The Importance of Appropriate Controls, Animal Feed, and Animal Models in Interpreting Results from Low-Dose Studies of Bisphenol A

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Interpreting results of studies that report only negative effects is problematic. A number of published studies to determine whether chemicals with estrogenic activity can cause effects at low doses have not taken into account the possibility that the commercial animal feed being used can mask effects of even potent estrogenic drugs such as diethylstilbestrol (DES). In addition, the sensitivity of the strain of animal being used for the specific category of chemical being tested has not always been described. For environmental chemicals, such as the estrogenic polycarbonate plastic monomer bisphenol A, DES is an appropriate positive control for estrogenic effects, and using an appropriate low dose of DES can eliminate the possibility of false-negative conclusions of safety when the above or other variables contribute to the negative outcome. Only when simultaneous positive effects of low doses of a positive control chemical such as DES and negative effects of environmentally relevant low doses of the test chemical are demonstrated within the same experiment are conclusions of no effect of the test chemical warranted, and this has not been reported for bisphenol A in any study. Instead, more than 90 refereed journal publications have reported effects due to exposure to low doses of bisphenol A in a wide variety of animals (for references see: http://rcp.missouri.edu/endocrinedisruptors/vomsaal/vomsaal.html). However, due to lack of attention to the importance of appropriate positive controls, a small number of studies reporting negative effects of bisphenol A have created a false sense of controversy regarding low-dose effects of bisphenol A. *Birth Defects Research (Part A) 73:140–145, 2005.*

Key words: endocrine disruptors; estrogen; fetal development; prostate

INTRODUCTION

Recently, the issue of unexpected "low-dose" effects in toxicology has received considerable attention. The "low-dose" issue is based on findings from endocrine studies that low doses within a physiological range of hormonal activity of drugs and environmental chemicals referred to as endocrine disruptors can exert unique effects not observed at higher doses, similar to nonmonotonic dose-response curves commonly observed in experiments with naturally occurring hormones (Welshons et al., 2003). "Low-dose" effects of environmental endocrine disrupting chemicals refer to effects being reported for chemicals at doses lower than the dose previously thought to be the no effect level (NOEL) based on traditional "high-dose" tests used in toxicological studies conducted for risk assessment purposes (vom Saal and Welshons, 2000; NTP, 2001).

The possibility of effects of endocrine disrupting chemicals at doses below those associated with systemic toxicity is considered plausible, since there is now extensive evi-

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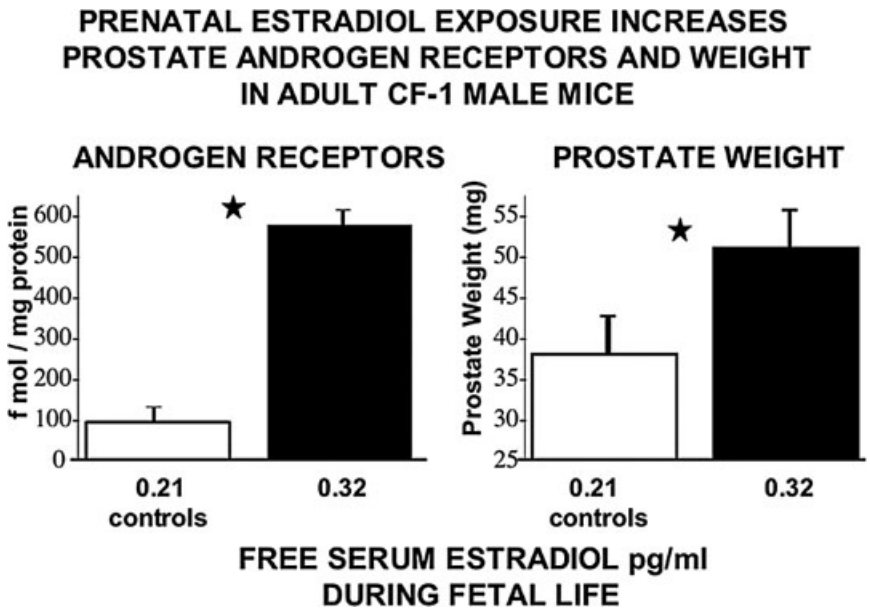

Figure 1. Prostate androgen receptors and prostate weight in adult CF-1 male mice. From GD 13 to GD 18, pregnant females were implanted with a Silastic capsule containing vehicle (controls) or estradiol. Free serum estradiol was measured in male fetuses by ultrafiltration dialysis in combination with radioimmunoassay in randomly selected litters on GD 18, and related to adult prostate androgen receptors and prostate weight in other males from the same treatment groups (vom Saal et al., 1997). *indicates that the groups are significantly different ($p < 0.05$).

dence that during fetal life, organogenesis is altered by very small changes in hormone concentrations. For example, prostate gland epithelial hyperplasia, associated with a permanent increase in prostate size and a permanent increase in prostate androgen receptors, is induced in CF-1 male mouse fetuses by a very small increase relative to controls of about 50 pg/ml total serum estradiol (estradiol was administered to pregnant females via Silastic capsules (Dow) implanted subcutaneously; this corresponds to an increase of approximately 0.1 pg/ml free (bioactive) serum estradiol (Fig. 1), because only a very small proportion (~0.2%) of the total circulating estradiol in mouse fetuses is free (unconjugated and not bound to plasma proteins). A permanent increase in prostate androgen receptors will increase the response of the prostate to stimulation by androgen throughout life, and this is a risk factor for prostate disease during aging in men (Richter et al., 2004). This is just one example of how sensitive fetal tissues are to gonadal steroids and other endocrine signaling compounds (vom Saal et al., 1997; Welshons et al., 2003). Even small changes in gonadal steroids during fetal life can have a significant impact on the life history of the individual (vom Saal, 1989).

We have confirmed the above findings of a very high sensitivity of the developing prostate to estrogen in primary culture of fetal urogenital sinus (UGS) mesenchymal tissue collected from CD-1 fetal mice on GD 17; the prostate begins differentiating on GD 17 in mice. The cranial UGS differentiates into the prostate in males and a portion of the vagina in females, and differentiation of the epithelium lining the ducts is controlled by adjacent mesenchyme. Against a constant background of 0.7 nM dihydrotestosterone (DHT), estradiol stimulated a dose-related increase in androgen receptor (AR) mRNA. A significant increase in AR mRNA was observed at 1 pM (0.28 pg/ml), consistent with our in vivo findings (Fig. 1), with a maximum response at 100 nM, after which the response decreased. Nonmonotonic dose-response curves can thus occur when examining the activity of individual genes (Richter and vom Saal, unpublished observations).

Taken together, these findings suggest that there is a permanent "imprinting" of prostate androgen receptor gene activity by estrogen, with very low levels of estrogens permanently increasing AR gene activity and AR protein. The consequence is an adult prostate that is more sensitive to stimulation by androgen and is thus permanently enlarged; these males also have a malformed and abnormally small bladder neck (as well as other malformations of the urethra), which may be involved in bladder outlet obstruction and difficulty urinating (vom Saal et al., 1997). Along with benign prostate hyperplasia (BPH), difficulty with bladder control is a common disorder in aging men. These findings with estradiol, and the confirmation that very low doses of DES (0.02 μg/kg/day fed to pregnant CF-1 females and 0.5 pg/ml DES in primary organ culture from CD-1 fetuses) produce identical stimulating effects on the fetal mouse prostate, are important with regard to establishing that the fetal mouse prostate is an appropriate organ for examination of endocrine disrupting effects caused by estrogenic endocrine disrupting chemicals in both the CF-1 and CD-1 mouse (vom Saal et al., 1997; Gupta, 2000a).

REDUCED BINDING TO PLASMA PROTEINS PREDICTS BIOLOGICAL ACTIVITY OF BISPHENOL A AT LOW DOSES

We will describe here data from experiments with bisphenol A, the monomer used to manufacture polycarbonate plastic and resins and as an additive in other products, in order to illustrate issues that are important in studies of endocrine disrupting chemicals. We have found that many estrogenic endocrine disrupting chemicals, including bisphenol A, show reduced binding to plasma proteins and thus exhibit an elevated free concentration in blood relative to estradiol (Nagel et al., 1997, 1999). Chemicals that can bypass the barrier created by plasma binding proteins (which regulate the free fraction of natural or manmade estrogen) have a greater biological activity and thus pose a

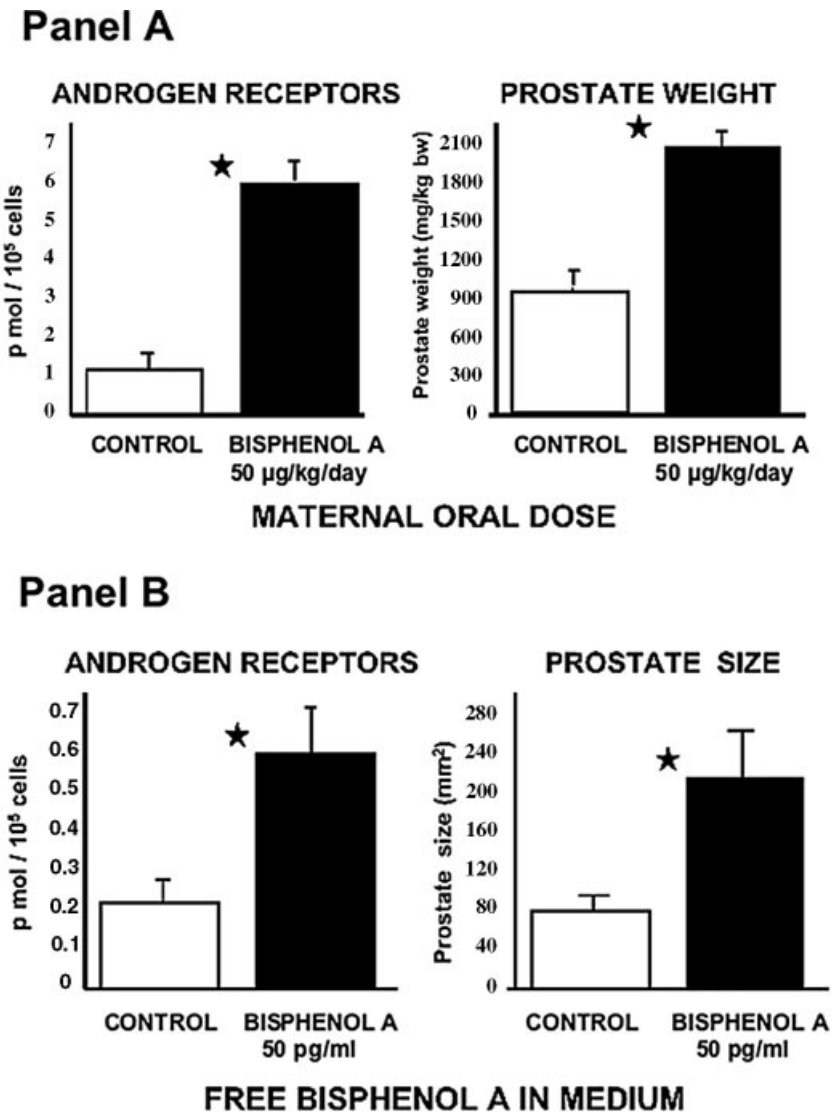

Figure 2. A: Prostate androgen receptors and prostate weight in adult CD-1 male mice exposed to bisphenol A during fetal life. From GD 14 to GD 18 pregnant females were fed either oil (control) or 50 μg/kg/day bisphenol A dissolved in oil using a pipetter. **B:** Prostate androgen receptors and size in CD-1 fetal mouse prostates in primary culture. Prostates were removed from fetuses on GD 17 and maintained in serum-free culture medium without (controls) and with 50 pg/ml bisphenol A. A,B: From data in Gupta (2000a). *indicates that the groups are significantly different ($p < 0.05$).

greater hazard than would be predicted if this were not taken into account.

Based on our initial study with bisphenol A in which we found limited binding to plasma estrogen-binding proteins, we predicted that a very low (~20 μg/kg/day) dose of bisphenol A would produce effects similar to effects caused by the very small increase in serum estradiol or by administration of a low dose of a well-characterized estrogenic drug, such as DES, at a dose of 0.2 μg/kg/day (Nagel et al., 1999). Our prediction was thus that bisphenol A would be biologically active at doses far below those predicted to produce no effect based on traditional high-dose toxicological testing methods. Our "low dose" prediction has now been confirmed in more than 90 peer-reviewed journal publications using different experimental animals and a wide range of outcomes; a list of these "low dose" bisphenol A references and a brief description of findings can be downloaded at: http://rcp.missouri.edu/endocrinedisruptors/vomsaal/vomsaal.html.

We chose as the initial estrogen-responsive bioassay to test this "low dose" hypothesis enlargement of the prostate in male mice, since as shown above, we had demonstrated that fetal exposure to very low doses of both estradiol and DES caused this response (vom Saal et al., 1997). We confirmed our prediction that feeding pregnant mice very low doses (2 and 20 μg/kg/day) of bisphenol A from GD 11 to GD 17 resulted in a permanent increase in prostate size (Nagel et al., 1997), similar to the increase in prostate size caused by 0.02 and 0.2 μg/kg/day DES (vom Saal et al., 1997). This finding has been confirmed by Gupta (2000a), who reported that oral administration to pregnant CD-1 mice of a 50 μg/kg/day dose of bisphenol A from GD 14 to GD 18 caused permanent enlargement of the prostate associated with a permanent increase in prostate androgen receptors in male offspring examined between the neonatal period and adulthood (Fig. 2); Gupta (2000a) also reported virtually identical results for DES at a maternal dose of 0.1 μg/kg/day. Thus, virtually identical findings of increased prostate size and prostate androgen receptors have been reported due to exposure to very low doses of estradiol, DES and bisphenol A in independent studies with mice (vom Saal et al., 1997; Gupta, 2000a).

In addition, Gupta (2000a, 2000b) showed that in primary organ culture of the fetal prostate, DES at a dose of 0.1 pg/ml serum-free culture medium and bisphenol A at a dose of 50 pg/ml stimulated an increase in prostate gland number and size and also increased androgen receptors (Fig. 2); the no effect concentration (NoEC) for bisphenol A was 5 pg/ml (5 parts per trillion). Of interest, this is close to the NoEC for bisphenol A determined in an extensive series of experiments describing a wide range of abnormalities caused by bisphenol A in snails (Schulte-Oehlmann et al., 2001). Gupta's (2000a, 2000b) findings thus confirmed both in vivo and in vitro our prior finding that bisphenol A is approximately 100-fold less potent than DES as a disrupter of prostate development (Nagel et al., 1997; vom Saal et al., 1997), which is a far greater potency than had previously been assumed for bisphenol A. The lowest dose of bisphenol A examined in prior toxicological studies had been 50 mg/kg/day, and the acceptable daily intake (ADI) for humans was predicted to be 50 μg/kg/day (IRIS, 2004). An important related finding is that subcutaneously (s.c.) injecting pregnant female mice with a 25 μg/kg dose of bisphenol A resulted in 4.20, 0.48, and 0.13 ng/gm (ppb) parent (unconjugated) bisphenol A levels in fetuses at 0.5, 2, and 24 hr, respectively, after administration (Zalko et al., 2002). The levels of unconjugated bisphenol A reported in human fetal serum collected at parturition were in the range of 0.1–10 ng/ml (0.1–10 ppb), and the mean bisphenol A concentration in human male fetuses was 3.5 ng/ml (Schonfelder et al., 2002). This finding suggests that human exposure to bisphenol A likely exceeds the ADI of 50 μg/kg/day, which is already higher than doses that can cause permanent effects in the offspring of female mice and rats exposed during pregnancy (see website for publications: http://rcp.missouri.edu/endocrinedisruptors/vomsaal/vomsaal.html).

COMPONENTS OF ANIMAL FEED INTERFERE WITH STUDIES OF ENDOCRINE DISRUPTING CHEMICALS

Estrogens are generally assumed to have inhibitory effects on the rate of growth. An interesting consequence of fetal exposure to a very low (2 μg/kg/day) dose of bisphenol A in mice is an increase in the rate of postnatal growth in both males and females, as well as an accelerated onset of puberty in females (Howdeshell et al., 1999). Exposure to low (1 μg/kg/day) doses of DES during fetal or neonatal development also resulted in an increase in body weight in offspring that became apparent in adulthood (Newbold et al., 2005). We have found that there are components of some commercial mouse feeds, such as soy-based Purina 5002 certified diet (Purina-Mills, St. Louis, MO), which relative to other feeds, such as soy-based Purina 5008 pregnancy diet, significantly increase endogenous estradiol in CD-1 mouse fetuses (vom Saal, unpublished observation). This is associated during later life in both males and females fed Purina 5002 throughout life with accelerated postnatal growth, an increase in the deposition of abdominal fat, and early onset of puberty in females. Male mice fed Purina 5002 diet also had reproductive abnormalities, including an increase in prostate size and a decrease in daily sperm production (all of the above were relative to animals whose mothers were fed Purina 5008 during pregnancy and lactation, followed by Purina 5001 after weaning).

All of the above diets, Purina 5008, 5001, and 5002, are soy-based commercial rodent feeds. Thigpen et al. (2003) observed that variation in phytoestrogen content of Purina 5002 feed was related to the masking of DES effects, but this was not examined for any other type of feed and is thus unlikely to be an issue only with regard to Purina 5002. A previous report of disruption of an experiment due to variability between batches of feed involved a feed produced by Harlan Teklad (Boettger-Tong et al., 1998). It is important to note that most batches of both Purina 5008 and 5001 show higher overall estrogenic activity than Purina 5002 in our MCF-7 breast cancer (MCF)-cell bioassay system (Welshons, unpublished observation), and yet both Purina 5008 and 5001 feeds have not interfered with our ability to find low-dose effects of estrogenic chemicals. It is thus possible that differences between these feeds other than phytoestrogen content (such as the source of fat) might mediate the marked difference in fetal serum estradiol described above. A change in endogenous estradiol may be far more significant than differences in estrogenic activity in the feeds due to phytoestrogens. Clearly, these are important issues that need to be resolved for researchers to be able to produce consistent findings. A standard feed that is not highly variable from batch to batch in hormonally active compounds or in components that can alter endogenous hormones needs to be agreed upon for use in studies of endocrine disrupting chemicals involving rodents and other laboratory animals. Our findings suggest that it would be a mistake to assume that the only components of feed about which there should be concern are the phytoestrogens.

POSITIVE CONTROLS ARE REQUIRED IN ALL STUDIES OF ENDOCRINE DISRUPTING CHEMICALS

Our observation that Purina 5002 feed, through some mechanism, interferes with the endogenous estradiol and alters the reproductive system in mice is consistent with the results of a study in which effects of prenatal exposure to both DES (included as a positive control) and bisphenol A were examined and no effects of either chemical were found (Cagen et al., 1999). An important issue is that this study by Cagen et al. (1999) has been incorrectly reported as being an exact replication of research on bisphenol A and DES done in our laboratory (Nagel et al., 1997; vom Saal et al., 1997). Cagen et al. (1999) used Purina 5002 feed in their experiment, while we used Purina 5008 during pregnancy and lactation and Purina 5001 after weaning. As described above, Cagen et al. (1999) used the feed that has been reported to mask the effects of DES (Thigpen et al., 2003). Another industry-sponsored study (Ashby et al., 1999) also presented what was claimed to be an exact replication of our research, used yet another type of animal feed and again reported no reproductive effects of either bisphenol A or the positive control chemical, DES, in either male or female mice.

That there was a problem with the animals in the Cagen et al. (1999) or Ashby et al. (1999) studies was immediately obvious based on the fact that the animals exposed during fetal life to the positive control chemical, DES, were not different from the negative control or bisphenol A–exposed animals in traits shown to be affected by DES (vom Saal et al., 1997; Gupta, 2000a; Alworth et al., 2002; Honma et al., 2002; Newbold et al., 2004a, 2005). In the experiments

by Cagen et al. (1999) and by Ashby et al. (1999) in which the positive control (DES) failed to show an effect different from the negative controls or test chemical (bisphenol A), the conclusion by these authors that bisphenol A did not cause any effects in the face of an absence of any significant difference between the negative and positive control groups violates the standard that when the positive control fails, the experiment should be considered to have failed (NTP, 2001).

The use of low doses of DES as a positive control for studies of the effects of low, environmentally relevant doses of estrogenic chemicals such as bisphenol A is appropriate, since there is considerable information regarding its mechanisms of action of both chemicals. Both of these chemicals were shown in the 1930s by Dodds and Lawson (1936) and Dodds et al. (1938) to have full estrogenic activity, and these chemicals have been extensively studied since that time (Welshons et al., 1999, 2003). For studies such as ours with bisphenol A in which we feed the chemical to animals to mimic a major route of human exposure, estradiol would be inappropriate as a concurrent positive control, since similar to all of the natural steroids, it has very low (<5%) oral activity (Faigle and Schenkel, 1998) and would require very high oral doses relative to DES to yield any detectable effects.

Experiments concerning the health hazards posed by chemicals for which there is considerable information about mechanisms of action, such as bisphenol A, should include appropriate positive controls at appropriate relevant low doses in order to provide the ability to interpret purely negative findings (Welshons et al., 2003), which is often not done (Ema et al., 2001; Tyl et al., 2002). The importance of including a positive control for interpreting negative findings for bisphenol A was revealed in a study by Yamasaki et al. (2002). They found that their rats (Charles River Laboratories CD Sprague-Dawley, CD-SD) required a very high (~600 mg/kg/day) dose of bisphenol A to cause effects, and this strain of rat also required a very high (50–200 μg/kg/day) dose of the potent estrogenic drug ethinyl estradiol to show effects. The CF-1 mouse showed significant effects of ethinyl estradiol at a dose of 0.002 μg/kg/day (Thayer et al., 2001) and significant effects of bisphenol A at 2 μg/kg/day (Nagel et al., 1997; Howdeshell et al., 1999). The lack of response to low doses of bisphenol A by the CD-SD rat was thus accurately predicted by a lack of response to low doses of ethinyl estradiol (Yamasaki et al., 2002); levels of ethinyl estradiol in oral contraceptives are about 0.5 μg/kg/day. The CD-SD rat thus requires a dose of ethinyl estradiol 100- to 400-fold higher than doses in oral contraceptives to show responses. The CD-SD rat strain was selected over a 40-year period by Charles River for very large litters and large body size and are no longer similar in phenotype to the Sprague Dawley rats that were purchased by Charles River in 1950.

A study conducted by Tyl et al. (2002) for the American Chemistry Council (ACC) provides an interesting example of the potential for false conclusion of safety of bisphenol A that can be drawn from a study in which there are only negative results in the low-dose range and no positive control. The findings by Yamasaki et al. (2002) suggest that the CD-SD rats used by Tyl et al. (2002) are so insensitive to exogenous estrogen that they will not show effects of low-dose exposure to any orally active estrogen, regardless of the animal feed that is used. However, Tyl et al. (2002) used Purina 5002 feed, and as discussed above, there is evidence that this feed can mask the effects of exogenous estrogenic chemicals in mice, and this possibility is thus also likely in rats. Had this study incorporated appropriate positive controls, such as DES or ethinyl estradiol at doses approximately 100- to 1000-fold lower than bisphenol A, it would be possible to determine whether, similar to mice, the CD-SD rats in the Tyl et al. (2002) study were rendered unresponsive to low doses of any estrogenic chemical due to the Purina 5002 feed or due to the strain being unresponsive to low doses of even potent estrogenic drugs. Tyl et al. (2002) concluded that "bisphenol A should not be considered a selective reproductive toxicant" and subsequently argued that no further research concerning the health effects of bisphenol A was warranted (Tyl, 2003). These conclusions are not justified based on negative findings for low doses of bisphenol A while not simultaneously including appropriate positive controls (Welshons et al., 2003).

The expectation is that the most sensitive outcome in the most sensitive animal will be used in the process of selecting animal models to estimate the risk to humans posed by the chemical (U.S. E.P.A., 2004). We chose to use CF-1 and CD-1 mice in our studies due to the fact that they are sensitive enough to estradiol and testosterone to respond to very small differences in these hormones due to the intrauterine position phenomenon (vom Saal, 1989; Vandenbergh, 2003). For example, in outbred CF-1 mice (vom Saal, 1989), females that develop in utero between males (2M females) are exposed to the highest levels of testosterone and show longer and more irregular cycles (five to seven days) relative to females that develop in utero between other females (2F females) that are exposed to the highest levels of estradiol and show shorter and more regular cycles (four to five days) (vom Saal, 1989). In Sprague Dawley rats purchased from Sprague Dawley (Madison, WI), 2F female rats showed cycles that were 4.6 ± 0.1 days long (vom Saal, unpublished observation). As discussed above, a commonly used rat in toxicological studies is the CD-SD rat, which was used in a number of studies that found no low-dose effects of bisphenol A. This rat was reported to have a mean litter size of 15 for all groups in two studies (Ema et al., 2001; Tyl et al., 2002), which means this rat is hyperfertile relative to Sprague-Dawley rats. The CD-SD female rat also shows little variation in estrous cycle length. Mean cycle length for all groups in the Tyl et al. (2002) study was ~4.5 days; in the Ema et al. (2001) study mean cycle length regardless of treatment was ~4.1 days, with a very low coefficient of variation for groups of about 5%. Both the control and bisphenol A–treated CD-SD female rats used in the Ema et al. (2001) and the Tyl et al. (2002) studies appeared have a phenotype characteristic of animals exposed to elevated levels of estrogen and low levels of androgen during fetal life. Strain comparisons of the intrauterine position phenomenon in mice have shown that the phenomenon is absent in inbred mice (such as C57 mice) and present in outbred stocks, such as R-S, CD-1, and CF-1 (Svare et al., 1984; vom Saal, 1989; Vandenbergh, 2003). Clearly, the CD-SD rat no longer shows the phenotypic variability (nor the average phenotype) of the original Sprague-Dawley stock. The continued use of CD-SD rats in studies of low doses of estrogenic chemicals, only because this has been

an accepted model in toxicology studies of other types of chemicals, was criticized by the NIH Low Dose Peer Review Panel (NTP, 2001) and clearly needs to be reevaluated.

Taken together, these findings show that investigators and regulators need to pay close attention to the traits of the animals being used in toxicological studies designed to assess the safety of chemicals at low doses that are relevant to exposures by wildlife and humans. The inability to find effects of low doses of chemicals in some selected rat or mouse strains, while effects of bisphenol A at low doses are being reported in many other strains, as well as other vertebrates and invertebrates, shows that the animal model selected to test for low-dose effects is critical. There should also be concern about the use of polycarbonate plastic animal cages and water bottles, which leach increasing amounts of bisphenol A as a function of repeated washings at levels that can disrupt experiments (Howdeshell et al., 2003; Hunt et al., 2003). In addition, the issue of batch-to-batch variability in components of commercial feeds, with some batches being able to mask effects of DES, shows the need to use a feed that will result in a consistent phenotype and repeatable responses to environmental chemicals. Only by including appropriate positive controls in research conducted with chemicals such as bisphenol A can the public be protected against false negative findings due to these and other variables that can interfere with obtaining positive results in research.

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