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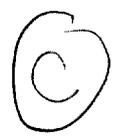
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The enclosed report titled A PHYSIOLOGICAL AND ETHOTOXICOLOGICAL APPROACH TO THE STUDY OF ESTROGENIC ENDOCRINE DISRUPTORS ON REPRODUCTIVE ORGANS AND BEHAVIOR is supplemental to General Electric Company's submissions of Bisphenol-A dated January 29, 1996 and September 3, 1996 (copy attached).

Please do not hesitate to contact me if you have any questions, (413) 448-4853.

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**A PHYSIOLOGICAL AND ETHOTOXICOLOGICAL APPROACH TO THE STUDY OF ESTROGENIC
ENDOCRINE DISRUPTORS ON REPRODUCTIVE ORGANS AND BEHAVIOR**

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ABSTRACT

Two chemicals with estrogenic activity, bisphenol A and octylphenol, which are used in the manufacture of plastics and other products and have been detected in food and water consumed by animals and people, were examined for their effects on accessory reproductive organs and sperm production in male offspring of mice fed these chemicals during pregnancy. Pregnant female mice were fed a concentration (dissolved in oil) of bisphenol A (2 ng/g body weight, 2 parts per billion), below that reported to be swallowed during the first hour after application of a plastic dental sealant (up to 900 μg); a 20 ng/g dose of bisphenol A was also administered. Based on our prior findings concerning the estrogenic potency of bisphenol A and octylphenol relative to estradiol, and the increase in the serum concentration of estradiol previously shown to alter fetal development, we predicted that bisphenol A, but not octylphenol, would alter development of the reproductive system at the doses administered. Our prediction was confirmed in that the 2 ng/g dose of bisphenol A resulted in changes in the size of a number of accessory reproductive organs. Specifically, bisphenol A permanently increased the size of the preputial glands, but reduced the size of the seminal vesicles and epididymides, which both differentiate from the Wolffian ducts. At 20 ng/g, there was a decrease in daily sperm production per g testis (by 20%) relative to control males. None of these effects was observed in response to similar doses of octylphenol. A new approach to studying physiologically relevant doses of environmental endocrine disruptors is discussed, particularly with regard to the development of the reproductive organs, the brain and behavior.

The widespread presence in the environment of chemicals with the capacity to disrupt the functioning of the endocrine system is now established (Colborn et al., 1993). Previously, the primary focus of research regarding the effects of man-made chemicals has been on their capacity to act as mutagens or to induce gross abnormalities after administration of a dose which has typically been much higher than would be encountered in food, water or air. Recently, there has been an increased concern with the consequences of exposure to environmentally relevant doses of endocrine disrupting chemicals during critical periods in organ system development, since alteration of the developmental program can lead to irreversible changes in the functioning of organ systems without altering the genetic code.

As yet there are few published studies in which environmental estrogens or other categories of endocrine disrupting chemicals that mimic endogenous hormones have been examined at environmentally relevant concentrations, although this is likely to change (Nagel et al., 1997). The doses that have been used in most toxicological studies (that have followed federal guidelines for testing systemic toxicants) have not been based on estimates of *in vivo* bioactivity relative to the endogenous hormone that the xenobiotic is mimicking (vom Saal et al., 1997). Of central importance with regard to the experiment we describe here is that the doses of chemicals used are within the range of current human exposure. The doses we used were also based on a new *in vitro* assay designed to predict *in vivo* bioactivity of xenoestrogens (Nagel et al., 1997). In this assay, the potency relative to estradiol of xenoestrogens was assessed and a dose of xenoestrogen was equated with an increase in estradiol previously shown to alter development of the reproductive organs and behavior in mice.

Chemicals that have the capacity to disrupt the endocrine system act via many different mechanisms (Colborn et al., 1993). For example, the contaminant of commercial DDT, o,p' DDT, binds to estrogen receptors and is an estrogen agonist (Fry et al., 1987; Johnson et al., 1988; vom Saal et al., 1995), while the *in vivo* metabolite of DDT, p,p' DDE, binds to androgen receptors and acts as an androgen antagonist (Kelce et al., 1995). Also, some PCB's can bind to transthyretin, a serum transport protein for thyroid hormone, thus altering bioavailable levels of thyroid hormone (Brouwer et al., 1995). Since the actions of the hormones being disrupted by these endocrine disrupting chemicals are mediated via binding to intracellular receptors that are all members of the steroid receptor

superfamily (Evans, 1988), our prediction is that the methods that we have developed using environmental estrogens to determine low doses to administer in animal experiments will apply to endocrine disruptors whose effects are mediated by these other receptor systems.

At this time the best characterized endocrine disrupting chemicals are those able to bind to estrogen receptors and act as either estrogen agonists or antagonists. One such chemical, bisphenol A [4,4'-(1-methylethylidene)bisphenol], is an essential component used in the manufacture of resins and polycarbonate, and yearly production of bisphenol A in the USA alone is well over one-billion pounds (Krishnan et al., 1993; Jennings, 1994). Bisphenol A is also a component of plastic used in dental fillings, which are often used to protect teeth in children (Olea et al., 1996). Bisphenol A has been reported to be released from the resins used to coat the interior of food cans when the cans are autoclaved: in commercial practice cans are autoclaved after the addition of the food (Brotons et al., 1995). In another study, when MCF-7 breast cancer cells were grown in media prepared with water autoclaved in polycarbonate flasks, the cells began proliferating and progesterone receptors were induced. This finding demonstrated a significant estrogenic response to amounts of bisphenol A released during autoclaving of plastic made with bisphenol A (Krishnan et al., 1993). Another environmental estrogen is octylphenol [1,1,3,3-tetramethylbutyl]phenol], which is an industrial additive used in a wide variety of detergents and plastics. Octylphenol has been reported to have estrogenic activity in both *in vitro* and *in vivo* studies (White et al., 1994; Sonnenschein et al., 1995; Nagel et al., 1997).

The focus of the studies described here is on development of the reproductive organs. The transition from embryonic to fetal life is marked by the onset of differentiation of organs from embryonic tissues, which occurs in mice at about gestation day 11-12. At this time the testes in male embryos differentiate and begin secreting testosterone (Block et al., 1971). Morphological organization of the testes (the formation of the spermatogenic cords) also begins at this time, while development of the accessory reproductive organs in males begins on gestation day 15-16. Beginning at puberty, the continuous production of mature sperm from germ cells in the seminiferous tubules occurs in regular cycles (maturation of sperm in mice takes 16 days) that normally continue to the end of life (vom Saal et

al., 1994).

We will describe here an experiment that revealed significant changes in development of reproductive organs in male mice due to fetal exposure to environmentally relevant doses of bisphenol A, but not octylphenol. Bisphenol A decreased daily sperm production (per g testis) and also decreased the size of two organs that form from the Wolffian ducts, the epididymides and seminal vesicles. The epididymides play an important role in sperm maturation and storage prior to ejaculation, while fluid from the seminal vesicles constitutes the major portion of the ejaculate in mice. In contrast, bisphenol A increased the size of the preputial glands, which are involved in social communication. We will relate the results of these studies to our previous findings that changes in adult socio-sexual behavior can occur due to exposure of mouse fetuses to very small changes in endogenous sex hormones and low, environmentally relevant, concentrations of environmental chemicals that have estrogenic activity.

GENERAL METHODS

Animals. CF-1 mice were purchased from Charles River Laboratories (Wilmington, MA) in 1979 and have been maintained as an outbred stock in a closed colony. Mice were housed in standard polypropylene mouse cages on corn cob bedding. Pregnant and lactating mice were fed Purina breeder chow (#5008) and, after weaning, males were maintained on Purina #5001 standard lab chow. Rooms were kept at 23°C, with 12 h light and 12 h dark, and lights on at 1200 h.

Determination of Dose of Bisphenol A and Octylphenol. The concept that environmental estrogens are "weak" has not taken into account evidence that there is a limited capacity for some of these chemicals to bind to plasma hormone binding proteins, which determines the proportion of the chemical (or endogenous hormone) that is free to pass from blood into cells (Skalsky et al., 1978; Sheehan et al., 1979; Akpoviroro et al., 1980; Sheehan et al., 1984; Arnold et al., 1996; Nagel et al., 1997). To address this issue, we have used MCF-7 breast cancer cells incubated in human serum to establish values for the relative binding affinity (RBA) for estrogen receptors of bisphenol A, octylphenol, and numerous other xenobiotic estrogens (RBA values are expressed relative to estradiol).

We then compared these RBA values to values generated when MCF-7 cells were incubated in serum free medium. In this *in vitro* assay, referred to as the relative binding affinity-serum modified access (RBA-SMA) assay, the relative bioactivity of the xenobiotic that is calculated takes into account the action of components of serum, which has increased our ability to predict *in vivo* bioactivity (vom Saal et al., 1995; Nagel et al., 1997).

When the RBA of bisphenol A was examined in serum-free medium, it appeared to be a less potent estrogen (by about 10-fold) than octylphenol. This conformed to prior published reports that, in a variety of assays *in vitro*, bisphenol A was measured to be weaker in estrogenic activity than octylphenol. However, when tested in 100% serum in our RBA-SMA assay, the estrogenic potency of bisphenol A was predicted to be over 500-fold greater than the potency of octylphenol in mouse fetuses. Our assay led to the prediction that bisphenol A would have approximately 0.1% (1/1000) the estrogenic activity of estradiol in mouse fetuses, while octylphenol was predicted to have approximately 0.0002% (1/500,000) the activity of estradiol (Nagel et al., 1997).

The final essential piece of information that we used to calculate the doses of these chemicals to feed to pregnant mice was the increase in serum concentration of estradiol that we previously observed to alter development of the fetal reproductive system in male mice (Nonneman et al., 1992; vom Saal et al., 1997). Based on this information, in the present study, we administered a dose of bisphenol A (20 ng/g body weight) that our *in vitro* assay predicted would be bioactive in mouse fetuses. We also predicted that the same doses of octylphenol would not produce a detectible response. A 10-fold lower dose of bisphenol A and octylphenol (2 ng/g body weight) was also administered. In summary, in the present experiment we administered pregnant female mice doses of 2 and 20 ng/g body weight/day of both bisphenol A and octylphenol from gestation day 11-17.

Mating Procedure and Treatment of Pregnant Females with Bisphenol A and Octylphenol. Virgin females were paired with stud males for 4 h at the end of the dark phase of the L:D cycle. Mating was verified the presence of a copulatory plug (gestation day 0), and females were separated from the stud male.

Bisphenol A and octylphenol were dissolved in tocopherol-stripped corn oil (Cat# 901415, ICN, Aurora, OH), and 30 μ l containing 2 different concentrations was fed to pregnant female mice (n = 7/group) one time per day (at 1000 h) from gestation day 11-17. There were also two control groups: vehicle controls (n = 6) and females that remained unhandled throughout pregnancy (n = 5). An electronic micropipetter enabled delivery of an accurate volume of corn oil into the mouth. Mice readily consume corn oil, and this procedure was used instead of gavage to reduce stress, which can interfere with sexual differentiation (vom Saal et al., 1990).

Females delivered their litters normally on gestation day 19, and pups were weaned on postnatal day 23. Male littermates were housed 3 per cage until they were 5 months old.

Collection of Reproductive Organs. At 5 months of age one randomly selected male from each litter was individually housed. One month later at 6 months of age, the males were killed, body weights were recorded, and the testes, epididymides, preputial glands and seminal vesicles were removed and weighed. The prostate was also removed and weighed. The data for prostate weight are reported elsewhere along with detailed information concerning the methods used to determine the doses of bisphenol A and octylphenol which were administered (Nagel et al., 1997).

Daily Sperm Production (DSP). Daily sperm production was determined for the right testis from 8 control males (4 vehicle controls and 4 unhandled controls) and 5 randomly selected males per treatment group by a procedure that has been previously described (Robb et al., 1978; Cooke et al., 1991; Joyce et al., 1993). Briefly, after removal and weighing of testes and epididymides, the tissues were placed in liquid nitrogen, and subsequently kept at -70°C until being examined. The tunica albuginea were removed, and the testes were reweighed. Testes were then homogenized for 3 min in 25 ml of physiological saline containing 0.05% (vol/vol) Triton X-100 (Sigma, St. Louis, MO) using a semimicro Waring blender (Robb et al., 1978).

Step 14-16 spermatids (stage II-VIII) survive this homogenization, and their nuclei can then be counted using a hemacytometer. To count the spermatids, a 200 μ l sample of homogenate were diluted

with 300 μ l of saline and 500 μ l of 4% trypan blue, which stains spermatids and facilitates counting (Cooke et al., 1991). Sample aliquots of 5.5 μ l were placed on the hemacytometer and counted twice under a microscope to determine average number of spermatids per sample. These values were used to obtain total number of spermatids per testis, then divided by the testis weight to give spermatids per g of testis. Developing spermatids spend 4.84 days in steps 14-16 during spermatogenesis in the mouse. Thus, the values for the number of spermatids per testis and spermatids per g testis were divided by 4.84 to obtain daily sperm production and efficiency of sperm production (per g testis), respectively (Robb et al., 1978; Joyce et al., 1993).

Statistical Analyses. The unit for statistical analysis in developmental toxicology studies is the dam, which provided one experimental animal per litter. As described above, results using our new *in vitro* assay led to the prediction that bisphenol A but not octylphenol would produce detectable changes in the weight of reproductive organs (Nagel et al., 1997). We thus analyzed the data for octylphenol and bisphenol A separately. Analysis of covariance (ANCOVA) was first conducted to determine whether organ weight and daily sperm production measures needed to be corrected for body weight. If body weight accounted for a significant component of the variance, then group means were adjusted for body weight. The correlation between organ weight and body weight was also determined using Pearson's Correlation analysis. If body weight did not account for a significant component of the variance, then the data were reanalyzed by ANOVA, and group means were not adjusted for body weight. Planned comparisons were made using the LS Means Test, and means and SEM values for each group were taken from the LS Means comparisons after either ANCOVA or ANOVA. The Statistical Analysis System (SAS) on the University of Missouri mainframe IBM computer was used for these analyses.

RESULTS

Long-Term Effects of Fetal Exposure to Bisphenol A or Octylphenol on Daily Sperm Production and Efficiency. We analyzed daily sperm production without correction for testis

weight and after correction for testis weight, referred to as efficiency (daily sperm production per g testis). Vehicle and unhandled controls did not differ and were combined as one group. The 20 ng/g dose of bisphenol A significantly ($P < 0.01$) reduced efficiency (Fig. 1) and tended ($P = 0.06$) to reduce daily sperm production relative to controls (Table 1). There was no significant effect of either dose of octylphenol on daily sperm production or efficiency ($P > 0.1$).

Long-Term Effects of Fetal Exposure to Bisphenol A or Octylphenol on Testes, Epididymides, Preputial Gland and Seminal Vesicle Weight. Prior to comparing males from the different treatment groups, unhandled and vehicle-exposed control males were compared. These two groups did not differ significantly on any measure and were combined into one control group ($n = 11$). As reported previously for these males (Nagel et al., 1997), when examined at 6 months of age, there was a significant effect of prenatal treatment on adult body weight ($P < 0.05$). For animals exposed to the 2 μg dose of either bisphenol A or octylphenol, body weight was significantly lower than for controls ($P < 0.05$), while body weight of males exposed to the 20 μg dose of either chemical did not differ significantly from controls (Table 2).

For bisphenol A, neither testes nor epididymidal weight was significantly correlated with body weight, and the data were thus analyzed by ANOVA. In contrast, for octylphenol, both testes and epididymidal weight were significantly correlated with body weight ($P < 0.01$), and group means shown in Table 2 for octylphenol were adjusted for effects of body weight by ANCOVA, since body weight accounted for a significant component of the variance for both testis and epididymidal weight ($P < 0.01$).

Neither dose of bisphenol A or octylphenol had a significant effect on testis weight ($P > 0.1$). In contrast, the 2 ng/g dose of bisphenol A significantly ($P < 0.05$) reduced epididymidal weight, while the 20 ng/g dose tended ($P = 0.08$) to reduce epididymal weight relative to control males. Neither dose of octylphenol had a significant effect on epididymal weight ($P > 0.1$).

For both bisphenol A and octylphenol, body weight was not correlated with the weight of seminal vesicles or preputial glands ($r < 0.1$), and based on ANCOVA, body weight did not account for a

significant component of the variance in the weight of the seminal vesicles or preputial glands ($p > 0.5$). We previously reported that for these same males, body weight also did not account for a significant component of the variance in prostate weight, and prostate weight and body weight were not significantly correlated (Nagel et al., 1997). The effects of octylphenol and bisphenol A on the weight of the seminal vesicles and preputial glands was thus compared by ANOVA, and means for these organs are presented in Table 2 without correction for body weight.

Seminal vesicles in males exposed *in utero* to 2 μg dose of bisphenol A tended to be smaller ($P = 0.08$) than seminal vesicles in control males. Seminal vesicles in males treated with either dose of octylphenol did not differ significantly from controls ($P > 0.1$).

Males exposed to the 2 μg dose of bisphenol A had preputial glands that were significantly larger than controls ($p < 0.05$). Males exposed to the 20 μg dose of bisphenol A tended to have enlarged preputial glands, although the difference was not statistically significant ($p = 0.1$). In contrast, males exposed *in utero* to either the 2 or 20 μg dose of octylphenol did not differ significantly from controls.

DISCUSSION

We report here that in adult male mice, daily production of sperm and three accessory reproductive organs, the epididymides, preputial glands and seminal vesicles, were permanently altered by prenatal exposure to environmentally relevant doses of bisphenol A. We did not observe changes in any reproductive organ in response to either dose of octylphenol that we administered. We previously reported that these same doses of bisphenol A, but not octylphenol, permanently altered prostate size (Nagel et al., 1997).

Daily Sperm Production. There has been considerable interest generated by the report of a dramatic (50%) decline in sperm count over the past 50 years (Carlsen et al., 1992). Support for the hypothesis that sperm count has declined in this century was recently provided by a study of French men over a 20-year period (Auger et al., 1995). The absence of a decline in sperm count over time in some other studies, coupled with marked regional variation in sperm counts, has led to the hypothesis

that environmental estrogenic chemicals are interfering with spermatogenesis, possibly via an impact on Sertoli cell proliferation and/or function during testicular differentiation (Sharpe et al., 1993). Sertoli cells in the mouse are proliferating rapidly at birth, then their mitogenic activity decreases steadily during postnatal life, finally ceasing by postnatal day 15 (Vergouwen et al., 1991). Our findings provide support for the hypothesis that levels of estrogenic chemicals in the environment are sufficiently high to permanently alter sperm production when exposure occurs *in utero*. Males whose mothers had consumed the 20 ng/g dose of bisphenol A for 7 days during pregnancy had significantly (20%) lower daily sperm production (per g testis), referred to as efficiency, relative to control males.

Accessory Reproductive Organs. The preputial glands secrete pheromones which are involved in social communication in mice. Fetal exposure to the 2 ng/g dose of bisphenol A significantly increased (by 35%) the size of the preputial glands relative to untreated males. We previously reported that the prostate in these same male mice, that were exposed to either the 2 or 20 ng/g dose of bisphenol A, was also significantly increased (by about 30%) relative to untreated males (Nagel et al., 1997)

The seminal vesicles tended to be smaller in males exposed *in utero* to the 2 ng/g dose of bisphenol A. The seminal vesicles are the largest of the accessory sex organs in male mice and contribute the bulk of the fluid in the ejaculate, and their removal reduces fertility in mice (Pang et al., 1979; Peitz et al., 1986). Disease of the seminal vesicles is a cause of infertility during aging in mice (Bronson et al., 1982; vom Saal et al., 1994), and changes in this organ due to exposure during fetal life to environmental chemicals might influence fertility and/or the incidence of disease during aging

The epididymal duct is a coiled duct lying next to each testis that transports sperm from the efferent ducts to the ductus deferens. The final phase of sperm maturation occurs during passage through the caput and corpus regions of the epididymis, and the caudal region of this duct serves as a storage area for mature sperm. Each epididymis and seminal vesicle develop from a Wolffian (mesonephric) duct in response to testosterone from the adjacent testis (vom Saal et al., 1992). The 2 ng/g dose of bisphenol A decreased the size of both the epididymis and seminal vesicles, suggesting that this low dose of bisphenol A interfered with the normal development of the Wolffian ducts. The Wolffian duct

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expresses estrogen receptors during prenatal development in the mouse (Cooke et al., 1991). Therefore, these organs can potentially be directly affected by compounds that bind to estrogen receptors, such as bisphenol A. In addition, early DES treatment has been shown to result in epididymal abnormalities in both experimental animals (Newbold et al., 1985) and humans (Wilcox et al., 1995). The effects on seminal vesicles and epididymides observed here may therefore reflect direct effects of bisphenol A on the organs themselves. While at this time systemic effects of bisphenol A, such as alterations in the hypothalamo-pituitary-testis axis, cannot be ruled out, we have previously shown that the effects of estradiol on prostate size reflect direct effects on the developing prostate (vom Saal et al., 1997).

In rats exposed during fetal life to dioxin (via the dam), a decrease in epididymal sperm content is much more severe than that observed in daily sperm production (Gray et al., 1995). Thus, while a significant decrease in daily sperm production per g testis was observed in response to bisphenol A, an even greater decrease in epididymal sperm content may occur, which could contribute to the decrease in overall weight of the epididymides that we observed in response to bisphenol A.

Critical Life Stages. Whereas effects of hormones in adulthood are typically transient (referred to as activational effects), during fetal life hormones regulate the course of differentiation of neurons in the brain and spinal cord as well as reproductive tissues and tissues in other organs. The developmental effects of hormones are permanent (referred to as organizational effects). A central issue, then, is that there are critical life stages during which exposure to endocrine disruptors are most likely to lead to permanent adverse effects. During critical developmental periods in organogenesis, regardless of whether an endocrine disrupter acts as an agonist or antagonist after binding to receptors for endogenous signalling molecules, there will be a unique biological response that will not be observed when these receptors are not occupied by the endocrine disruptor, which has led to the concept of the "fragile fetus" (Bern, 1992).

Importance of Dose in Studies Involving Endocrine Disruptors. An issue that is now

recognized to be of central importance with regard to understanding the effects of environmentally relevant concentrations of endocrine disrupting chemicals is the choice of dose used in laboratory studies. While part per billion up through part per million concentrations of endocrine disrupting chemicals, such as bisphenol A, are typically encountered in food and water, the focus of toxicological research has been, and continues to be, on effects of much higher doses of these chemicals. Our unique approach has involved determining in prior experiments: 1. the potency relative to estradiol-17 β of estrogenic environmental chemicals using a new *in vitro* assay; it is this information that led us to predict that we would find effects of bisphenol A but not octylphenol in this study (Nagel et al., 1997), and 2. the levels of free estradiol (not bound to plasma estrogen-binding proteins and thus biologically active) in the serum of mice during sexual differentiation, as well as the increase in serum estradiol associated with a significant change in fetal development (vom Saal et al., 1997).

During fetal life in mice, the concentration of free (bioactive) estradiol is extremely low [0.2×10^{-12} g/ml serum; 0.2 parts per trillion (ppt); 0.8 pM]. It is this concentration of estradiol with which environmental chemicals, such as bisphenol A and octylphenol, are presumed to act additively in leading to total estrogenic bioactivity in the blood. A 50% increase in free serum estradiol of 0.1×10^{-12} g/ml (0.1 ppt) in male fetuses (due to implanting an estrogen-containing Silastic capsule in pregnant mice) significantly increased the formation of prostate glands in the fetus and permanently enlarged the prostate (vom Saal et al., 1997). This 0.1 ppt increase serves as the reference increase in free estradiol in the serum of fetuses for producing a significant change in the course of fetal development, and establishes the very high sensitivity of fetuses with regard to developmental effects of estrogen. This is important with regard to the concept that environmental estrogens, such as bisphenol A, are "weak". While our studies show that bisphenol A is about 1000-times less potent than estradiol (Nagel et al., 1997), it is difficult to consider a chemical that can significantly alter fetal development when 2 parts per billion are consumed by a pregnant female one time per day for 7 days as being weak.

Up to now, only very high concentrations of endocrine disrupting chemicals have been tested in laboratory studies, based on the assumption that responses to toxicants would increase or stay the same as dose increased (toxicants were predicted to exhibit a monotonic function), but responses would

never increase and then decrease as a function of dose (a non-monotonic function). However, in studies in which we administered pregnant female mice a 5-log range of doses of DES, we obtained a non-monotonic, inverted-U dose-response function for adult prostate size in male offspring: that is, as dose increased we first saw an increase in prostate size (at 0.02, 0.2 and 2 ng/g body weight/day), but with a further increase in dose, prostate size significantly decreased (at 200 ng/g body weight/day). The finding that exposure during fetal life to a high dose of DES permanently decreased prostate size in male mice while lower doses produced the opposite effect, an increase in prostate size, provides evidence that results from studies in which only high doses of endocrine disrupting chemicals have been used may not predict low-dose effects.

We have also examined the effect of the same doses of DES described above and an insecticide used widely around the world, DDT, on territorial marking behavior in CF-1 male mice. We found that the estrogenic contaminant of the insecticide DDT, o,p'-DDT, which comprises about 20% of commercial DDT, increased territorial marking in mice at the environmentally relevant dose of 20 ng/g body weight (20 parts per billion) when fed to pregnant female mice using the same procedures as described in the present study (vom Saal et al., 1995). At the same dose, another currently used insecticide, methoxychlor, produced a similar increase in territorial marking behavior. In addition, the same effect was seen at a dose of 0.02 ng/g DES (20 parts per trillion). DES was thus approximately 1,000-times more potent than o,p'-DDT or methoxychlor in terms of producing an effect on territorial behavior.

The rate of urine marking in males exposed prenatally to a 2 ng/g dose of DES was greater than with the 0.02 ng/g dose of DES. However, at the highest dose of DES administered, 200 ng/g, we did not see a further increase in territorial marking behavior, but rather, a significant decrease in marking behavior relative to the 2 ng/g dose. The dose-response curve for territorial marking in male mice as a function of prenatal dose of DES was thus non-monotonic and formed an inverted U, similar to the effect of these doses of DES on prostate size.

While a non-monotonic, inverted-U dose-response may not occur for all toxicants, numerous examples of non-monotonic functions have been reported (Davis et al., 1990). The conclusion from

our studies, as well as these other findings, is that responses to endocrine disruptors cannot be assumed to be monotonic across a wide dose range. Our findings suggest that unique outcomes may occur in response to low, environmentally relevant doses of endocrine disruptors that will not be observed at higher doses. The possibility of qualitatively different outcomes as dose increased was anticipated by a panel of experts at an Environmental Protection Agency workshop on endocrine disruptors, but has not, as yet, been incorporated into EPA regulations for testing endocrine disrupting chemicals in the environment (Kavlock et al., 1996).

With the exception of our studies with pregnant mice, to our knowledge there have been no other low-dose studies with bisphenol A, similar to the situation for virtually all other environmental endocrine disruptors and estrogenic drugs, including DES. Bisphenol A has been examined in high-dose studies in rats in which 1,250 mg/kg (1.25 parts per thousand) was administered (Morrissey et al., 1987). This dose is 625,000-times higher than our 2 ppb dose that altered development of the reproductive organs. The 2 ppb dose of bisphenol A that we administered to pregnant mice is similar to the concentration found to have estrogenic effects in human MCF-7 breast cancer cells (Krishnan et al., 1993; Nagel et al., 1997). This 2 ppb dose is also 25,000-times lower than the no adverse effect dose of 50 mg/kg body weight/day that was previously predicted (using currently accepted risk assessment procedures) based on studies that only examined very high doses of bisphenol A (Society of the Plastics Industry, 1995).

High-dose selection in developmental toxicology studies is typically based on some measure of maternal toxicity, for example, a 10% reduction in body weight. Several lower doses are then selected to generate a dose-response curve for fetal toxicity measures. The no adverse effect level (NOAEL) is the dose which shows no statistically significant difference from controls for fetal toxicity. Doses below the NOAEL are not required to be examined for regulatory purposes, and the potential toxicity of low, but environmentally relevant, doses of chemicals (below the NOAEL) is very rarely examined. Regulatory agencies involved in risk assessment use results from high-dose studies to calculate a dose of an environmental chemical which is predicted (but not directly shown) to be safe to consume. Our study here, as well as prior studies (vom Saal et al., 1995; Nagel et al., 1997; vom Saal et al., 1997).

indicate that this process can lead to false conclusions concerning safety, and this is cause for concern.

As yet, we do not know the lowest dose of bisphenol A that can permanently alter development of fetal reproductive system and thus the subsequent functioning of reproductive organs in mice (or any other species). Our findings suggest that since effects in mice and human breast cancer cells are seen at 2 ppb, an alteration in the course of development of reproductive organs could also occur in human fetuses carried by pregnant women who consume this amount of bisphenol A in canned products (Brotons et al., 1995) or foods heated in polycarbonate containers (Krishnan et al., 1993). Also, as much as 900 μ g of bisphenol A migrates out of plastic dental sealant during the first hour after application (Olea et al., 1996), suggesting that pregnant women may be placing their fetuses at risk by exposing them to bisphenol A.

Ethotoxicology. It is generally accepted that the underlying mechanisms of action of hormones, such as estradiol, are fundamentally similar across vertebrates. For example, the hormone estradiol, which is the most potent endogenous estrogen in vertebrates, is identical in fish, amphibians, reptiles, birds and mammals, including women. In addition, the interaction of estrogen receptors with different ligands, thus leading to the initiation of transcription of genes associated with the receptor, is remarkably similar across all vertebrates that have been studied, again including women (vom Saal, 1995). One consequence of this observation is that if an endocrine disruptor can bind to the estrogen receptor in one vertebrate, it should bind to the estrogen receptor in any other vertebrate. This is important with regard to the degree to which animal models (laboratory animals or wildlife) predict the possibility that changes will occur in humans when exposed during development to environmentally relevant concentrations of endocrine disrupting chemicals. With regard to estrogenic chemicals, the conclusion is that if an estrogenic chemical can pass through the body and enter a cell with an estrogen receptor, whether that receptor is in a cell in a fish or a human, it will bind to the receptor and alter the rate of transcription of genes associated with the receptor. While the specific genes transcribed will differ from species to species, as well as tissue to tissue within a species, disruption of the development of estrogen-responsive tissues in fetuses due to interfering with the normal regulation of these genes

will occur following exposure to the chemical. Some degree of disruption will occur at any dose, although a particular measurement may not reveal a change, since there can be no threshold for response in a system (such as the estrogen response system in fetuses) that is already operating above threshold.

The development of the nervous and endocrine systems of vertebrates shows substantial similarities across the various classes of vertebrates (vom Saal, 1995). Animals with a common phylogeny show similar morphological, physiological and behavioral traits which share common adaptive functions and are thus presumed to have been subjected to the same selection pressures, although there exist numerous variations on the common "theme", due to particular adaptive functions which are characteristic of the diverse taxa. During fetal life, endogenous steroid hormones, such as testosterone and estradiol, have marked effects on the development of the brain and reproductive organs in all vertebrates (vom Saal et al., 1992). For example, across a wide variety of vertebrate species, testosterone influences aggression and sexual behavior in males, while estradiol influences sexual behavior in females. What has been less clear is the degree to which testosterone influences the normal development, subsequent regulation of organ function, and expression of behavior in females and estradiol influences the normal development, subsequent regulation of organ function, and expression of behavior in males (vom Saal, 1989; vom Saal et al., 1994).

We propose that natural selection has operated on developmental processes such that fitness is maximized: that is, animals have evolved an optimum phenotype for the environment that they inhabit. Perturbation of systems that differentiate under endocrine control will result in disruption of the normal course of development, and the consequence will be that the fitness of affected individuals will be reduced. While these effects are often studied at the level of the individual, effects due to developmental exposure to endocrine disruptors that are detectable at the population level have been described in wildlife. In fact, the original hypothesis concerning effects of exposure to endocrine disrupting chemicals came from studying natural populations of animals living around the Great Lakes in North America (Colborn et al., 1993).

Based on our evolutionary perspective of reproductive function and behavior, our interest has been

in determining the degree to which endogenous hormones, as well as environmental toxicants that act as endocrine disruptors, can perturb development, thus impacting reproduction and social behaviors. One primary concern is the long-term effects of endocrine disruptors on the behavioral interactions within the species and with their environments (referred to as ethotoxicology). In social species, such as house mice, intrasex aggression serves to regulate the density of animals, leading to an appropriate spacing. Since sex steroids play a critical role in regulating the development of the neural areas mediating aggression, as well as the expression of aggression in adulthood (in species that have the genetic predisposition for aggressiveness), environmental chemicals that interfere with the normal actions of sex steroids have the potential to alter levels of aggressiveness in exposed animals. An assumption in ethotoxicology is that environmental chemicals that alter aggressiveness or other social behaviors will lead to changes in social interactions, which will be reflected by changes in population dynamics (vom Saal, 1984; Parmigiani et al., 1993).

In mice, preputial gland pheromones are involved in social communication between males and females (Caroom et al., 1971) and influence aggressiveness between males (Mugford et al., 1972; Ingersoll et al., 1986). Preputial gland secretions pass through ducts which empty into the prepuce, which is specially adapted in mice for depositing urine marks (Maruniak et al., 1975). The placing of these pheromones into a male mouse's environment is thus via urine marking behavior, which is influenced by dominance status (Bronson, 1979). This finding concerning effects of estrogenic chemicals, such as bisphenol A, on preputial gland size is particularly interesting given our finding described above that the rate of depositing urine marks in a novel environment was increased by maternal ingestion of a low dose of DES, o,p'-DDT, and methoxychlor (vom Saal et al., 1995).

We subsequently examined aggressive behavior in male mice exposed prenatally to 0.02 and 0.2 ng/g doses of DES and 2 and 200 ng/g doses of o,p'-DDT (administered using the same procedures described for the current study). Importantly, in these studies we used a different stock of mice (CD-1) in order to determine whether similar effects would occur in response to these chemicals in different stocks of mice. Males were examined during a 10-min test and categorized as to whether or not they attacked (bit and chased) a male intruder into the home cage of the resident experimental male. The two

groups of control males (produced by oil exposed and unhandled mothers) did not differ, and 14/26 (53%) of the control males attacked the intruder. In contrast, for the 0.02 and 0.2 ng/g DES doses, 12/14 (86%) and 13/13 (100%) of the males attacked the intruder, respectively ($P < 0.05$ relative to controls for both comparisons). Similarly, for males exposed to the 20 ng/g dose of DDT, 10/12 (83%) of males attacked the intruder ($P = 0.08$ relative to controls), while exposure to 200 ng/g DDT resulted in 9/14 (64%) of the males attacking the intruder ($P > 0.1$) (P. Palanza, unpublished observation).

These recent findings confirm in another strain of mouse that very low doses of the estrogenic chemicals DES and o,p' DDT influence the development of adult socio-sexual behaviors. When viewed in conjunction with other findings concerning effects of exposure to low doses of estrogenic chemicals on development of reproductive organs, we predict that many other systems whose normal course of development is influenced by estrogen, such as liver and kidney p450 enzymes, bone and blood vessels, will also be shown to be altered by low doses of estrogenic chemicals.

Taken together, our findings show that exposure during fetal life to low doses of endocrine disrupting estrogenic chemicals can alter the development of socio-sexual behaviors as well as the size of reproductive organs. We propose that natural selection operates to create a phenotype that is optimum for a particular environment. If exposure to endocrine disruptors changes that phenotype, leading to a less than optimum set of traits, such as an altered level of aggressiveness or functioning of the preputial glands, seminal vesicles and prostate, for that environment, a negative impact on the individuals in the population is likely to occur, and changes in population dynamics will likely follow.

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TABLE 1. DAILY SPERM PRODUCTION

Daily sperm production and efficiency (daily sperm production per g testis) for 8 control males and 5 randomly selected males per experimental group. Only the right testis was analyzed.

* indicates $P < 0.01$ relative to controls

** indicates $P = 0.06$ relative to controls

	DAILY SPERM PRODUCTION	EFFICIENCY
CONTROL	$5.26 \pm 0.18 \times 10^6$	$4.66 \pm 0.17 \times 10^7$
BISPHENOL A		
2 ng/g	$5.25 \pm 0.24 \times 10^6$	$4.50 \pm 0.22 \times 10^7$
20 ng/g	$4.65 \pm 0.24 \times 10^6$ **	$3.76 \pm 0.22 \times 10^7$ *
OCTYLPHENOL		
2 ng/g	$4.56 \pm 0.34 \times 10^6$	$3.90 \pm 0.42 \times 10^7$
20 ng/g	$5.01 \pm 0.34 \times 10^6$	$5.42 \pm 0.42 \times 10^7$

TABLE 2. BODY WEIGHT AND REPRODUCTIVE ORGAN WEIGHTS

Body weight (in g) and weight of both preputial glands, seminal vesicles, testis and epididymides (in mg) in adult male mice produced by females fed bisphenol A, octylphenol or no chemical (control) from gestation day 11-17. For the males exposed to bisphenol A, none of the organ weights were correlated with body weight and were thus not corrected for body weight. For the males exposed to octylphenol, testes and epididymides were significantly correlated with body weight and means are adjusted for body weight by ANCOVA.

* indicates significant difference from controls ($P < 0.05$).

** indicates $P = 0.08$ relative to controls.

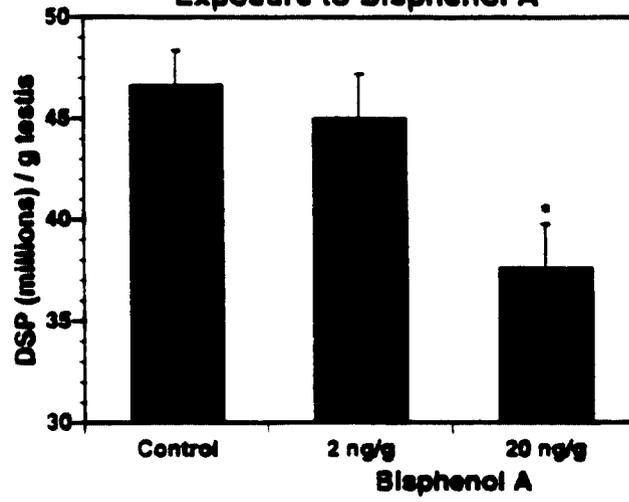
*** indicates $P = 0.1$ relative to controls.

	BODY WT (g)	PREPUTIAL WT (mg)	SEMINAL VESICLE WT (mg)	TESTES WT (mg)	EPIDIDYMIDAL WT (mg)
CONTROL	37.9±0.8	39.3±3.7	48.9±2.0	233.0±6.5	94.4±2.5
BISPHENOL A 2 ng/g	34.6±1.1*	53.3±4.6*	43.1±2.6**	216.1±8.2***	83.3±3.4*
20 ng/g	36.7±1.1	49.5±4.6***	49.5±2.6	234.7±8.2	88.8±3.4
OCTYLPHENOL 2 ng/g	33.4±1.1*	43.4±4.5	48.1±2.9	231.1±7.5	90.3±2.6
20 ng/g	37.3±0.7	47.8±4.5	48.2±2.7	236.9±6.4	90.0±2.2

FIGURE LEGEND

FIGURE 1. The mean (+SEM) daily production (in millions) of sperm per g testis (efficiency) in control adult male mice and the male offspring of pregnant females fed bisphenol A at 2 or 20 ng/g body weight. * indicates $P < 0.01$ relative to controls.

**Daily Sperm Production (DSP)
Per Gram Testis after Prenatal
Exposure to Bisphenol A**



bcc: Lynne Harris - SPI
Dick Sayad - Dow
Tom Gardner - Shell
Sondra O'Block - Aristech

Judy Babcock
Steve Dimond
Ray Hiley
Doug Johns
Tim Ullman