A PHYSIOLOGICALLY BASED APPROACH TO THE STUDY OF BISPHENOL A AND OTHER ESTROGENIC CHEMICALS ON THE SIZE OF REPRODUCTIVE ORGANS, DAILY SPERM PRODUCTION, AND BEHAVIOR

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Two chemicals previously shown to have estrogenic activity, bisphenol A and octylphenol, were examined for their effects on accessory reproductive organs and daily sperm production in male offspring of mice fed these chemicals during pregnancy. These chemicals are used in the manufacture of plastics and other products, and have been detected in food and water consumed by animals and people. From gestation day 11–17 female mice were fed an average concentration (dissolved in oil) of bisphenol A or octylphenol of 2 ng/g body weight (2 ppb) and 20 ng/g (20 ppb). The 2 ppb dose of bisphenol A is lower than the amount reported to be swallowed during the first hour after application of a plastic dental sealant (up to 931 μg; 13.3 ppb in a 70 kg adult). We found that the 2 ng/g dose of bisphenol A permanently increased the size of the preputial glands, but reduced the size of the epididymides; these organs develop from different embryonic tissues. At 20 ng/g, bisphenol A significantly decreased efficiency of sperm production (daily sperm production per g testis) by 20% relative to control males. The

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2. Abbreviations: ANCOVA, analysis of covariance; ANOVA, analysis of variance; DES, diethylstilbestrol; DSP, daily sperm production; L:D, light:dark cycle; LS, least squares; NOAEL, no-observed-adverse-effect-level; PCB, polychlorinated biphenyl; RBA, relative binding affinity; SAS, statistical analysis system; SMA, serum modified access.

3. Key words: bisphenol A, developmental toxicology, dose-response, octylphenol.

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only significant effect of octylphenol was a reduction in daily sperm production and efficiency of sperm production at the 2 ng/g dose. 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.

INTRODUCTION

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. However, with regard to endocrine-disrupting chemicals, the major concern is with exposure to low, physiologically relevant doses during critical periods in organ development. Endocrine signals coordinate cell differentiation and organogenesis, and many of these signaling molecules, such as estradiol, regulate the course of development at much lower concentrations than had been appreciated (vom Saal et al., 1997). Alteration of the developmental program can occur as a result of changing, even slightly, the concentrations of endocrine signaling molecules that are available to bind to receptors in target cells (either due to exposure to chemicals that mimic or antagonize hormones). This disruption of the normal developmental program can lead to irreversible changes in the functioning of organ systems throughout the remainder of life. This occurs without altering the genetic code in cells by mutations but, instead, involves altering the processes that turn on and off specific genes, as well as setting the rate of activity of genes that are turned on during the developmental period of cell differentiation.

Effects of hormones in adulthood are typically transient (referred to as activational effects), and exposure to an environmental chemical that interferes with signaling molecules can modulate the functioning of systems while the chemical is present, but the effects disappear when the chemical is not present (assuming exposure levels were not high enough to cause permanent damage to cells). To refer to environmental chemicals that can cause these types of effects as "endocrine modulators" is thus appropriate with regard to transient effects in adults. Webster's New Collegiate Dictionary (1980) defines modulate as "to adjust." In marked contrast, environmental chemicals that alter gene activity during sensitive developmental periods when cell differentiation is occurring, irreversibly disrupt the functioning of exposed cells. Medical science has, as yet, not developed techniques to "adjust" or reset the functioning of these genes in living organisms once this genetic imprinting has occurred. Referring to chemicals that can produce these permanent effects as "endocrine disruptors" emphasizes that the greatest threat posed by these chemicals is the disruption of development, with the result that the functioning of cells, organs, and organ systems is irreversibly changed.

There are critical life stages during which exposure to endocrine disruptors is most likely to alter the course of development, thus leading to lifetime changes in the functioning of organs. During critical developmental periods in organogenesis, regardless of whether an endocrine disruptor acts as an agonist or antagonist after binding to receptors for endogenous signaling molecules, there will be an unique biological response that will not be observed when these receptors are not occupied by the endocrine disruptor. There may also be unique responses observed due to exposure to combinations of chemicals that are not observed when the chemicals are present individually. The fact that this can occur with very low doses of natural hormones or man-made hormone mimicking chemicals (Nagel et al., 1997; vom Saal et al., 1997) supports the concept proposed by Howard Bern in "The fragile fetus" (Bern, 1992).

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). The doses of estrogenic endocrine-disrupting chemicals that we use in our studies are based on a new *in vitro* assay designed to predict *in vivo* bioactivity of xenoestrogens (Nagel et al., 1997). Specifically, in this assay, the relative binding affinity-serum modified access (RBA-SMA) assay, the potency of xenoestrogens relative to estradiol is determined in the presence of serum, which alters estimates of bioactivity relative to serum-free medium (Nagel et al., 1997). A dose of xenoestrogen is then administered to pregnant mice that is equated with an increase in estradiol previously shown to alter development of the reproductive organs in fetal mice (vom Saal et al., 1997).

Of considerable importance with regard to the experiment we describe here is that the doses of chemicals used, based on our physiological approach to determine low doses that are predicted to produce a biological effect, are also within the range of current human exposure. For example, during the first hour after application of plastic dental sealant, 931 μg of bisphenol A (the monomer used to make the plastic polymer) was detected in saliva (Olea et al., 1996); for a 70 kg person, this would represent 13.3 μg/kg (13.3 ppb), and for the average 8-year old child, who weighs 25 kg, a dose of 37.2 ppb. Our prediction, based on our *in vitro* assay, was that a dose of bisphenol A within this range of estrogenic activity would alter fetal development. This was confirmed when pregnant mice were fed 2 or 20 ppb of bisphenol A for 7 days, and the prostate in male offspring was enlarged relative to untreated males (Nagel et al., 1997). As yet, however, there are few other published studies in which environmental estrogens or other categories of endocrine-disrupting chemicals that mimic or antagonize endogenous hormones have been examined at either physiologically relevant or environmentally relevant concentrations.

Chemicals that have the capacity to disrupt the endocrine system act via many different mechanisms (Colborn et al., 1993). For example, o,p'-DDT, a contaminant in commercial DDT, binds to estrogen receptors and is an estrogen agonist (Fry and Toone, 1987; Johnson et al., 1988; vom Saal et al., 1995), whereas 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 PCBs can bind to transthyretin, a serum transport protein for thyroid hormone, thus altering bioavailable levels of thyroid hormone (Brouwer et al., 1995). 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). Therefore, our prediction is that the methods we have developed to determine the low doses of environmental estrogens 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 one of the top 50 chemicals produced in the US, where yearly production is over 1.6 billion pounds (Jennings, 1994; Kirschner, 1996). Bisphenol A is the monomer used in the manufacture of the resin used to line food and drink cans and is the monomer from which polycarbonate plastic is made. 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; Sharpe et al., 1995; Sonnenschein et al., 1995; Jobling et al., 1996; Nagel et al., 1997).

The focus of this study 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 days 11–12 of gestation. At this time the testes in male fetuses 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).

In this experiment we examined effects on development of reproductive organs in male mice as a result of fetal exposure to environmentally relevant doses of bisphenol A and octylphenol. We examined daily sperm production (measured as sperm production per g testis) and 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. We also examined the size of the preputial glands, which are involved in social communication. We relate the results of this study to prior and recent findings concerning changes in adult sociosexual behavior 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, Massachusetts) 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 (so that timed matings could occur at the end of the dark phase of the light:dark cycle).

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, along with other factors, determines the proportion of the chemical (or endogenous hormone) that is free to pass from blood into cells (Skalsky and Guthrie, 1978; Sheehan and Young, 1979; Akpoviroro and Fotherby, 1980; Sheehan et al., 1984; Arnold et al., 1996; Nagel et al., 1997). To address this issue, we 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 (Nagel et al., 1997). 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. The RBA-SMA 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 detectable 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.

Treatment of Pregnant Females with Bisphenol A and Octylphenol and Mating Procedure
Bisphenol A and octylphenol were dissolved in tocopherol-stripped corn oil (Cat# 901415, ICN, Aurora, Ohio), and 30 μL containing two different amounts (0.1 μg and 1.0 μg) were fed to pregnant female mice (n = 7/group) one time per day (at 1000 h) from gestation day 11–17. Based on an average body weight of 50 g for the pregnant females during the period of administration of these xenobiotics, as reported previously (vom Saal et al., 1995), the average maternal dose for each of these two treatment groups throughout the 7 days of treatment was 2 ng/g and 20 ng/g body weight. There were also two control groups: vehicle controls (n = 6) and females that remained unhandled throughout pregnancy (n = 5). An electronic micropipette 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).

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 by the presence of a copulatory plug (gestation day 0), and females were separated from the stud male. Females delivered their litters normally on gestation day 19, and pups were weaned on postnatal day 23. Male littermates were housed three per cage until they were 5 months old.

Collection of Reproductive Organs
At 5 months of age, randomly selected males were 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 by a procedure that has been previously described (Robb et al., 1978; Cooke et al., 1991a; 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 minutes in 25 mL of physiological saline containing 0.05% (vol/vol) Triton X-100 (Sigma, St. Louis, Missouri) 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 μL sample of homogenate was diluted with 300 μL of saline and 500 μL of 4% trypan blue, which stains spermatids and facilitates counting (Cooke et al., 1991a). Sample aliquots of 5.5 μL 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 the total number of spermatids per testis, then this number was 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

Data for daily sperm production and efficiency (daily sperm production per g testis) were analyzed by analysis of variance (ANOVA). For organ weights, analysis of covariance (ANCOVA) was first conducted to determine whether organ weight 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. For both ANOVA and ANCOVA, planned comparisons were made using the LS Means Test. Where appropriate to produce the greatest degree of homogeneity of variance for the different groups, data were log transformed prior to conducting the analysis. 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), in a randomly chosen subset of five males from each experimental group and eight control males. For the octylphenol 2 ng/g group, testes from an additional three animals were examined. After removing the testes, the remainder of the reproductive tract from these three males was removed and placed in fixative for histological analysis, and organs from these males were thus not included in the organ weight analyses. The 20 ng/g dose of bisphenol A significantly ($p < 0.05$) reduced efficiency (Figure 1; Table 1). The 2 ng/g dose of octylphenol significantly reduced both daily sperm production and efficiency ($p < 0.05$).

Long-Term Effects of Fetal Exposure to Bisphenol A or Octylphenol on Testis, Epididymal, 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 with both bisphenol A (n = 7/dose) and octylphenol (n = 7/dose) on adult body weight ($p < 0.05$). For animals exposed to the 2 ng/g 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 ng/g dose of either chemical did not differ significantly from controls (Table 2).

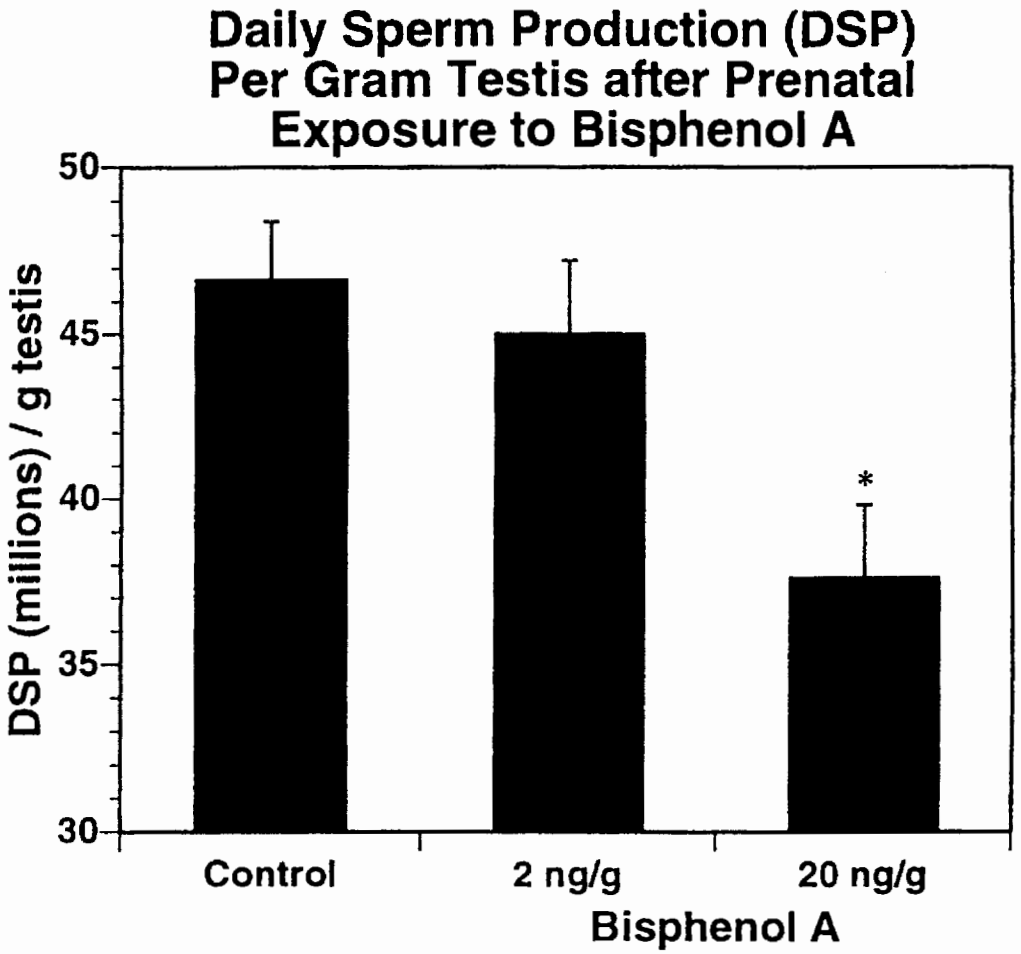

FIGURE 1. The mean (± SEM) daily production (in millions) of sperm per g testis (efficiency of sperm production) in control adult male mice and the male offspring of pregnant females fed bisphenol A at 2 or 20 ng/g body weight. * indicates $p < 0.05$ relative to controls.

Testis and epididymal weights were significantly correlated with body weight. Group means for these organ weights shown in Table 2 were adjusted for effects of body weight by ANCOVA, since body weight accounted for a significant component of the variance for both testis and epididymal weight ($p < 0.01$). Neither dose of bisphenol A or octylphenol had a significant effect on testis weight ($p \geq 0.1$). In contrast, the 2 ng/g dose of bisphenol A significantly ($p < 0.05$) reduced epididymal weight, while the 20 ng/g dose tended ($p = 0.06$) to reduce epididymal weight relative to control males. Neither dose of octylphenol had a significant effect on epididymal weight ($p > 0.1$).

TABLE 1. Daily Sperm Production

	Daily Sperm Production	Efficiency
Control	$5.26 \pm 0.18 \times 10^6$	$4.66 \pm 0.16 \times 10^7$
Bisphenol A		
2 ng/g	$5.25 \pm 0.24 \times 10^6$	$4.50 \pm 0.25 \times 10^7$
20 ng/g	$4.65 \pm 0.28 \times 10^6$	$3.76 \pm 0.22 \times 10^{7*}$
Octylphenol		
2 ng/g	$4.23 \pm 0.28 \times 10^{6*}$	$3.74 \pm 0.21 \times 10^{7*}$
20 ng/g	$5.01 \pm 0.54 \times 10^6$	$5.42 \pm 0.74 \times 10^7$

Daily sperm production and efficiency (daily sperm production per g testis) for the right testis from 8 control males, 8 octylphenol exposed males (2 ng/g), and 5 randomly selected males in the other experimental groups. * indicates $p < 0.05$ relative to controls

TABLE 2. Body Weight and Reproductive Organ Weights

	Body wt (g)	Preputial wt (mg)	Seminal vesicle wt (mg)	testes wt (mg)	epididymal wt (mg)
Control	37.9 ± 0.8	39.3 ± 2.9	48.9 ± 2.3	229.3 ± 6.9	94.3 ± 2.5
Bisphenol A					
2 ng/g	$34.6 \pm 1.1^*$	$53.3 \pm 4.7^*$	$43.1 \pm 2.7^{\dagger\dagger}$	216.9 ± 8.4	$83.3 \pm 3.1^*$
20 ng/g	36.7 ± 1.1	49.5 ± 5.9	49.5 ± 1.7	232.9 ± 7.4	$87.2 \pm 2.7^{\dagger}$
Octylphenol					
2 ng/g	$33.4 \pm 1.1^*$	43.4 ± 6.1	51.2 ± 4.3	231.0 ± 10.5	92.9 ± 3.8
20 ng/g	37.3 ± 0.7	47.8 ± 3.9	48.2 ± 1.4	229.6 ± 8.8	90.3 ± 3.2

Body weight (in g) and weight of paired preputial glands, seminal vesicles, testes and epididymides (in mg) in adult male mice produced by females fed bisphenol A (n = 7/dose), octylphenol (n = 7/dose) or no chemical (combined oil exposed and unhandled controls; n = 11) from gestation day 11–17. For the males exposed to bisphenol A and octylphenol, testes and epididymides were significantly correlated with body weight, and means were adjusted for body weight by ANCOVA. Seminal vesicles and preputial glands were not correlated with body weight and these data were analyzed by ANOVA. * indicates significant difference from controls ($p < 0.05$). † indicates $p = 0.06$ relative to controls. †† indicates $p = 0.08$ relative to controls.

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, prostate weight and body weight were also not significantly correlated, and body weight did not account for a significant component of the variance in prostate weight (Nagel et al., 1997). The effects of octylphenol and bisphenol A on the weight of the seminal vesicles and preputial glands were 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 a 2 ng/g 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 or the 20 ng/g dose of bisphenol A did not differ significantly from controls ($p > 0.1$).

Males exposed to the 2 ng/g dose of bisphenol A had preputial glands that were significantly larger than controls ($p < 0.05$), while for the males exposed to the 20 ng/g dose of bisphenol A, this trend was not statistically significant ($p = 0.11$). Preputial glands in males exposed *in utero* to either the 2 or 20 ng/g dose of octylphenol did not differ significantly from controls. We previously reported that both of these doses of bisphenol A, but not octylphenol, permanently increased prostate size in these same male mice (Nagel et al., 1997).

DISCUSSION

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), as well as an increase in testicular cancer (Bergstrom et al., 1996). 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). Another study compared sperm concentration of men living in three different cities (New York, New York, Los Angeles, California, and Roseville, Minnesota) in the United States and found marked differences (80% higher values in New York relative to Los Angeles), suggesting an environmental influence, although no decrease in sperm concentration over a 25-year period was found (Fisch et al., 1996). Sharpe and Skakkebæk (1993) have hypothesized that environmental estrogenic chemicals are interfering with spermatogenesis, possibly via an impact on Sertoli cell proliferation and/or function during testicular differentiation. Sertoli cells in the mouse proliferate rapidly at birth, then their mitogenic activity decreases steadily during postnatal life, finally ceasing by postnatal day 15 (Vergouwen et al., 1991).

Males whose mothers had consumed a 20 ng/g dose of bisphenol A for 7 days during pregnancy had significantly (20%) lower efficiency of sperm production (daily sperm production per g testis) relative to control males, while daily sperm production uncorrected for testis weight was not significantly different. There was also a significant reduction in both daily sperm production and efficiency for males exposed to the 2 ng/g dose of octylphenol, but not the 20 ng/g dose. These findings provide preliminary evidence that exposure of male mice to the environmental estrogens bisphenol A and octylphenol during fetal life can reduce daily sperm production in adulthood, and lend support for the hypothesis that exposure to environmental estrogens during testicular differentiation could account for a decrease in sperm count in men. The basis for the finding that effects were only observed at one of the two doses administered for each chemical (the higher dose for bisphenol A and the lower dose for octylphenol) requires further investigation. Whether a greater decrease in sperm count will be seen with lifetime exposure to environmentally relevant doses of environmental estrogens also remains to be determined.

Accessory Reproductive Organs

The seminal vesicles are the largest of the accessory sex organs in male mice and contribute the bulk of fluid in the ejaculate, and their removal reduces fertility in mice (Pang et al., 1979; Peitz and Olds-Clarke, 1986). Disease of the seminal vesicles is a cause of infertility during aging in mice (Bronson and Desjardins, 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 that lies next to each testis and 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 diffusion of testosterone from the adjacent testis (vom Saal et al., 1992). The 2 ng/g dose of bisphenol A significantly decreased the size of the epididymis and tended to decrease the size of the seminal vesicles, suggesting that this low dose of bisphenol A interfered with the normal development of the Wolffian ducts.

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 glands in these same male mice, exposed to either the 2 or 20 ng/g dose of bisphenol A, were also significantly increased (by about 30%) relative to untreated males (Nagel et al., 1997). Organs that develop from tissues in the embryonic urogenital sinus and perineum, such as the prostate, preputial glands, penis, and scrotum, express the enzyme 5α-reductase during fetal life. Testosterone in the systemic circulation serves as the substrate for 5α-reductase and the formation of 5α-dihydrotestosterone, which is a more potent androgen than testosterone. Wolffian ducts do not express this enzyme during early organogenesis, and sufficiently high levels of testosterone are achieved in cells as a result of diffusion from the adjacent testis. For normal differentiation of the prostate or preputial glands to occur, sufficient conversion of testosterone to 5α-dihydrotestosterone via the action of 5α-reductase is required (vom Saal et al., 1992).

The Wolffian ducts and urogenital sinus express estrogen receptors during prenatal development in the mouse (Stumpf et al., 1980; Cooke et al., 1991b). Therefore, these organs can potentially be directly affected by compounds that bind to estrogen receptors, such as bisphenol A. The effects on testes, preputial glands, seminal vesicles, and epididymides observed here may therefore reflect direct effects of bisphenol A on the organs themselves. At this time systemic effects of bisphenol A, such as alterations in the hypothalamus-pituitary-testis axis, cannot be ruled out. However, in a previous study in which male mice were exposed to different doses of estradiol during fetal life and then in adulthood were castrated and implanted with capsules containing testosterone, we found a significant effect of estradiol on prostate size (vom Saal et al., 1997). This finding shows that permanent effects on accessory reproductive organs due to fetal estrogen exposure can be seen when the levels of testosterone in the circulation at the time of organ collection are held constant.

The finding that an elevation in estrogen during fetal life tended to decrease seminal vesicle size in adulthood was predicted by our prior finding that male mice who developed *in utero* between two female fetuses (2F males), and were thus exposed to elevated estradiol via diffusion from the adjacent females (vom Saal, 1989; Even et al., 1992), had smaller seminal vesicles in adulthood than their siblings who developed *in utero* between two male fetuses (2M males) (Nonneman et al., 1992). Subsequent studies suggested that this effect was mediated by a permanent "imprinted" decrease in seminal vesicle 5α-reductase activity in 2F males relative to 2M males.[1] In contrast, these same adult 2F males showed enlarged prostates, associated with a permanent increase in prostatic androgen receptors, relative to their 2M male siblings (Nonneman et al., 1992).

1. Vom Saal, F., Nonneman, D., Ganjam, V. (1997). Unpublished observation, University of Missouri–Columbia.

It is possible that there is also an increase in androgen receptors in the preputial glands of males exposed during fetal life to low doses of natural or man-made estrogens, thus leading to a permanent increase in sensitivity to the growth promoting action of androgen, although this remains to be examined. Exposure to supplemental estrogen (natural or man-made) during fetal life can thus produce opposite effects (increase vs. decrease in size and increase vs. decrease in sensitivity to hormonal stimulation) on organs that form from different embryonic tissues, with these effects being mediated via different mechanisms. With regard to different effects occurring at different doses, at this time we do not have an explanation for the finding that changes in all accessory reproductive organs that were examined occurred in response to the 2 ppb dose of bisphenol A, while only the testes, epididymis, and prostate glands were altered by the 20 ppb dose. Also unclear is why the 2 ng/g dose of octylphenol would result in a significant decline in daily sperm production and not affect any other reproductive organ. However, there is consensus that "the same hormone and the same receptor can produce quantitatively and qualitatively different responses depending on cell type, age, and other factors" (Kavlock et al., 1996).

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 and octylphenol, an even greater decrease in epididymal sperm content may occur. This could contribute to the decrease in overall weight of the epididymides that we observed in response to bisphenol A. Developmental exposure to diethylstilbestrol (DES) has also been shown to result in epididymal abnormalities in both experimental animals (Newbold and McLachlan, 1985) and humans (Wilcox et al., 1995).

The Physiological Range for Estradiol and the 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. In many cases, part per billion concentrations of endocrine-disrupting chemicals, such as bisphenol A, are encountered in food, water and air. In contrast, the focus of dose-response assessment in toxicological research has been, and continues to be, on effects of much higher doses of these chemicals, i.e., up to a part per thousand and rarely below a part per million (Morrissey et al., 1987). The use of doses in toxicology that are much higher than doses encountered in the environment is based on the incorrect assumption that as dose increases, responses should increase or stay the same, and that response will not first increase and then decrease.

Our new approach to determining the physiologically relevant dose range for xenoestrogens is dependent upon determining in prior experiments: (1) the potency relative to 17β-estradiol of estrogenic environmental chemicals using a new *in vitro* assay (Nagel et al., 1997); (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; and (3) 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 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 or synergistically 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; this was associated with a 6-fold increase in prostatic androgen receptors (vom Saal et al., 1997).

The 0.1 ppt increase in the serum concentration of free estradiol that permanently increased prostate size and prostatic androgen receptors serves as the reference increase in free estradiol in the serum of fetuses for producing a significant disruption of normal 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." It is difficult to consider a chemical as being "weak" when that chemical can significantly alter fetal development after 2 parts per billion are consumed by a pregnant female one time per day for 7 days.

Estradiol regulates the expression of receptors for a number of hormones, such as uterine oxytocin receptors and both uterine and brain progesterone receptors (Challis and Lye, 1994; Clark and Mani, 1994), and prior studies have also shown that estradiol influences hypothalamic androgen receptors in adult male rats (Roselli and Fasasi, 1992). Taken together, these findings clearly indicate that the physiological effects of exposure to estrogenic endocrine disruptors can include changes in the functioning of tissues in a variety of organs, including the brain, due to changes in the response mechanisms for other hormones that regulate these tissues.

The underlying mechanisms of action of hormones, such as estradiol, are fundamentally similar across vertebrates. For example, the hormone 17ß-estradiol, which is the most potent endogenous estrogen in vertebrates, is identical in fish, amphibians, reptiles, birds, and mammals, including humans; the name of a steroid hormone identifies its exact structure, which is not the case for protein hormones. 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, including humans (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. The conclusion is that if an estrogenic chemical can pass through the body and enter a cell that contains estrogen receptors, whether the receptors are in a cell in a fish or a human, the chemical will bind to the receptors and alter the rate of transcription of genes associated with the receptors. The specific genes transcribed will differ from species to species, as well as from tissue to tissue within a species. However, disruption of the development of estrogen-responsive tissues in fetuses, due

to interference with the normal regulation of these genes, will inevitably occur following exposure to a sufficient concentration of the chemical to occupy even one additional estrogen receptor; since endogenous estradiol is already above the threshold for response in estrogen-responsive cells, there can be no threshold for environmental estrogens that act additively with endogenous estrogen.

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 the dose increased (toxicants were predicted to exhibit a monotonic dose-response 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 effects of the same doses of DES described above and an insecticide found in human tissue 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 ppb) 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, also produced a significant increase in territorial marking behavior. In addition, the same effect was seen at a dose of 0.02 ng/g DES (20 ppt). DES was thus approximately 1000 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 doses 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 curve may not occur for all responses to endocrine disruptors, numerous examples of non-monotonic functions have been reported for responses mediated by receptors for hormones and other intercellular signaling molecules (Amara and Dannies, 1983; Davis and Svendsgaard, 1990; Bigazzi et al., 1992; vom Saal et al., 1995, 1997). 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 different outcomes across the dose-response curve was addressed by a panel of experts on dose-response issues at an Environmental Protection Agency workshop on endocrine disruptors. It was recommended that "doses should span a wide range, including environmentally relevant doses." The objective was "to identify both toxic and mechanistic endpoints" (Kavlock et al., 1996). The recognition that the study of acute toxic effects of chemicals at high doses does not provide information about effects of the same chemicals when the dose is within a physiologically relevant (mechanistic) range of hormone-mimicking activity has not, as yet, been incorporated into EPA regulations for testing endocrine-disrupting chemicals in the environment.

With the exception of our studies with pregnant mice and a recent study with adult rats (Steinmetz et al., 1997), to our knowledge there have been no other published low-dose studies of bisphenol A in animals, which also appears to be the case 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 doses as high as 1250 mg/kg (1.25 parts per thousand) were administered (Morrissey et al., 1987). The 2 ppb dose that altered development of the reproductive organs in our study is 625 000-times lower than this dose. The 2 ppb dose is also 25 000-times lower than the 50 mg/kg body weight/day dose that was previously reported to be the no effect level for bisphenol A based on these high dose studies (Society of the Plastics Industry, 1995, 1996).

High-dose selection in developmental toxicology studies is typically based on some measure of acute 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 (3 doses covering a 5-fold to 10-fold range are typical). Dose-response evaluation in risk assessment thus does not fully assess the relationship between dose and response, and the effect of dose on response is only examined in a very small region of the dose-response curve where acute toxicity occurs. The no-observed-adverse-effect-level (NOAEL) is the dose that shows no statistically significant difference from controls for acute fetal toxicity. Doses below the NOAEL are not required to be examined for regulatory purposes.

A critical issue is that functional changes, such as changes in number of prostatic androgen receptors or in daily sperm production, as opposed to acute gross toxicity or teratology, have not typically been examined in toxicological studies. The possibility that exposure to a chemical would result in a prostate that is hyperplastic and hyperresponsive to testosterone, or result in a testis with a decreased rate of sperm production, would not have been detected in a study in which only acute gross toxicity was examined; however, these clearly represent adverse outcomes that should be of concern with regard to human health. Our findings suggest that in studies involving endocrine disruptors, the traditional approach of testing only for acute gross toxicity with very high doses needs to be replaced with a focus on effects of physiologically and environmentally relevant doses of endocrine disruptors on organ function. In addition, the possibility of a long latency between fetal exposure, identification of functional changes, and disease occurrence needs to replace the current emphasis on gross abnormalities (teratology) observed at birth.

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, 1997; Nagel et al., 1997), indicate that this process can lead to false conclusions concerning safety, and this is cause for concern. There is no extrapolation procedure that will accurately predict effects across a wide range of doses of endocrine disruptors when the dose-response curve forms an inverted U. As yet, we do not know the lowest dose of bisphenol A that can permanently alter development of fetal brain and reproductive systems and thus produce subsequent effects on behavior and the functioning of reproductive organs in mice or any other species. Our assumption is that some degree of disruption of endogenous endocrine signals will occur at any dose of bisphenol A, although a particular measurement may not reveal a change, since there can be no threshold for responses in a system (such as the estrogen response system in fetuses) that is already operating above threshold.

Effects of bisphenol A are seen in human breast cancer MCF-7 cells at 10^{-8} M (10 nM) or 2.3 ppb (molecular weight = 228) (Krishnan et al., 1993; Olea et al., 1996), and in rat GH_3 pituitary cells that secrete prolactin at 1 nM (0.23 ppb) (Steinmetz et al., 1997). Our finding that feeding pregnant female mice a 2 ng/g dose of bisphenol A (Nagel et al., 1997) resulted in a similar increase in prostate size in male offspring as did a 0.02 ng/g dose of diethylstilbestrol (DES) (vom Saal et al., 1997), showed that bisphenol A is approximately 100-times less potent than DES when fed to pregnant mice. In another study (Steinmetz et al., 1997), estradiol and bisphenol A were administered to rats via subcutaneous Silastic capsules, and serum prolactin, which is elevated by estrogen treatment, was measured (along with numerous other responses). In this study bisphenol A appeared to be within 100-fold less potent than estradiol *in vivo* (for a discussion see Feldman, 1997), which is consistent with our finding comparing bisphenol A and DES in fetal mice. There are now similar results from three independent *in vitro* experiments using cell culture assays and two independent *in vivo* experiments using rats and mice showing that bisphenol A is bioactive within the range of human exposure.

Taken together, these findings suggest that an alteration in the course of development of reproductive organs could also occur in human fetuses carried by pregnant women who consume amounts of bisphenol A found in canned products (Brotons et al., 1995; Society of the Plastics Industry, 1996) or foods heated in polycarbonate containers (Krishnan et al., 1993). Also, as much as 931 μg of bisphenol A migrates out of plastic dental sealant during the first hour after application (Olea et al., 1996), which represents a dose of 13.3 ppb for a 70 kg pregnant woman. This suggests that women may be placing their fetuses at risk by having dental sealant applied during pregnancy. Whether other chemicals in dental sealants, such as bis-GMA, have estrogenic activity after being swallowed, similar to bisphenol A or bisphenol A dimethacrylate, is unknown. Also, whether these other chemicals can be metabolized to bisphenol A or other estrogenic metabolites after being swallowed requires further investigation (Olea et al., 1996).

Effects of Estrogenic Endocrine Disruptors on Behavior: An Ethotoxicological Approach

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 numerous similarities in morphological, physiological, and behavioral traits which share common adaptive functions, and it is assumed that these traits have been subjected to similar selection pressures. For example, 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). 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 the degree to which estradiol influences the normal development, subsequent regulation of organ function, and expression of behavior in males (vom Saal, 1989; vom Saal et al., 1994).

It is generally accepted that natural selection operates 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., 1994).

In mice, preputial gland pheromones are involved in social communication between males and females (Caroom and Bronson, 1971) and influence aggressiveness between males (Mugford and Nowell, 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; dominant males mark at high rates, and subordination inhibits this behavior (Bronson, 1979). Our current finding concerning effects of estrogenic chemicals, such as bisphenol A, on preputial gland size is particularly interesting given our prior 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). Taken together, these findings suggest that exposure to low doses of estrogenic chemicals during fetal life in mice can increase the rate of urine marking as well as change the functioning of the preputial glands that produce the pheromonal signals deposited into the urine.

Since we found that exposure during fetal life to low doses of DES and estrogenic pesticides increased the rate of territorial urine marking behavior in male mice, we recently examined aggressive behavior in male mice exposed prenatally to 0.02 and 0.2 ng/g doses of DES and 20 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 minute test and categorized as to whether or not they attacked (bit and chased) a male intruder placed 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). 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 ($p > 0.1$) (manuscript in preparation).

These recent findings provide additional evidence in another stock of mice that exposure during fetal life to low doses of the estrogenic chemicals DES and o,p'-DDT influence the development of adult sociosexual behaviors. When viewed in conjunction with the findings reported here 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 p-450 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 sociosexual behaviors as well as the size and functioning of reproductive organs. It is generally assumed 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 testes, 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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