

## Developmental Exposure to Bisphenol A: Interaction with Endogenous Estradiol During Pregnancy in Mice<sup>1</sup>

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**SYNOPSIS.** Individual variability in endogenous hormones can confound the interpretation of effects of developmental exposure to endocrine disrupting chemicals. In single-birth species, such as humans, there are many sources of variability in fetal sex hormone levels, such as birth order or race. In litter-bearing species a source of fetal variability in serum levels of estradiol and testosterone is the sex of adjacent fetuses due to fetus-to-fetus steroid transport (called the intrauterine position phenomenon or IUP). Distinct phenotypes of reproductive physiology and behavior are due to IUP in house mice and other litter-bearing animals. We review here the effects of background levels of sex steroids in fetuses due to IUP in an experiment in which pregnant mice were exposed to an environmentally relevant low dose of the estrogen-mimicking chemical, bisphenol A. Bisphenol A is the monomer used to make polycarbonate plastic products (such as baby bottles), the resin lining of food and beverage cans, dental sealants, and a host of other products. Fetal exposure via the mother to bisphenol A increased the rate of postnatal growth in males and females and also advanced the timing of puberty in females. However, the greatest response to bisphenol A occurred in males and females with the highest background levels of endogenous estradiol during fetal life due to their IUP, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A treatment. This finding suggests that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development.

### INTRODUCTION

Environmental endocrine disruptors are synthetic chemicals (*i.e.*, components of plastics, pesticides, and other industrial products and by-products) and naturally produced compounds (such as phytoestrogens) that are released into the environment and have the ability to disrupt the normal functioning of the endocrine system. This has been demonstrated in wildlife, laboratory animals, and humans. The major concern regarding endocrine-disrupting chemicals is with exposure during critical periods in organ development in the embryo, fetus, and newborn (Colborn *et al.*, 1993). The basis for this concern is that development is coordinated by hormonal signals

and disruption of these signals can lead to irreversible changes in organ function.

Our research concerns the interaction between the endogenous estrogen, estradiol, and environmental estrogen-mimicking chemicals on development in both male and female mice. It is well known that significant variability among individuals within a species can occur in response to variability in hormone levels during critical periods in development. In the house mouse (*Mus musculus domesticus*), there are significant strain differences in endocrine function, including responsiveness to estrogen (Roper *et al.*, 1999). There are also epigenetic sources of variation in development. A primary focus of this review is the epigenetic factor of the position of a fetus within a uterine horn in relation to the sex of adjacent fetuses, which is known as the intrauterine position phenomenon (Fig. 1).

The intrauterine position phenomenon (IUP) occurs in litter-bearing animals due to the diffusion of sex hormones between

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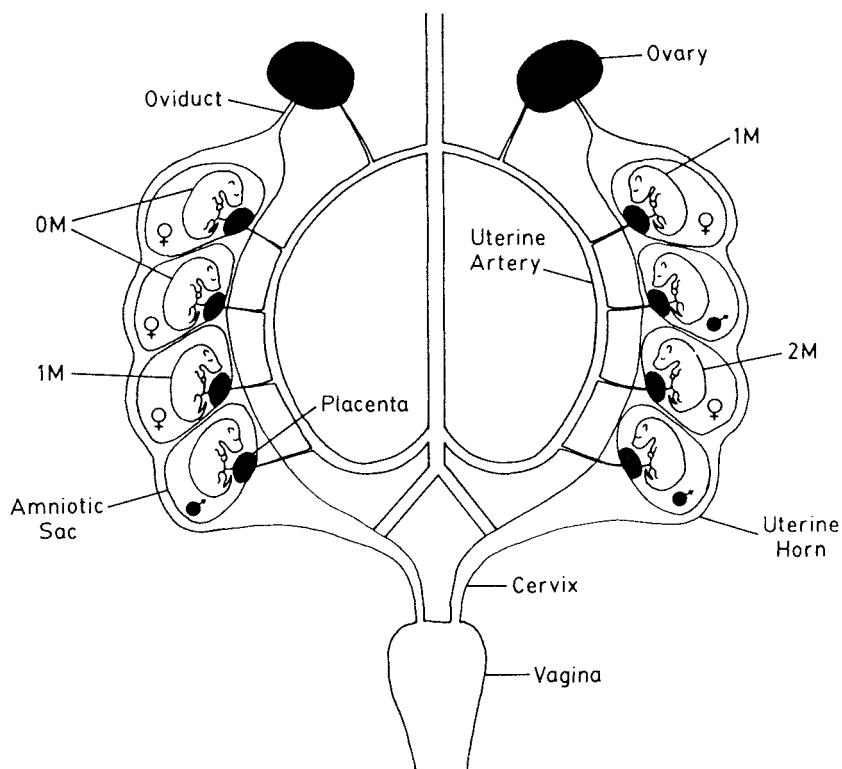


FIG. 1. The intrauterine position of fetuses as identified in the uterine horns of a pregnant mouse at term. 2M refers to fetuses between two male fetuses. 1M refers to fetuses with only one male neighbor, and 0M identifies fetuses between two female fetuses or between one female fetus and the end of the uterine horn.

adjacent fetuses. This phenomenon has been described in mice, rats, gerbils and pigs, as well as in humans carrying twins (vom Saal *et al.*, 1999). As in most species, male mouse fetuses have higher serum levels of testosterone than do females. In mice, female fetuses have higher serum levels of estradiol than do males (vom Saal, 1989b). With regard to estradiol, 0M male mouse fetuses show a 23 pg per ml serum increase in total serum estradiol relative to 2M male fetuses. Similarly, 0M female fetuses show a 32 pg/ml increase in total serum estradiol relative to 2M female fetuses (vom Saal, 1989b). It has been experimentally demonstrated (vom Saal *et al.*, 1997) that these small differences in estradiol due to IUP are sufficient to result in significant differences in the course of development and subsequent morphological, physiological and behavioral characteristics (vom Saal *et al.*, 1999).

The finding that very small differences in fetal serum estradiol due to intrauterine position were correlated with significant differences in phenotype during later life revealed the exquisite sensitivity of fetuses to hormonal signals during the critical periods in the development of the brain and genitals. This realization led to a reevaluation of the generally held belief that estrogenic endocrine-disrupting chemicals would not produce effects at levels encountered by wildlife and humans in most environments (Colborn *et al.*, 1993).

Estrogenic endocrine-disrupting chemicals typically have an affinity for estrogen receptors within the range of 1,000 to 100,000-times lower than estradiol (Welshons *et al.*, 1999), and thus, are often described as being "weak" estrogens. While these chemicals are not as potent as estradiol, what is critical is the minimum or reference dose of estradiol capable of produc-

ing an effect during development (that is, the minimum change in serum estradiol relative to baseline levels *in vivo* (vom Saal *et al.*, 1997). Our knowledge of the reference dose of estradiol that alters development, together with information about absorption, plasma binding, cell uptake and affinity for estrogen receptors for a number of estrogenic chemicals (Nagel *et al.*, 1998), led to predictions that much lower doses of these chemicals than had previously been tested would result in changes in fetal development (Welshons *et al.*, 1999). Of particular importance is that the doses of chemicals that we are studying fall within the range of human exposure (Olea *et al.*, 1996), including exposure of human fetuses (Kuriyayashi *et al.*, 1999) and neonates (Takao *et al.*, 1999). Thus, we refer to these exposures as “environmentally relevant.”

#### THE ESTROGENIC CHEMICAL, BISPHENOL A

Bisphenol A is the monomer that is used in the production of polycarbonate plastic, plastic dental sealants, the interior resin lining of food and beverage cans, and many other products. Bisphenol A was synthesized in the 1930s along with other synthetic estrogens, such as the drug diethylstilbestrol (DES), by Dodds and colleagues (Dodds and Lawson, 1936); they reported that bisphenol A acted as a full estrogen agonist in ovariectomized rats. Decades later, polymer chemists used this highly reactive phenolic compound to make plastics and resins. Bisphenol A is known to leach out of products (Olea *et al.*, 1996; Takao *et al.*, 1999).

Bisphenol A has a lower affinity for plasma binding proteins relative to endogenous estradiol (Nagel *et al.*, 1997), which results in reduced ability of the body to regulate the interaction of bisphenol A with the estrogen receptor in estrogen-responsive cells. The consequence is that bisphenol A is less regulated and thus more potent (when potency is expressed relative to estradiol) *in vivo* than predicted by *in vitro* assays which do not take into account serum binding.

Our prior research has demonstrated that feeding pregnant female mice doses of bisphenol A within the range of human exposure significantly alters reproductive or-

gan development in male offspring by increasing the size of the prostate and preputial glands (which originate from the urogenital sinus) and decreasing the weights of the seminal vesicles and epididymides (which are derived from the Wolffian ducts) (Nagel *et al.*, 1997; vom Saal *et al.*, 1998). Prenatal exposure to bisphenol A also decreased daily sperm production (per g testes) (vom Saal *et al.*, 1998). Other studies on female mice and rats have reported estrogenic effects of bisphenol A on breast (Colerangle and Roy, 1997) and uterine tissues (Steinmetz *et al.*, 1998) at doses below those previously assumed to produce no effect based on studies that only examined very high doses (NTP, 1985). In standard toxicological studies, only a few very high doses are tested and “safe” exposure levels are estimated based on mathematical models used in the risk assessment process by the Environmental Protection Agency (EPA) and the Food and Drug Administration.

#### *Effects of prenatal bisphenol A exposure on postnatal growth and the timing of puberty in female mice*

We conducted a study to examine the effects of prenatal exposure to an environmentally relevant dose of bisphenol A on postnatal growth and the subsequent timing of puberty in female mice of known intrauterine positions. The objective of the experiment was to determine whether individual differences in background levels of estradiol would alter the response of female fetuses to an estrogen-mimicking chemical due to maternal ingestion of bisphenol A during pregnancy.

Time-mated pregnant mice were fed 0 or 2.4  $\mu\text{g}/\text{kg}/\text{day}$  bisphenol A dissolved in tocopherol-stripped corn oil on days 11–17 of pregnancy, during the initial period of differentiation of the urogenital system (mating was day 0 of pregnancy). The dose was administered one time each day directly into the mouth of the pregnant mice. The pups were delivered by Cesarean section a few hours before natural parturition on day 19 to determine the intrauterine position of each fetus (Fig. 1); pups were identified within a litter using a toe-clipping pattern.

Each litter was placed with an untreated foster mother that had given birth within the prior 72 h. The foster mother's natural pups were removed immediately before introducing the experimental litter. The day of birth was considered postnatal day 1.

**Litter survival.**—Prenatal exposure to bisphenol A reduced pup survival between birth and weaning. Bisphenol A litters (6/21 complete litters dying before weaning) were significantly more likely to die before weaning than control litters (1/21 complete litters dying before weaning). The decreased survival rate of the bisphenol A-treated pups was not due to the fostering procedure, which does not significantly affect litter survival in CF-1 mice (vom Saal, 1981). Our prior study found that maternal exposure to bisphenol A had no effect on survival of offspring (Nagel *et al.*, 1997). In other studies, pup mortality was reported only at a very high dose that caused acute maternal toxicity (NTP, 1985). Thus, we do not have an explanation for the apparent increase in pup mortality due to prenatal exposure to bisphenol A in this study. Equal proportions of females from each intrauterine position died from both the control and bisphenol A-treated groups. All statistical analyses described below included adjustment for litter (maternal) effects, since we examined more than one individual per litter.

**Body weight at birth and weaning.**—In females, prenatal treatment with bisphenol A significantly increased body weight at weaning, but only in 0M and 1M animals. For all intrauterine positions combined, bisphenol A-treated females weighed significantly more at weaning than control females (Fig. 2). Bisphenol A-treated 0M females showed the greatest increase in body weight at weaning relative to 0M control females (Fig. 2). Bisphenol A-treated 1M females showed a moderate increase in body weight over control 1M females. In contrast, bisphenol A-treated 2M females showed no difference in body weight at weaning relative to control females of the same intrauterine position. The body weight at weaning of control females did not differ based on intrauterine position, confirming prior findings (vom Saal, 1989a).

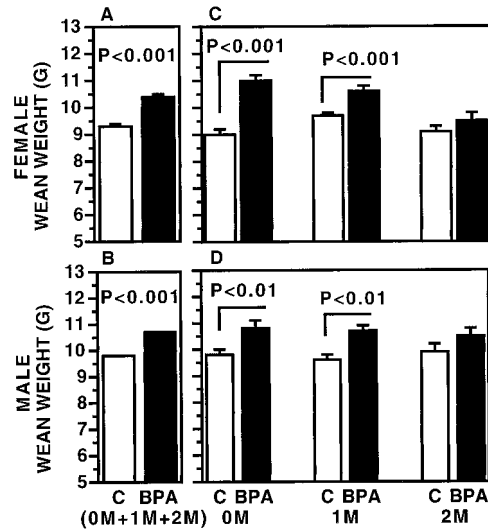


FIG. 2. Mean ( $\pm$ SEM) body weights at weaning of all females (A) and males (B) combined and by intrauterine position (C, D) for animals surviving to weaning. All body weight data were adjusted for litter to control for maternal effects. Wean weights of females were calculated on 41 0M, 47 1M, and 23 2M control (open bars) animals and 20 0M, 43 1M, and 12 2M bisphenol A-treated animals (closed bars). Wean weights of males were calculated on 32 0M, 71 1M, and 20 2M control (open bars) animals and 26 0M, 43 1M, and 14 2M bisphenol A-treated animals (closed bars).

The body weight at weaning for bisphenol A-treated males was also significantly greater than control male pups (Fig. 2). As was true for the females, bisphenol A-treated males with the highest background levels of estradiol *in utero* (0M males) showed the greatest body weight gain at weaning compared to 0M control males (Fig. 2). Bisphenol A-treated 1M males showed an intermediate increase in body weight relative to control 1M males; while bisphenol A-treated and control 2M males showed no significant difference in body weight at weaning. There were no significant differences in body weight at birth based on intrauterine position or prenatal treatment for either the male or female pups (data not shown).

**Timing of puberty.**—On postnatal day 26, control and bisphenol A-exposed females from each intrauterine position were housed individually to examine the timing of puberty. The timing of puberty in female

mice is regulated by pheromonal cues produced by both male and other female mice, and female mice isolated from males exhibit a marked delay in puberty (Vandenbergh, 1994). The pheromones in the urine from an intact adult male advance the timing of puberty and stimulate subsequent normal estrus cycles in female mice (when they are not allowed to mate) by causing the release of luteinizing hormone and then estrogen in females. This cascade of events subsequently triggers the preovulatory LH surge, which results in the first ovulation, signaling the end of the pubertal transition to adolescence (Bronson and Desjardins, 1974).

Female mice housed together produce an inhibitory pheromone that delays the onset of puberty and interferes with the accelerating action of the pheromone produced by males (Vandenbergh, 1994). In prior studies, 0M females were found to produce a more potent inhibitory pheromone and to also be more sensitive to the presence of the inhibitory pheromone relative to 2M females. The result is that 0M females enter puberty at a younger age than 2M females when housed individually with a male, but 0M females enter puberty at an older age than 2M females when housed around other females (again, in the presence of a male). Thus, the timing of puberty in untreated 0M, 1M and 2M female mice is highly dependent on the social environment with 0M females exhibiting increased sensitivity to such social cues (vom Saal, 1989a).

The potency of the male puberty accelerating pheromone is directly related to proximity of males to females, as the pheromone appears to have very low volatility (Bacchini *et al.*, 1992). Relative to previous studies of puberty conducted with untreated control females, our bisphenol A study did not allow the males to be in direct contact with the female (by using a wire-mesh partition). In fact, we placed the males at a greater distance (approximately 10 cm) away from the females relative to any of our previous studies of puberty (vom Saal, 1989a). The consequence of our male caging arrangement was that the potency of the male pheromone was sufficient to stimulate all of the females to enter puberty, but the differences between 0M, 1M and 2M fe-

males, observed when females are in close proximity to males, were not observed. The purpose of our housing procedure with submaximal male stimulation was to allow either an advance or delay in the timing of puberty to be observed due to prenatal bisphenol A treatment in females from any intrauterine position.

The method of determination of puberty in rodents is dependent upon the animal model being studied. In rodents, the vaginal canal remains closed until just prior to puberty. In rats, the age at vaginal opening is closely correlated with the first ovulation and thus is considered a reliable marker of puberty (Ojeda and Urbanski, 1994). However, in mice, canalization of the vagina is not a predictor of the age at the first ovulation and is less dependent on environmental cues than is the case in rats. The timing of puberty in house mice can only be determined by monitoring the vaginal cytology, observing mating, or monitoring the production of offspring after vaginal opening has occurred (Cooper *et al.*, 1993). In CF-1 mice, the appearance of nearly 100% cornified epithelial cells (also called vaginal estrus) is highly correlated with ovulation, as confirmed by the presence of ova in the distal segment of the fallopian tubes (vom Saal and Bronson, 1980). We maintained our animals on a 12 hr:12 hr light:dark schedule with lights on at 08:00 and collected vaginal smears beginning at 08:00 hr through 10:30 hr.

As predicted, we found that the age at vaginal opening did not differ based on prenatal treatment or intrauterine position (Fig. 3). However, for all females combined, prenatal treatment with bisphenol A shortened the number of days between vaginal opening and first vaginal estrus compared to controls. As was the case with body weight, 0M females showed the greatest response to prenatal bisphenol A exposure by an advancement of the interval between vaginal opening and first vaginal estrus of approximately 5 days in bisphenol A-treated 0M females relative to 0M controls (Fig. 3). Prenatal treatment with bisphenol A tended to advance the timing of puberty in 1M females compared to 1M controls. In contrast, bisphenol A-treated 2M females showed a

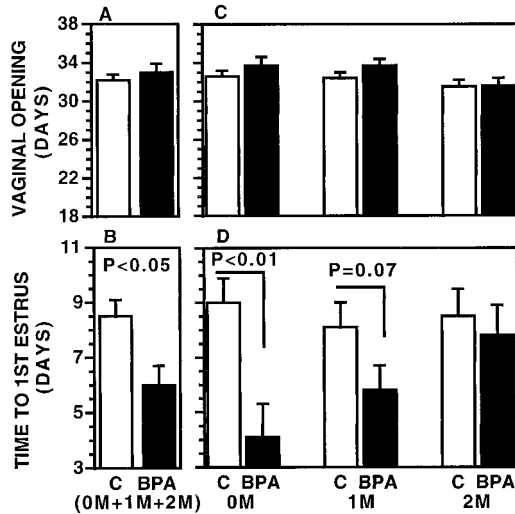


FIG. 3. Mean ( $\pm$ SEM) age at vaginal opening and interval between vaginal opening and first vaginal estrus for all females combined (A, B) and as a function of intrauterine position (C, D). All data were adjusted for litter to control for maternal effects. Data were also corrected by analysis of covariance for body weight at weaning. Vaginal opening and interval data were calculated on 19 0M, 20 1M and 19 2M control females (open bars) and 19 0M, 21 1M and 11 2M bisphenol A-treated females (closed bars).

similar timing of puberty as the 2M controls. Thus, the first vaginal estrus occurred at a significantly younger age ( $P < 0.05$ ) in bisphenol A-treated 0M females compared to 0M controls. These differences in the timing of puberty were based on an analysis of covariance that adjusted for effects due to body weight at weaning, revealing that the effect of bisphenol A on the timing of puberty was independent of its effect on body weight. This is critical since body weight is a significant predictor of puberty in mice, as well as many other species (Bronson and Desjardins, 1974).

#### IMPORTANCE OF EFFECTS OF BISPHENOL A ON GROWTH

In our study of the effects of bisphenol A on prepubertal growth and timing of puberty in females, prenatal exposure to a very low dose significantly increased the body weights at weaning of both male and female mice that had the highest endogenous levels of estradiol. Bisphenol A-exposed 0M females were 22% heavier than

0M female controls and bisphenol A-exposed 0M males were 10% heavier than 0M male controls, while 2M animals of either sex appeared to be unresponsive to maternal consumption of bisphenol A. This body weight difference was not present at birth and could not be attributable to bisphenol A-induced alterations in maternal behavior or lactation, because all litters were fostered to untreated dams. Thus, prenatal treatment with bisphenol A appeared to render 0M animals more responsive to postnatal growth-promoting factors.

The mechanism by which prenatal bisphenol A exposure interacts with background levels of estradiol to alter postnatal growth rate is unknown. The pattern of growth hormone (GH) secretion is influenced by gonadal steroids (Gatford *et al.*, 1998). Testosterone and estradiol are known to have stimulatory effects on the GH/IGF-1 axis in prepubertal animals and humans (Clark and Rogol, 1996; Gatford *et al.*, 1998). The increase in postnatal growth rate in bisphenol A-exposed 0M animals, as well as the early onset of puberty, could be related to changes in GH and IGF-1, as well as other signaling molecules implicated in both growth and the functioning of the neuroendocrine system involved in controlling puberty. Signaling molecules implicated in coordinating the timing of puberty include not only IGF-1 (Hiney *et al.*, 1996), but also leptin and neuropeptide Y (Aubert *et al.*, 1998).

One interesting aspect of these findings is that rate of growth and timing of puberty are factors that have a profound impact on population dynamics. Specifically, the age at which females begin producing young, and the number of young produced during the first pregnancy, are major determinants of population growth. At the individual level, body size is a major determinant of reproductive success (Clutton-Brock, 1988). It is thus possible that exposure of mice and other wildlife to environmental chemicals with estrogenic activity could markedly impact the reproductive fitness of selected individuals and also alter population dynamics.

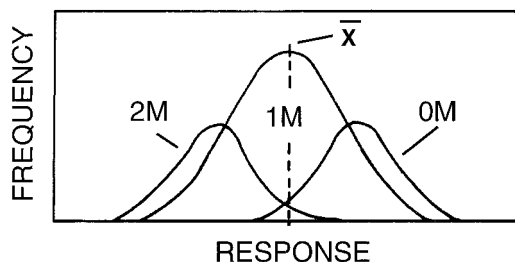


FIG. 4. This schematic represents the response of mice from different intrauterine positions to estrogenic endocrine disruptors. Serum estradiol levels are highest in 0M fetuses, intermediate in 1M fetuses and lowest in 2M fetuses. The 0M mice represent the sub-population that are most sensitive to the estrogenic chemical, bisphenol A, while 2M mice appear to be unresponsive to low, environmentally relevant doses of bisphenol A. We propose similar sensitive and insensitive sub-populations exist in other animal and human populations.  $\bar{x}$  indicates population mean.

#### CONCLUSIONS

It is generally accepted that there is variability among individuals within human and animal populations in responsiveness to endocrine-disrupting chemicals. In the current study, we demonstrate that one source of variability in the response of both male and female mouse fetuses to the estrogen-mimicking chemical, bisphenol A, is their background levels of endogenous sex hormones (Fig. 4). Background levels of sex hormones vary among individual human fetuses due to a variety of factors, such as the first versus subsequent pregnancy, placental size, twin membership versus singleton pregnancy and race (Henderson *et al.*, 1988; Hsieh *et al.*, 1992). Birth order effects on gonadal hormone levels have also been reported in birds (Schwabl, 1993). Our findings suggest that even a very small increase in the level of endogenous estradiol may substantially increase the susceptibility of fetuses to estrogen-mimicking chemicals consumed or absorbed through the skin or lungs by pregnant animals and humans.

One problem with the current toxicological literature is that laboratory experiments conducted to examine the effects of developmental exposure to estrogenic endocrine disruptors have studied only very high doses. The doses of such chemicals used in toxicological studies have been much higher than those tested in our studies of male

reproductive organs and behavior, as well as the study of puberty in females described above. While the differences in dose make comparisons difficult, prior studies have shown that developmental exposure to high doses (mg/kg amounts) of estrogenic chemicals disrupts the timing of puberty and subsequent reproductive function in female rodents (Bulger and Kupfer, 1983; Gray *et al.*, 1989). However, these findings have generally been interpreted as indicating that only with exposure levels much higher than would be encountered due to use of these estrogenic endocrine-disrupting chemicals by the general public would safety be a concern.

In contrast to gross malformations, which are the standard endpoint in toxicological studies, less obvious damage to organ function can occur at very low doses of estrogenic chemicals (vom Saal *et al.*, 1997, 1998; Welshons *et al.*, 1999), although this type of damage is more difficult to detect. A prime example of the lack of detection of the developmental effects of estrogenic chemicals is the DES tragedy. In the late 1940s through the 1960s, millions of pregnant women were administered DES to prevent miscarriage. As there were no grossly observable malformations in most offspring, the doses of DES administered were erroneously thought to be safe. We now know that significant internal damage due to prenatal DES exposure, which was undetected at birth, became apparent in adulthood. Furthermore, the adverse effects of DES in mice and humans were remarkably similar (Newbold, 1995).

Fetuses exposed to elevated endogenous estradiol may be at particularly high risk for abnormalities and diseases that may not be detectable until much later in life. Our findings of accelerated timing of puberty in 0M female mice suggest that an interaction between endogenous levels of estrogen and exposure to environmental estrogenic chemicals might contribute to the reported secular trend over the last century for an earlier age at sexual maturation in girls (Styne and Grumbach, 1986; Herman-Giddens *et al.*, 1997), including a change over the last two decades (Kaplowitz *et al.*, 1999). There has also been a doubling of

the incidence of childhood obesity over the last 20 years (Yanovski and Yanovski, 1999). These findings provide a compelling reason to conduct prospective studies with humans and wildlife to determine whether exposure to estrogenic endocrine disruptors during fetal life is contributing to these trends.

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