Hormones and Behavior 40, 252–265 (2001)
doi:10.1006/hbeh.2001.1697, available online at http://www.idealibrary.com on IDEAL®

Effects of Prenatal Exposure to Low Doses of Diethylstilbestrol, o,p'DDT, and Methoxychlor on Postnatal Growth and Neurobehavioral Development in Male and Female Mice

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Received August 9, 2000; accepted May 1, 2001

We examined effects of a wide range of doses of three man-made estrogenic chemicals during fetal life on neurobehavioral changes during early postnatal life in mice. Pregnant mice were fed a 4-log range of o,p'DDT, methoxychlor (MXC), and the drug diethystilbestrol (DES) from gestation days 11 to 17. Offspring were examined for changes in postnatal growth and the development of neuromuscular reflexes. Fetal exposure to the estrogenic chemicals altered the number of live pups per litter, the sex ratio of the litters, the anogenital distance of male and female offspring at birth (a bioassay for fetal androgen action), and the body weight of offspring at birth and during the first 5 days of postnatal life. In most cases, however, the dose–response relationships were complex (non-monotonic), with effects at the highest dose examined being opposite to effects seen at lower doses. The two markers of neurobehavioral development, righting and cliff avoidance reflexes, were not sensitive indicators of prenatal estrogen exposure. Only maternal exposure to the lowest MXC dose produced an increase in reactivity in righting and cliff avoidance tests in offspring. © 2001 Academic Press

Key Words: **endocrine disruptors; environmental estrogens; development; neurobehavioral development.**

During the last 50 years a large number of man-made chemicals have been released into the environment. Only recently has attention been drawn to the fact that a number of these chemicals have the potential to disrupt the endocrine system of animals, including humans, and these chemicals are thus referred to as endocrine disruptors (Colborn *et al.*, 1993). During fetal life, developing organs, including the brain, are vulnerable to the disruptive effects of endocrine disrupting chemicals (vom Saal *et al.*, 1992).

The focus of our research is on the subclass of environmental endocrine disrupting chemicals that have the capacity to bind to intracellular estrogen receptors, which mediate the effects of endogenous estrogen. While estrogens are critical hormones with regard to the functioning of the reproductive system in adult females, estrogen is now also recognized to play an important role in normal fetal development and the functioning of the brain and reproductive organs in males as well as females (vom Saal *et al.*, 1983, 1997; Nonneman *et al.*, 1992; Hess *et al.*, 1997).

In the studies described here, we examined morphological changes and the development of reflexes in male and female mice whose mothers consumed the insecticides o,p'DDT or MXC. These chemicals have previously been shown to have estrogenic activity both *in vitro* and *in vivo* (Gray, 1992; Soto *et al.*, 1995; vom Saal *et al.*, 1995). DDT (dichlorodiphenyl trichloroethane) is still widely used in developing countries to control malaria. The *in vivo* metabolite of the insecticide DDT is p,p'DDE, which is one of the most commonly detected environmental pollutants in human tissues. This is due to the fact that p,p'DDE is a lipophilic molecule which is highly persistent in animal tissue. p,p'DDE accumulates in body fat (Kreiss *et*

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0018-506X/01 $35.00

al., 1981). However, the endocrine disrupting activity of p,p'DDE is as an androgen receptor antagonist, not as an estrogen agonist (Kelce *et al.*, 1995).

The chemical we examined in the present study is o,p'DDT, which is a contaminant (11–29%) found in commercial DDT. o,p'DDT is the primary estrogenic component of commercial DDT, but it has a much shorter half-life *in vivo* than p,p'DDE. The concern with chemicals such as o,p'DDT that are not highly persistent is that they can cause damage if exposure occurs during critical periods in the development of the brain and other organs, but because the chemical does not remain in the body for a long period of time, examination of tissues at later times in life will not reveal the presence of the chemical that produced the damage during fetal development.

Methoxychlor [bis-*p*-methoxy o,p'DDT; 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane] is an analog of o,p'DDT, but MXC and its *in vivo* metabolites are far less persistent than p,p'DDE and thus do not bioaccumulate in animal tissues. Methoxychlor exposure is due to its use as an insecticide for pets, gardens, crops, and livestock. Methoxychlor only has estrogenic effects *in vivo* after demethylation in the liver to monohydroxymethoxychlor (30% of administered dose) or bis-hydroxymethoxychlor (23% of administered dose) (Kapoor *et al.*, 1970), with the most potent estrogenic metabolite being bis-hydroxymethoxychlor (Welch *et al.*, 1969; Bitman and Cecil, 1970; Bulger and Kupfer, 1983). In addition, MXC has recently been reported to bind to androgen receptors and also acts as an antiandrogen, since it blocks androgen-induced transcription (Gray *et al.*, 1999). Thus, the bis-hydroxy *in vivo* metabolite of MXC has both estrogenic and antiandrogenic activity, which has led to increased concern regarding exposure during pregnancy, particular during critical periods in fetal life when the initial processes of masculinization of the male reproductive system are occurring.

Virtually all previous studies of o,p'DDT and MXC have involved the use of doses which are higher than those to which animals and humans are typically exposed. These studies have shown that exposure of female rats and mice to high doses of o,p'DDT during development results in precocious puberty as well as acceleration of the loss of fertility, referred to as the delayed anovulatory syndrome (Welch *et al.*, 1969; Heinrichs *et al.*, 1971; Bulger and Kupfer, 1983). In male rats, exposure to a high dose of o,p'DDT during early life led to marked impairment of fertility and reduced weight of the prostate and seminal vesicles (Gellert *et al.*, 1974). These effects are consistent with exposure to high doses of other estrogenic chemicals (vom Saal *et al.*, 1997, 1998; Gupta, 2000). Neurobehavioral effects have also been reported following developmental exposure to o,p'DDT, such as a change in locomotor activity associated with a change in muscarinic cholinergic receptors in the cerebral cortex (Eriksson *et al.*, 1992). We previously reported that exposure of male mouse fetuses to very low doses of o,p'DDT, which had previously been predicted to be below the MRL, led to an increase in territorial behavior, specifically an increase in marking a novel environment with urine (vom Saal *et al.*, 1995).

Estrogenic effects that have been observed after neonatal administration of high doses of MXC in female rats and mice are acceleration of puberty, persistent vaginal cornification, and acceleration of the loss of fertility. Also observed were abnormal cell types in the uterus and oviducts (Welch *et al.*, 1969; Gellert *et al.*, 1974; Gray *et al.*, 1989; Eroschenko and Cooke, 1990; Gray, 1992).

Exposure during development to MXC also leads to changes in the reproductive system and behavior in male rats and mice. In particular, a very low dose of MXC of 20 μg/kg/day (the current MRL) administered to pregnant female mice resulted in permanent enlargement of the prostate in male offspring (Welshons *et al.*, 1999). We previously described results of these fetal treatments on adult territorial marking behavior using a subset of the doses and males examined in the present study. The increase in the rate of territorial marking seen in response to prenatal exposure to low doses of MXC were similar to the effects of low doses of o,p'DDT and the estrogenic drug diethylstilbestrol (vom Saal *et al.*, 1995).

Diethylstilbestrol (DES) is a potent estrogenic drug that has been extensively studied for its developmental effects in animals. The interest in DES is based on the fact that millions of pregnant women were administered DES in the mistaken belief that it would prevent spontaneous abortion and promote the development of healthy, strong babies (Newbold, 1995; Mittendorf, 1995; Swan and vom Saal, 2000). While DES has thus been studied based on an interest in its developmental effects, it is also often commonly used as a positive control chemical in toxicological studies of putative estrogenic chemicals, since the action of DES as a ligand for the estrogen receptor has been well characterized across a wide dose range (Greco *et al.*, 1993; McLachlan, 1975, No. 11; vom Saal *et al.*, 1995, 1997). Here we used DES as a positive control for estrogenic activity to provide the basis for interpreting whether any behavioral effects seen as a result of

treatment across a wide range of doses o,p'DDT and MXC were consistent with effects seen across a wide range of doses (1000 times lower than those used for the pesticides) of this potent estrogenic drug.

We report here the results of an experiment in which pregnant mice were fed a wide range of doses of DES, o,p'DDT, or MXC during 7 days of fetal life (days 11–17 of pregnancy), when sexual differentiation of the brain and reproductive organs begins in mice (vom Saal *et al.*, 1992). Our purpose was to examine the dose–response relationships of these estrogenic chemicals in offspring when the chemicals were administered during only the fetal period of sexual differentiation, since to cause effects these chemicals would have to cross the placenta; it is now widely recognized that small lipophilic molecules, such as o,p'DDT and MXC, readily cross the placenta (Welshons *et al.*, 1999). We examined the offspring for changes in postnatal growth, the development of reflexes, and morphological landmarks.

The mouse is an altricial species, that is, the pups are born in a highly immature condition after a short pregnancy (18–20 days). Several reflexes and responses appear at successive postnatal stages in parallel with somatic changes, progressively increasing the pup's sensory and motor capabilities. The time of occurrence of specified somatic changes and the time of first appearance and subsequent complete maturation of various reflexes and responses show considerable regularity, thus providing an effective tool to assess whether somatic and neurobehavioral development are modified by prenatal exposure to hormone-mimicking drugs or chemicals (Bignami, 1996). The doses used were based on predictions of estrogenic potency of these chemicals using methods described in detail elsewhere (Nagel *et al.*, 1997, 1998; Welshons *et al.*, 1999).

METHODS

Animals, Husbandry, and Mating Procedures

CF-1 mice (*Mus domesticus*) have been maintained in a closed outbred colony at the University of Missouri since 1979. The light:dark cycle was 12 h light and 12 h dark, with lights on at 1000 h. Room temperature was 23 + 2°C. Adult, 3- to 4-month-old females were time mated by being placed into the cage of a stud male for 4 h beginning at 0800 h. When a vaginal plug was found (day 0 of pregnancy), females were housed three per cage.

Maternal Treatment

DES, o,p'DDT, and MXC were dissolved in tocopherol-stripped corn oil (Cat. No. 901415, ICN, Aurora, OH). With the exception of a group of females that were left undisturbed (unhandled group), all other pregnant females received daily administration of 30 μl of corn oil (with or without a chemical) from day 11 to day 17 of pregnancy. An electronic micropipetter (Rainin) enabled delivery of an accurate volume of corn oil into the mouth of an animal. In more detail, mice were picked up by the skin between the shoulders and held upright. The pipette tip was placed into the mouth with the pipette tip gently touching the roof of the mouth, and the oil was ejected from the pipetter. Mice readily consume corn oil, and this procedure is used to reduce maternal stress that can alter fetal development (vom Saal *et al.*, 1990). The last treatment was on day 17 to reduce the possibility that the higher doses of DES and pesticides would interfere with parturition. On day 17 of pregnancy, females were individually housed and left undisturbed until delivery.

On day 11 of pregnancy, females were randomly assigned to 1 of 16 groups (n = 6 to 10 females/group): Group 1, unhandled females were left undisturbed; Group 2, vehicle control females were administered 30 μl of corn oil per day; Groups 3–8, DES at 0.001, 0.01, 0.1, 1, 10, and 100 μg/30 μl; Groups 9–13, o,p'DDT at 1, 10, 100, 1000, and 5000 μg/30 μl; and Groups 14 to 18, MXC at 1, 10, 100, 1000, and 5000 μg/30 μl. Pregnant females were weighed on gestation days 11 and 17 in order to be able to calculate the average dose/kg of body weight. The doses presented in the remainder of the paper will thus be adjusted for average maternal body weight during the time of administration of chemicals and presented as micrograms per kilogram of body weight. For all chemicals, after calculating the average administered dose/kg of body weight, the dose was rounded off such that an administered dose of 0.182 μg/kg is presented as 0.2 μg/kg.

Measurements during Early Postnatal Development

Within 12 h of delivery on day 1 of postnatal life, the following variables were recorded by an investigator who was unaware of the prenatal treatment group of the animals: the number of pups per litter, ratio of male to total number of pups (sex ratio), as well as the anogenital distance (AGD) and body weight of each pup. Litters were culled to 8 pups (3–5 males and 3–5 females using a random selection procedure) to re-

duce variability in growth and development of pups during the postnatal period of nursing. Pups were then returned to their mothers. On postnatal days 2, 5, and 10, each pup was weighed and tested for reflexes. The day of eye opening was measured as an index of rate of maturation.

In more detail, *AGD* is the distance separating the posterior aspect of the genital papilla and the anterior aspect of the anus, which was recorded for each pup on the day of birth. AGD provides a bioassay of prenatal exposure to testosterone. This tissue becomes the scrotum in males. Measurements were made using an Olympus dissecting microscope with a micrometer lens (accurate to 0.05 mm). *Body weights* were measured daily with a digital balance accurate to 0.01 g. *Righting reflex* is the time taken for a pup which has been placed on its back to turn over with all four feet on the ground. This measure provides information concerning motor and vestibular maturation. Animals were tested for a maximum of 120 s. Animals that did not turn over within 120 s were assigned this score. In the *cliff drop aversion test*, each pup was placed on a table with the forepaws and face over the edge of the table. The time taken for the pup to turn until it was parallel to the edge of the table was recorded. Animals were given a maximum of 120 s to complete the test. If the animal fell from the "cliff," it was caught and assigned the maximum latency of 120 s. *Eye opening* was defined as the appearance of a separation in the skin covering the eye. The eyes of each animal were examined daily from day 13 until both eyes were open.

Statistical Analysis

For all measures the effect of feeding pregnant females oil (vehicle control group) alone was first compared with findings from offspring of unhandled mothers (unhandled control group). This comparison showed that the two control groups did not differ on any measure, and they were combined into one control group for comparison with chemical-exposed animals.

The effects of maternal treatment on the number of pups, sex ratio, and body weight were analyzed by ANOVA. To determine whether variation in AGD was accounted for by body weight at birth, AGD was analyzed by analysis of covariance (ANCOVA). If body weight did not account for a significant component of the variance in AGD, the data for AGD were then reanalyzed by ANOVA.

Maternal dose was examined separately for DES, o,p'DDT, and MXC, since there was a different dose range for DES and the other chemicals, which precluded an overall ANOVA for all chemicals that included dose as a variable. The same control group (consisting of oil-treated and unhandled controls) was included in each of these three analyses for comparison of dose effects.

Animals within a litter are not independent of each other, and this has to be considered in conducting ANOVA or ANCOVA, which assumes that all data are independent. To increase power we tested all animals within each litter and then adjusted for litter effects statistically by including litter as a main effect in the overall ANOVA or ANCOVA. The F value for each variable measured was the product calculated by dividing the F value obtained for the treatment variable by the F value associated with litter, thus correcting for variance associated with litter (due to maternal effects). Planned comparisons were conducted using the LS means test, again using an error term adjusted for variance due to litter. The P values reported are based on the LS means test and are thus adjusted for variance due to litter. The confidence level for rejecting the null hypothesis was 0.05.

A maximum testing time of 120 s was used in both the righting reflex and the cliff avoidance experiment, and the data were not normally distributed. The results were thus first expressed as a proportion of the total observation time and then analyzed by ANOVA.

RESULTS

There were no significant differences between the offspring of vehicle control and unhandled females on any of the measures. For all measures data for the animals in these two groups were combined. The term control group thus refers to all offspring not exposed via their mothers to an estrogenic chemical.

Maternal body weights averaged 55 g from gestation days 11 to 17 for all females used in the study. Maternal body weight was not influenced by any of the chemicals. With the exception of DES 10- and 100-μg-treated females, females delivered normally on day 19. However, none of the DES 100-μg-treated females ever showed any signs of impending parturition, and they were all euthanized at the end of gestation day 22, since prior studies have shown that all pups die if parturition is delayed more than 2 days (vom Saal and Moyer, 1985). Five of the 10 DES 10-μg-treated pregnant females delivered pups 1 day late during day 20 of pregnancy, and the other 5 females in

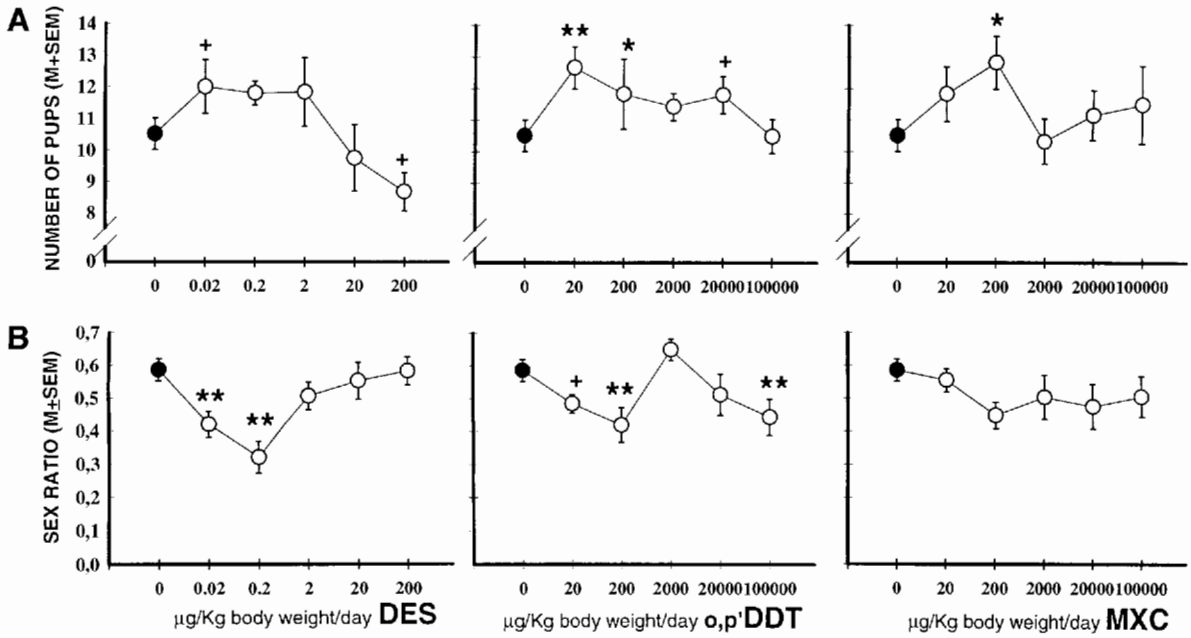

FIG. 1. Mean (±SEM) number of live pups (A) and sex ratio (number of males/total number of pups—B) of the offspring of mice fed DES, o,p'DDT, or methoxychlor (MXC) on gestation days 11–17. The doses indicated are per kilogram of body weight of the pregnant mice. * $P < 0.05$, ** $P < 0.01$, + $P = 0.06$–0.1 vs control.

this group delivered dead pups on day 22 of gestation or were euthanized on day 22, and all pups were dead. These findings have been previously described (vom Saal *et al.*, 1995).

Number of Surviving Pups and Sex Ratio

Figure 1A shows the mean (±SEM) number of live pups at birth for the different treatment groups. Disregarding dose, prenatal exposure to DES ($P < 0.05$) and o,p'DDT ($P < 0.05$), but not MXC ($P = 0.12$), significantly influenced the number of pups found alive within 12 h after delivery. Planned comparisons of controls versus animals exposed to each dose of a chemical showed that litters exposed to o,p'DDT 20 and o,p'DDT 200 were significantly larger than control litters; a similar trend was found for litters exposed to o,p'DDT 20,000 ($P = 0.07$) and the lowest dose of DES (DES 0.02; $P = 0.06$). Conversely, litters exposed to the highest dose of DES (DES 200) tended to be smaller than control litters ($P = 0.1$), although as described above, 50% of the pregnant females in this treatment group did not produce any live young, and the females in this group that did deliver live pups experienced a delay of 1 day in the onset of parturition.

The data shown in Fig. 1B suggest that an increase in litter size was associated with a decrease in sex ratio (a lower proportion of males). The overall ANOVA conducted on DES and o,p'DDT showed a significant effect on sex ratio (for both analyses, $P < 0.005$). Planned comparisons showed that relative to controls, pregnant females administered DES 0.02 and 0.2 ($P < 0.01$), o,p'DDT 20 ($P = 0.07$), o,p'DDT 200, and 100,000 ($P < 0.01$) produced litters with a lower sex ratio. With the exception of the o,p'DDT group, the lower, but not the higher, doses of these chemicals appeared to influence sex ratio. Non-monotonic dose–response relationships have been reported for DES (vom Saal *et al.*, 1995, 1997), and the data for DES could be interpreted as showing a U-shaped distribution; the shape of the dose–response curve for sex ratio for o,p'DDT does not appear consistent with results for DES.

Body Weight at Birth and on Days 2, 5, and 12

Body weight at birth. Without regard to chemical or dose, overall, male pups weighted significantly more than females ($P < 0.05$). For just males, there were no significant effects of DES or MXC on body weight at birth (Fig. 2). In contrast, there was a significant effect of maternal treatment with o,p'DDT on body weight in males ($P < 0.05$), with o,p'DDT-2000-exposed males showing lower body weights than controls.

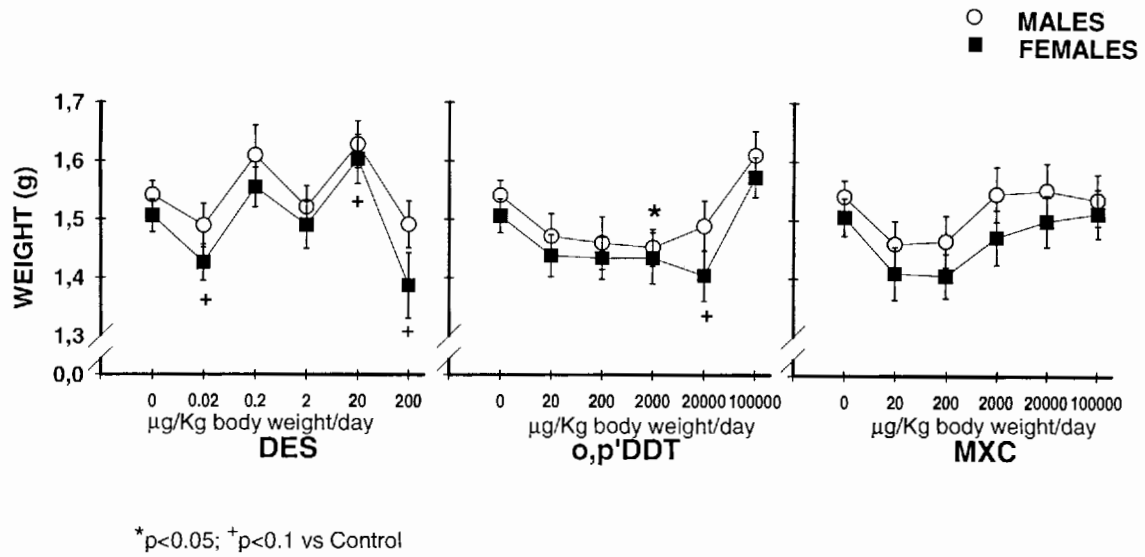

FIG. 2. Mean (±SEM) body weight at birth of the male and female offspring of mice fed DES, o,p'DDT, or MXC from days 11 to 17 of pregnancy. * $P < 0.05$, ⁻ $P < 0.06$–0.1 vs control.

Body weight at birth of females was affected by prenatal treatment with DES ($P < 0.01$) and o,p'DDT ($P < 0.05$). Females exposed to DES 0.02 ($P = 0.06$) and DES 200 ($P = 0.07$) tended to have lower body weights than controls. Females in the DES 20 group tended to be heavier than controls ($P = 0.07$). For o,p'DDT, only the o,p'DDT 20,000 females tended to have lower body weights than controls ($P = 0.06$). Prenatal exposure to MXC did not influence the body weight of female pups.

Body weight on days 2, 5, and 10. On days 2, 5, and 10, body weight did not significantly differ in relation to the sex of the pups in any treatment, and data for males and females are thus not presented separately (Fig. 3). As above, the data were corrected for litter effects. On day 2 body weight was significantly affected by prenatal exposure to DES ($P < 0.05$), but only DES-0.2-exposed pups were significantly heavier than controls. In contrast, DES 200 pups tended to show lower body weight than controls ($P = 0.07$). On day 5 DES also significantly affected body weight of pups ($P < 0.05$). The DES 0.2 animals tended to be heavier than controls ($P = 0.07$), while DES 200 animals were significantly lighter than controls. The findings for DES were thus consistent on days 2 and 5.

On day 2 there was a significant effect of maternal treatment with o,p'DDT ($P < 0.01$), with o,p'DDT 20,000 offspring tending to show lower body weight than controls ($P = 0.1$), while o,p' DDT 100,000 pups were significantly heavier than controls ($P < 0.01$). On day 5 the findings for o,p'DDT were virtually identical to those on day 2, but both the o,p'DDT 20,000 ($P < 0.05$) and o,p'DDT 100,000 ($P < 0.01$) animals were significantly different from controls. Prenatal exposure to MXC did not affect body weight of pups on either day 2 or day 5. On day 10, there were no significant effects of any chemical on body weight.

Anogenital Distance at Birth

At birth, male and female mice differ markedly in their AGD, and only comparisons within sex are of interest. To simplify the analysis, data for both AGD and body weight at birth were analyzed separately for male and female offspring (AGD should not be examined without also including body weight as a potential source of variation in the analysis). ANCOVA showed that body weight did not account for a significant portion of the variance in AGD for either males or females, confirming prior results in CF-1 mice (vom Saal and Dhar, 1992), and the data were thus reanalyzed using ANOVA. The data for the AGD measure shown in Fig. 4 are thus not adjusted for body weight.

Males. The overall ANOVAs showed that in male offspring there was a significant effect of each chemical on AGD. Prenatal treatment with DES ($P < 0.001$) significantly affected AGD, with AGD being significantly reduced at the DES 200 dose in comparison to control males ($P < 0.001$). This occurred even though the DES 200 males were 1 day older (from conception)

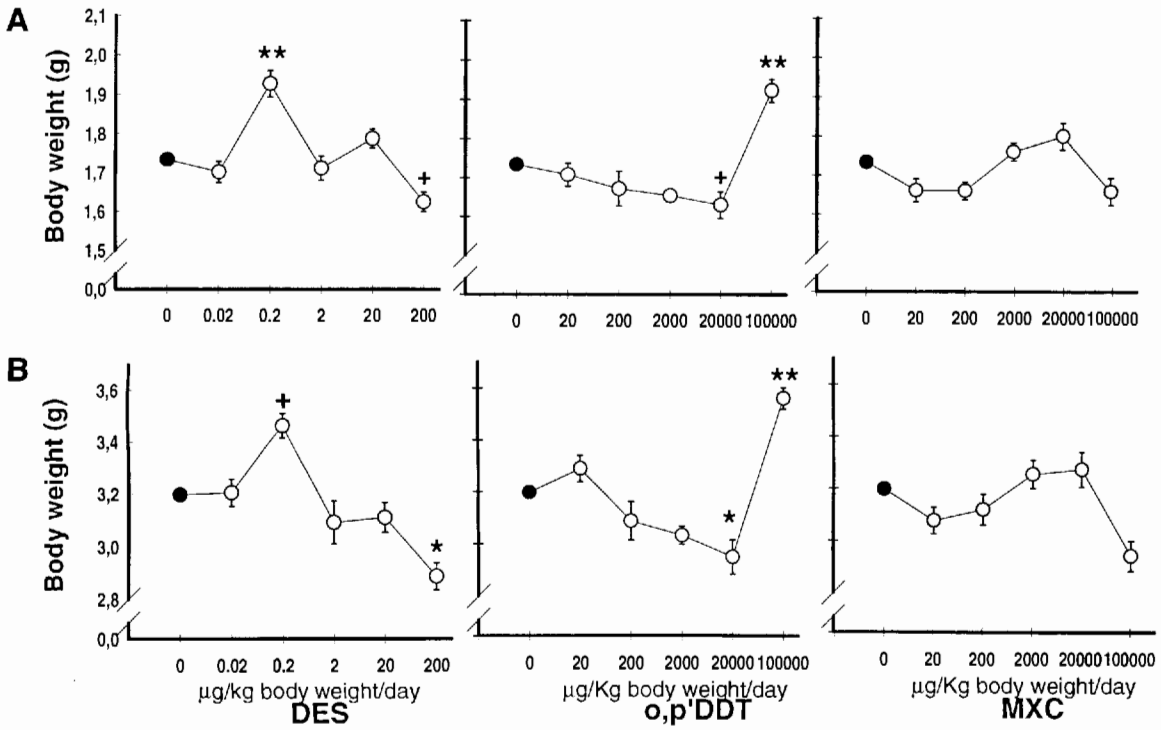

FIG. 3. Mean (±SEM) body weight on postnatal days 2 (A) and 5 (B) of the offspring of mice fed DES, o,p'DDT, or MXC during pregnancy. Data for male and female pups were combined, since they did not differ significantly on these days. * $P < 0.05$, ** $P < 0.01$, ˜ $P = 0.06$–0.1 vs control.

than the control males at the time of birth, due to parturition having occurred 1 day later with this dose of DES.

There was also a significant effect of o,p'DDT on AGD ($P < 0.05$). Males in the o,p'DDT 200 and 100,000 groups had significantly longer AGDs than control males. Conversely, AGD tended to be shorter for o,p'DDT 2000 males than for control males ($P = 0.08$).

Methoxychlor had a significant effect on AGD ($P < 0.05$), with MXC 20,000 males having a shorter AGD than controls ($P < 0.05$), while MXC 100,000 males had a longer AGD than controls ($P < 0.05$). This latter finding is consistent with the o,p'DDT findings.

Females. In female offspring, the overall analysis showed that prenatal treatment with DES was not statistically significant. In contrast, the overall analysis showed that prenatal treatment with o,p'DDT ($P < 0.005$) significantly altered AGD at birth. Specifically, o,p'DDT-100,000-exposed females had longer AGDs than controls ($P < 0.05$). For MXC, the overall analysis showed that prenatal treatment significantly affected AGD ($P < 0.001$), with MXC 200 and MXC 2000 (both Ps < 0.05) and MXC 20,000 ($P = 0.1$) having decreased AGDs, while the highest doses of MXC increased AGD relative to controls ($P < 0.05$).

Taken together, there is consistency in the effect of the 100,000 dose of both o,p'DDT and MXC in producing a lengthening of AGD in both male and female offspring. With regard to the effects of lower doses of MXC on AGD, the dose–responses, while unusual, were similar for both males and females.

Cliff Avoidance

There were no differences between males and females in the cliff avoidance response for pups in any treatment group, and data for males and females are thus not presented separately (Fig. 5). Data were corrected for litter effects as described above. Maternal treatment with o,p'DDT or DES did not influence cliff avoidance either on day 2 or day 5. In contrast, the time taken for the pup to turn and crawl away from the cliff was significantly influenced by prenatal treatment with MXC on postnatal day 2 ($P < 0.01$). Specifically, MXC 20,000 resulted in a significant increase in latency to respond relative to controls ($P < 0.01$). On day 5, the MXC 20 and MXC 100,000 groups showed a decrease in latency (both $P < 0.05$) relative to controls. The effects of MXC doses on cliff avoidance were thus

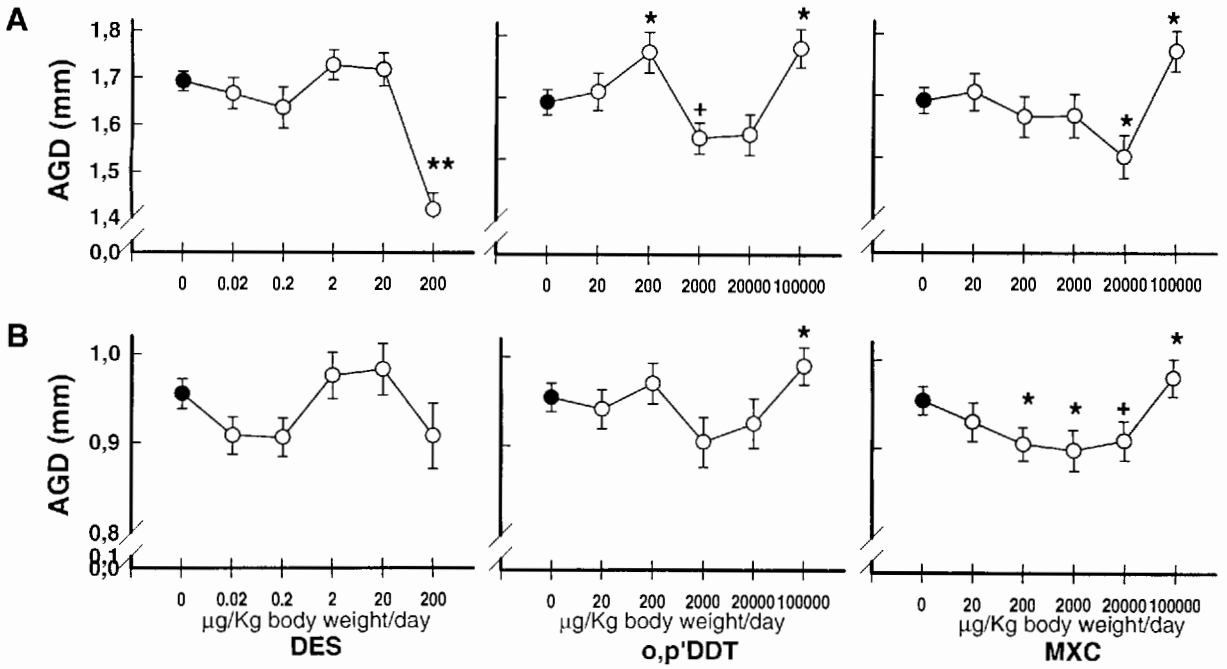

FIG. 4. Mean (±SEM) anogenital distance, which was measured within 12 h after birth with a micrometer disc accurate to 0.05 mm, in male (A) and female (B) offspring of mice fed different doses of DES, o,p'DDT, or MXC from days 11 to 17 of pregnancy. * $P < 0.05$, ** $P < 0.01$, $^{+}$ $P = 0.06$–0.1 vs control.

not consistent at these two time points. On day 10 there were no differences due to maternal treatment (data not shown).

Righting Reflex

Males and females did not differ in righting reflex in any treatment group, and data for males and females are thus not presented separately; as above, the data were corrected for litter effects (Fig. 6). Neither DES nor o,p'DDT influenced righting reflex on either day 2 or day 5. Righting reflex tended to be affected by maternal treatment on day 2 for MXC ($P = 0.07$), but only the MXC 20 animals showed decreased latency to perform the reflex relative to controls ($P < 0.01$); in contrast, there were no effects of MXC on day 5. On day 10 virtually all pups performed the righting reflex immediately, and there were no differences due to maternal treatment (data not shown).

Physical Landmarks

All the animals, both males and females, exposed prenatally to DES 200 had nipples on day 1. Testosterone normally results in the loss of nipples during fetal life in male mice. Animals in all other groups had normal nipples (data not shown).

Virtually all animals did not show evidence of eye opening prior to day 13, and virtually all animals had completed eye opening by day 15. There were no differences between males and females in eye opening regardless of treatment group, and data for males and females are thus not presented separately. Figure 7 shows the mean proportion of young in each litter that had both eyes open on day 14. The overall ANOVA conducted on these data showed that eye opening was significantly affected by prenatal treatment with DES ($P < 0.05$), but not DDT and MXC. Litters exposed to DES 0.2 ($P < 0.05$) and DES 20 ($P = 0.06$) showed a higher proportion of young with both eyes open than controls on day 14. The absence of an effect on eye opening for animals in the DES 2.0 group makes these findings difficult to interpret.

DISCUSSION

The major findings reported here relate to the fact that we examined the effects on physical landmarks and behavior during early postnatal life of fetal exposure (via feeding pregnant mice) to doses of DES,

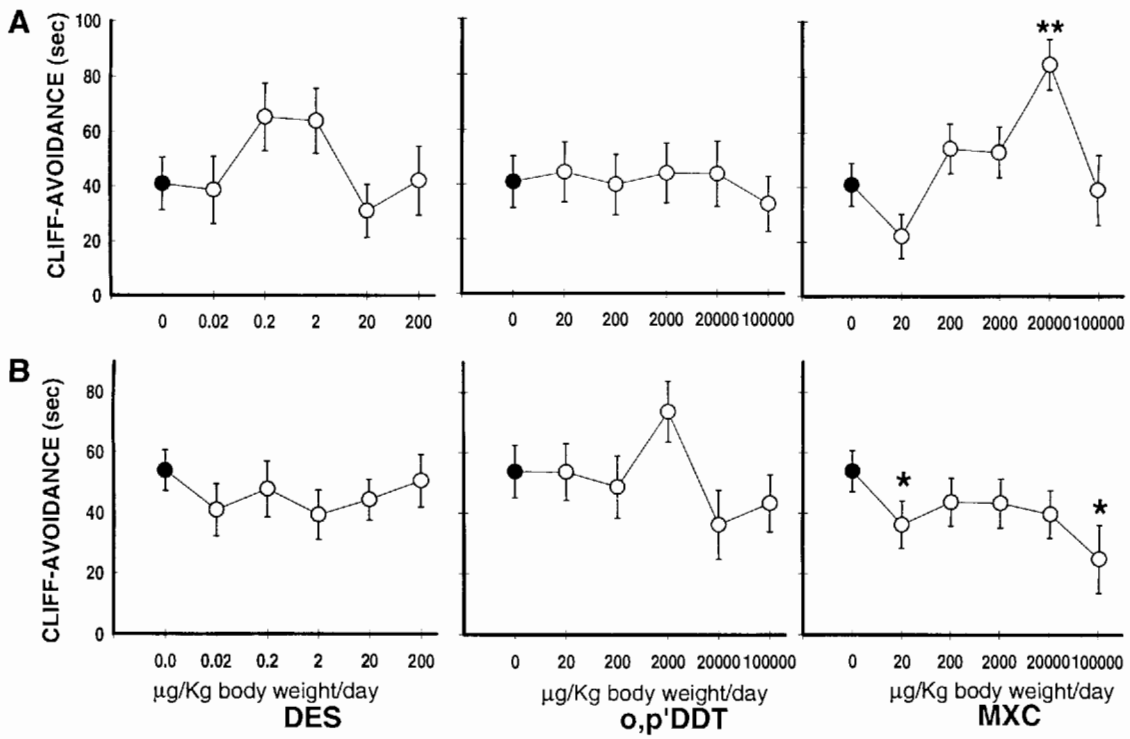

FIG. 5. Mean (±SEM) latency to perform the cliff avoidance reflex on postnatal day 2 (A) or day 5 (B) by pups whose mothers were fed DES, o,p'DDT, or MXC during days 11–17 of pregnancy. * $P < 0.05$, ** $P < 0.01$ vs control.

o,p'DDT, and MXC that were far below those typically examined in toxicological studies (Calabrese and Baldwin, 1997; vom Saal and Sheenan, 1998). For most variables measured, the dose–response curves were non-monotonic (they changed directions as a function of dose). In toxicological studies in which non-monotonic functions are observed, it is often stated that "there was no relationship to dose," since a basic assumption is that both stimulation and inhibition of responses will not be observed as a function of dose. As more research is done on "low-dose" effects of endocrine disrupting chemicals (where "low dose" is defined as being below the very high dose range typically examined in toxicological studies), we predict that many more examples will be reported of effects occurring within narrow dose ranges, while at other doses, significant effects might not be found or totally opposite effects will be found. We anticipate that until these effects that are only observed within a narrow dose range are understood at the cellular level, they will not be generally accepted as "real."

In these experiments DES was used as a positive control for estrogenic activity of both o,p'DDT and MXC. The doses of o,p'DDT and MXC that we examined were based on the relative estrogenic activity of these chemicals (based on the normal dose range for effects of estradiol as well as for effects of DES (vom Saal *et al.*, 1997; Welshons *et al.*, 1999), not on the doses that resulted in acute toxicity. We have referred to this as a physiologically based approach to conducting toxicological research (vom Saal *et al.*, 1998).

Very low doses of DES have now been shown in a number of assay systems to result in opposite effects at very low (physiologically relevant) doses and much higher, acutely toxic doses (vom Saal *et al.*, 1995, 1997; Welshons *et al.*, 1999; Gupta, 2000). For example, we previously reported that while an increase in territorial marking occurred as the prenatal dose of DES increased from 0.02 to 20 µg/kg/day, the 200 µg/kg/day dose resulted in a decrease in territorial marking relative to the 20 µg/kg/day dose. A similar inverted-U dose–response curve with fetal exposure to DES in mice was then found for prostate size and prostatic androgen receptors in male offspring (vom Saal *et al.*, 1997; Gupta, 2000). With regard to the experiments described here, our expectation was thus that a very wide dose range of DES would not produce a monotonic dose–response curve for all outcomes.

We used DES as a positive control based on the expectation that a similar pattern of effects would be

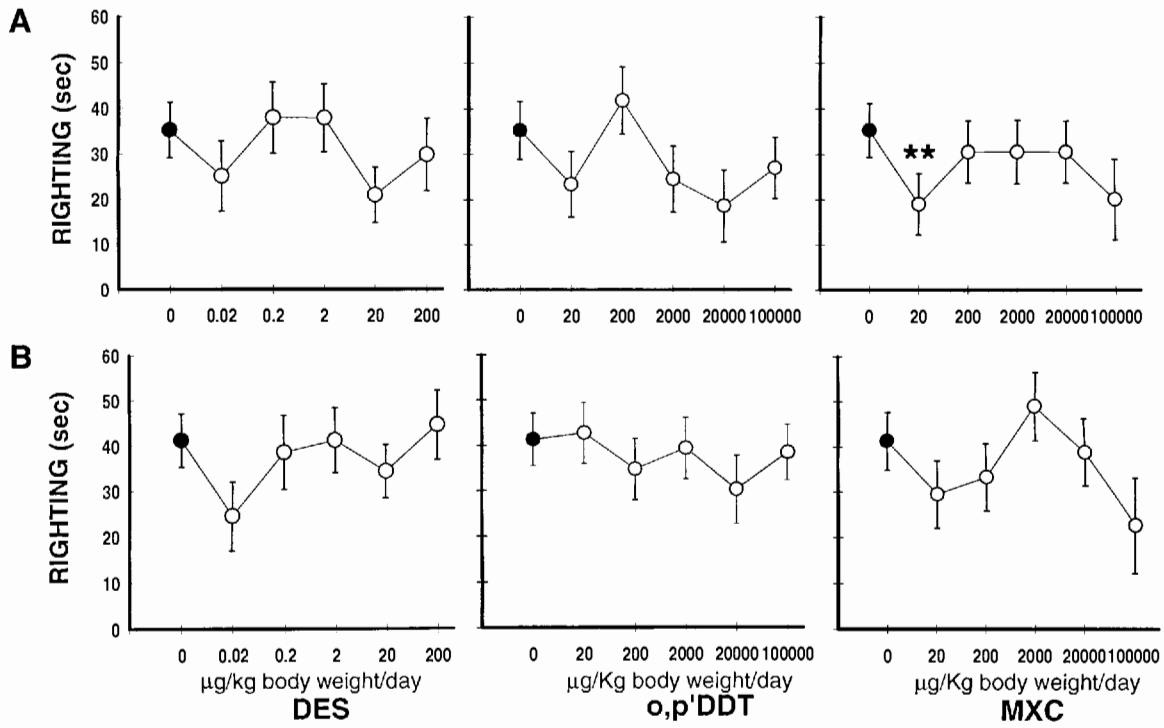

FIG. 6. Mean (±SEM) latency to perform the righting reflex on postnatal days 2 (A) or 5 (B) by pups whose mothers were fed DES, o,p'DDT, or MXC during days 11–17 of pregnancy. ** $P < 0.01$ vs control.

observed for o,p'DDT and MXC across a dose range 1000-fold higher than the dose range of DES. In fact, with the exception of the data for number of pups per litter and sex ratio (Fig. 1), this did not occur, and a 1000-fold lower dose of DES did not predict the effects of o,p'DDT and MXC.

Body Weight

We observed a complex relationship between the prenatal dose of DES and the body weight of pups, with an increase in body weight on both postnatals day 2 and 5 occurring in response to the DES 0.2 dose and a decrease in body weight occurring in response to the DES 200 dose. On both postnatal days 2 and 5 the dose–response curve for the effect of DES on body weight formed an inverted U. In contrast, the o,p'DDT 20,000 dose decreased body weight, while the o,p'DDT 100,000 dose increased body weight relative to controls on postnatal days 2 and 5. Unlike DES and o,p'DDT, MXC did not have a significant effect on the body weight of pups on any postnatal day examined.

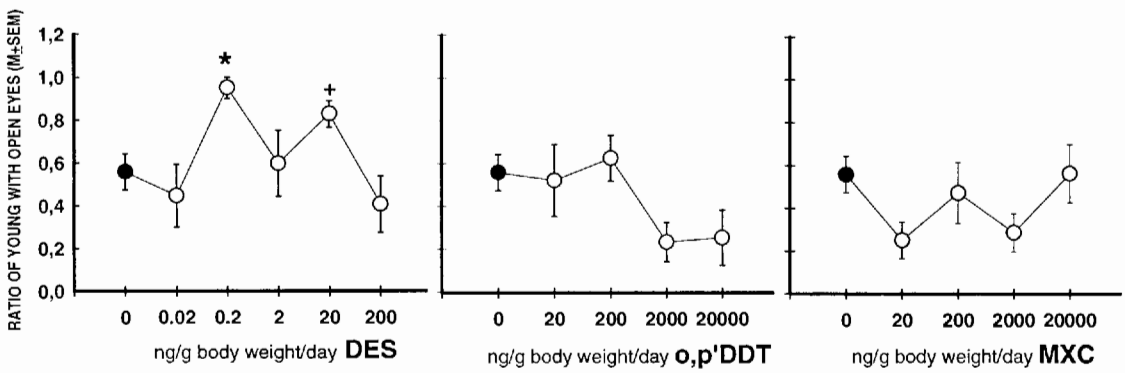

FIG. 7. Mean (±SEM) proportion of young in each litter that had both eyes open on postnatal day 14. Litters were produced by female mice fed DES, o,p'DDT, or MXC during gestation days 11–17. * $P < 0.05$ vs control; $^{+}$ $P < 0.07$ vs control.

Clearly, with regard to effects on body weight, there was a different dose–response relationship for these three chemicals.

Anogenital Distance

The elongation of the perineal tissue, which becomes the scrotum in males, is the AGD measure. This is a well described bioassay for prenatal exposure to androgen. There was a complex relationship between the prenatal dose of estrogenic chemicals and AGD in male mice, with some doses decreasing AGD and other doses increasing AGD. Interestingly, the highest doses of o,p'DDT and MXC that were tested (100,000 μg/kg body weight/day) increased AGD, while the highest, acutely toxic dose of DES (200 μg/kg/day) decreased AGD. While the inhibitory effect on masculinization of the high-dose effect of DES is consistent with the retention of nipples, the stimulatory effect of the highest doses of o,p'DDT and MXC (which has antiandrogenic activity) was unexpected. A similar trend was found in female offspring, where exposure to highest doses of the two pesticides (but not to DES) increased AGD, while low-intermediate doses decreased female AGD. The highest doses of o,p'DDT and MXC thus increased AGD in females as in males, while lower doses tended to decrease AGD.

The embryonic urogenital sinus tissue (from which the urethra and prostate differentiate) and perineal tissue share some of the regulatory mechanisms governing differentiation (vom Saal *et al.*, 1992), and low doses of estradiol and DES lead to an increase in androgen receptors, while high doses of DES lead to a decrease in androgen receptors in the urogenital sinus (vom Saal *et al.*, 1997; Gupta, 2000). There has thus been a mechanism identified by which estrogenic chemicals can result in an increase or a decrease in responsiveness of androgen-responsive tissues to endogenous androgen in developing tissues. Gupta (2000) also reported that low doses of DES and some other estrogenic chemicals resulted in a significant increase in AGD. It is thus possible that this could also account for the increase in the length of AGD in animals exposed to the o,p'DDT and MXC 100,000 dose. However, this stimulatory effect of estrogen on androgen receptors only occurred within a specific low-dose range for estradiol, DES, and ethinyl estradiol, and very high doses of DES can produce the opposite result (vom Saal *et al.*, 1997; Welshons *et al.*, 1999; Gupta, 2000; Thayer *et al.*, 2001). The inhibitory effect of very high doses of potent estrogens, such as DES, on androgen responsiveness could explain the decrease in anogenital distance at the highest DES dose that we examined.

The absence of a similar dose–response relationship between DES and AGD in either males or females in comparison to effects of exposure to the two pesticides o,p'DDT and MXC raises a question concerning whether the effects of the pesticides on AGD may not be due to their estrogenic activity as opposed to some other mechanism, such as effects on prostaglandins (Gupta and Goldman, 1986). Our finding that an increase in AGD occurred in males and females exposed to the MXC 100,000 dose was particularly unexpected, given that this chemical has both antiandrogenic and estrogenic activity (Gray *et al.*, 1999).

As described above, in a prior study prenatal DES exposure resulted in an inverted-U dose–response relationship between prenatal dose of DES and adult urine marking behavior in male mice. In contrast, prenatal exposure to the same doses of o,p'DDT and MXC described here produced a dose-dependent increase in urine-marking behavior, and no decrease in marking was observed at the o,p'DDT 100,000 and MXC 100,000 doses relative to lower doses (vom Saal *et al.*, 1995). Taken together with the data presented in the present study, our findings suggest that the o,p'DDT 100,000 and MXC 100,000 doses were not acutely toxic and were thus not equivalent to the DES 200 dose, which resulted in the death of 50% of the litters. The complex dose-related effects reported here suggest that considerable work remains to understand the stimulatory versus inhibitory effects of different doses of estrogenic chemicals which can modulate androgen action in target tissues, including the brain and thus behaviors.

Litter Size

In the present study an unexpected finding was that exposure during pregnancy to some of the low doses of DES, o,p'DDT, and marginally MXC resulted in an increase in litter size relative to controls, based on the number of live pups found within 12 h after delivery. The increase in the size of litters produced by females treated with nontoxic doses of estrogenic chemicals was generally associated with a decrease in sex ratio (a decrease in the proportion of males per litter). Since we randomly assigned pregnant females to treatment groups and began administering chemicals 11 days after conception, which precludes a direct effect on the fertilization of eggs by XX- or XY-bearing sperm, the basis for this finding is unknown.

In both house mice (Vandenbergh and Huggett,

1995) and Mongolian gerbils (Clark *et al.*, 1993), female mice exposed prenatally to elevated levels of estradiol produce litters containing a lower proportion of males than do females exposed prenatally to elevated levels of testosterone, based on being positioned *in utero* between female or male fetuses, respectively (vom Saal, 1989). However, in this naturally occurring "experiment," only sex ratio, but not litter size, was found to vary in females due to their prior intrauterine position relative to male and female fetuses. Whatever pre- or postnatal mechanisms are involved (such as differential fetal mortality or sex-biased infanticide by the mother), variation in sex ratio can significantly impact the behavioral ecology of a population. Mating patterns adopted by different species or even different populations of the same species are related to sex ratio. For example, female-biased sex ratios are considered to increase female–female competition and to decrease mating opportunities for females (e.g., Krebs and Davies, 1981). House mice populations can range from monogamous to polygamous to promiscuous mating systems, according socioecological conditions (Berry, 1989; Palanza *et al.*, 1996).

Righting and Cliff Avoidance Reflexes

We analyzed the effects of exposure to these chemicals on two reflexive responses, the righting reflex and cliff drop avoidance, which can provide information concerning physical and motor development as well as sensory function and/or processing. We found that only MXC-exposed animals showed changes in these reflexes relative to controls. In general, these reflexes did not appear to be sensitive indicators of prenatal exposure to DES, o,p'DDT, and MXC.

We recently reported (Palanza *et al.*, 1999) that prenatal exposure to the lowest MXC dose used in the present study (20 μg/kg/day) increased the reactivity of both male and female adolescent mice (25 days old) to novel environmental stimuli. In the present study, the results suggested that pups exposed to this same MXC 20 dose performed both righting and cliff avoidance reflexes somewhat more quickly than control pups. Since these results were not entirely consistent on postnatal days 2 and 5, it is possible that maternal treatment with this dose of MXC produces a nonspecific increase in motor activity in response to environmental stimuli in offspring. On the other hand, a more reactive profile could be related to differences in emotionality, since decreased reaction time responding to novel stimuli can be suggestive of a low anxiety profile. Cliff avoidance was retarded on day 2 by exposure to a high MXC dose (MXC 20,000). This response can be also used as a measure of anxiety as less fearful animals stay at the visual cliff longer, based on pharmacological studies of anxiety reducing drugs (e.g., Bignami, 1996). It is possible that this higher dose (MXC 20,000) had an opposite effect on emotionality than the lower MXC 20. Further experiments on more sensitive behavioral tests are needed to examine the levels of general activity and response to environmental stimuli of prenatally exposed animals.

Separation of Maternal Effects from Fetal Effects

In the present study we examined the developmental effect of estrogenic chemicals without a separation of the direct effects of treatment on the pups from those that can act through maternal behavior. Maternal behavior of the mouse is affected by the endocrine system, and maternal responses appear to be enhanced by elevated levels of estrogen and declining levels of progesterone (Bridges, 1990). It is well known that both variation in maternal care and the response of pups to maternal cues can be responsible for variation in the rate of maturation, such as differences in growth rate or neurobehavioral responses. The effects on offspring of administration of chemicals to pregnant females can thus be amplified due to an interaction of effects on the mother as well as effects on the offspring (Smotherman and Bell, 1980; Fleming *et al.*, 1999). A cross-fostering procedure is required to separate these effects. However, fostering per se may also exert an effect on maturation (e.g., Laviola *et al.*, 1990); the complex maternal–pup relationship is not fully understood, and the range of effects that fostering might have on development and behavior of offspring remains to be determined.

We have recently examined whether exposure to MXC during pregnancy would alter maternal behavior in lactating mice. We found that dams administered the lowest dose of MXC examined in the experiments described here (20 μg/kg/day) showed lower levels of nursing behavior compared to controls (Morellini *et al.*, 2001). A further step in our studies will be to clarify which part of the developmental effects we have described here can be attributed to the direct action on the fetus as opposed to effects mediated by an alteration of normal maternal behavior.

In summary, the conclusion from this and other studies that we have conducted 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 spe-

cific low, environmentally relevant doses of endocrine disruptors that will not be observed at higher doses.

ACKNOWLEDGMENTS

Support for this work was provided by NIH Grant ES08293 to F.v.S., by MURST (Italian Ministry of Scientific Research and University), and a CNR-NATO/Advanced Research grant to P.P.

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