to be arbitrary. Above, we addressed some of these criteria and next we will address additional issues.

1) Vehicle choice. The issue of DMSO has been discussed at length above. However, the panel's statements about other vehicles is still lacking: "Various oils each can bring their own potential issues, such as oxidative damage, but these were considered and discussed by a sub-team of the Panel and not considered to be consequential for this analysis." Elementary knowledge of oil vehicles indicates that many oils, but particularly corn oil, are at risk for contamination. One such contamination is a mold that grows on corn, which has **significant estrogenic effects**. Thus, in the dozens of cited studies that used unstripped oils (43% of studies cited in Section 3 alone), **it is plausible that the negative controls (oil injection alone) were, in fact, exposed to significant levels of estrogens**. While we do not suggest that this possibility alone is a reason to dismiss our colleagues' studies, it should be considered or discussed by the panel in view of its willingness to discount studies that used other vehicles, i.e. DMSO.

2) The choice of sample size. In the previous draft (April 2007), the panel stated the following about what it considered adequate for sample size:

> For in vivo studies, the Panel consensus was that n values of 7-8 or more were generally acceptable for many endpoints, with some significant exceptions. We would tend to believe and accept smaller n's for those studies such as highly-detailed tissue reconstructions or other approaches which involved detailed investigation of many cellular endpoints in a few animals. At the other end of the spectrum, even 20 animals is too few for a confident determination of serum Testosterone levels using terminal necropsy samples, while 10 is at the border of being unacceptable for assessment of fertility or epididymal sperm count. Thus, studies which measured these endpoints with fewer than these numbers of animals were generally deemed marginally adequate or inadequate, depending on the n used.

This paragraph alone illustrates the arbitrary nature of the sample size criteria, when the panel assumes an n of 7-8 is required, with a half dozen poorly described exceptions, which allow the panel to use different criteria when preferred. Rigorous knowledge of statistics indicates that **studies with great precision or measurements that reveal very marked differences between groups can be performed with fewer animals** depending on the amount of variability in the endpoint in question, while other studies of more error-prone or highly variable endpoints require more animals to achieve statistical significance. Thus, any specific requirement of 7-8, 10 or 20 animals, regardless of the endpoint, is capricious without additional information, which scientists are expected to gather and use *a priori* – i.e. before running their experiments.

In the current draft, the panel now states that "a minimum of 6 animals per treatment condition needed to be used to provide minimal confidence in results." Again, we are confronted by an arbitrary choice in number, now with no explanation at all from the panel about the selection of an 'n' of 6, or a discussion about acceptable deviations from this requirement.

3) Litter effect statistics. Studies continue to be criticized by the panel for improper use of statistics when the research papers for these studies clearly state that one animal per

litter was used for each endpoint. **If only one randomly chosen animal is used per litter, no additional statistical methods are needed to account for litter effect**. The panel's criticisms are thus unfounded. Additionally, in studies where intrauterine position was taken into account, several siblings from a single litter are useful, because they represent different positions within the uterus. These studies use ANOVA analysis to compare unexposed and BPA-exposed offspring at one of 3 intrauterine positions: 0M (between only females, and zero males), 1M (between one male and one female), 2M (between two males and zero females.)

As an additional statistical issue, some studies, including our own, were criticized for making "too many" measurements, implying that we were getting positive (significant) results simply by chance. This could perhaps be a relevant argument had we measured 20 endpoints, and found only one significant at $p<0.05$. In fact, in most of our studies, our results are highly significant (ranging from $p<0.01$ to $p<0.001$). Additionally, the number of endpoints examined is usually dictated by the complexity of the system being studied (i.e. in pubertal mammary gland development, at the tissue-level of organization alone, there are at least 5-10 independent endpoints that can be assessed; when assessing changes in expression levels of Gene X, there is only 1 independent endpoint to measure). In fact, we are often requested by peer reviewers to examine additional endpoints to add robustness to our findings; **these additional measurements are thus seen as enhancements, and not flaws**, by other scientists.

Assessment criteria were not set *a priori*

In our previous response to the panel's draft, we outlined evidence that the panel's criteria for determining adequacy of each study were not set prior to the evaluation of these publications. This issue has not been addressed, and in fact remains relevant in light of the panel's decision to change the (arbitrary) criteria for sample size from n=7-8 to n=6.

Additionally, as we stated in our last response, acceptable criteria were not outlined for the studies cited in the first two sections of this report. There was a paucity of information on many of these studies, which were lacking limits of detection, sources of funding, and methods of detection. Finally, the panel failed to state which studies were considered adequate or inadequate in these sections.

Conclusions

In summary, the analysis presented above indicates that the panel's latest review remains flawed. The errors and misrepresentations in their review need to be corrected to accurately reflect the true value and breadth of the currently available scientific evidence. This is absolutely essential if this document is to be used as a resource to assess the potential toxicity of BPA, and to allow meaningful conclusions regarding the safety of BPA exposure in the developing fetus and young children. We urge the NTP/CERHR panel to correct these errors and, in turn, reconsider dismissing the concerns of experienced researchers in this scientific community.

Request to Include Additional Publications

We wish to bring the following publication to the attention of the panel. This peer-reviewed study has not been included in previous drafts of the NTP/CERHR review.

Wadia PR, Vandenberg LN, Schaeberle CM, Rubin BS, Sonnenschein C, and Soto AM. Perinatal bisphenol-A exposure increases estrogen sensitivity of the mammary gland in diverse mouse strains. *Environmental Health Perspectives* 115: 592-598, 2007.

Abstract:

BACKGROUND: Studies of low-dose effects of xenoestrogens have yielded conflicting results that may be attributed to differences in estrogen sensitivity between the rodet strains examined. Perinatal exposure of CD-1 mice to low doses of the xenoestrogen bisphenol A (BPA) alters peripubertal mammary gland development. Future studies to assess the role of estrogen receptors as mediators of BPA action require estrogen receptor knock-out mice that were generated on a C57Bl6 background. The sensitivity of the C57Bl6 strain to estradiol and BPA is unknown. OBJECTIVES: In the present study we examined whether the mammary glands of CD-1 and C57Bl6 mice exhibited similar responses to 17beta-estradiol (E(2)) and whether perinatal exposure to BPA equally enhanced sensitivity of the mammary glands to E(2) at puberty. METHODS: Immature mice were ovariectomized and treated for 10 days with one of eight doses of E(2). Morphological mammary gland parameters were examined to identify doses producing half-maximal effects. Mice were exposed perinatally to 0 or 250 ng BPA/kg body weight (bw)/day from gestational day 8 until postnatal day (PND) 2. On PND25, female offspring were ovariectomized and given an estrogen challenge of 0, 0.5, or 1 microg E(2)/kg bw/day for 10 days. Morphometric parameters of the mammary gland were compared between strains. RESULTS: Both strains exhibited similar responses to E(2). Perinatal BPA exposure altered responses to E(2) at puberty for several parameters in both strains, although the effect in CD-1 was slightly more pronounced. CONCLUSION: Both mouse strains provide adequate models for the study of perinatal exposure to xenoestrogens.

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