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Fetal Effects on Sexual Behavior and Aggression in Young and Old Female Mice Treated with Estrogen and Testosterone

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During fetal life female mice (*Mus musculus*) that develop between two male fetuses (2M females) have higher blood concentrations of testosterone than do females that do not develop next to a male fetus (0M females). In the first experiment reported here, sexual receptivity and sexual attractiveness to males were examined in young (5 month old) and old (17 month old) ovariectomized, estrogen- and progesterone-treated 0M and 2M female mice that were placed in like-age pairs with a male. Most males inseminated the 0M female prior to inseminating the 2M female regardless of age. In addition, 0M females were more likely to exhibit lordosis when mounted than were 2M females. When the same young females were 9 months of age and the old females were 21 months of age, they were treated with testosterone and again placed together in pairs along with a sexually receptive female. Young 2M females exhibited more aggression toward the testosterone-treated female partner, and also exhibited more mounting of the receptive female, than did young 0M females. But, both old 0M and old 2M females were highly aggressive and exhibited mounting. An increase in sensitivity to the effects of testosterone on behavior thus occurs during aging in 0M females, which are relatively insensitive to testosterone in young adulthood. In contrast, when treated with estrogen and progesterone, 0M females were more attractive to males and were more sexually receptive than 2M females regardless of age.

Studies of aging in female rodents to date have concentrated on changes in reproductive capacity and estrous cycle characteristics concomitant with changes in circulating hormone concentrations (c.f., Peng, Chuong, and Peng, 1977; Lu, Hopper, Vargo, and Yen, 1979; Gray and Wexler, 1980; Butcher and Page, 1981; Nelson, Felicio, Osterburg, and Finch, 1981). To examine the role of steroids in the aging process, the concentrations of circulating steroids obviously need to be examined at various stages in adulthood. However, equally important, but often overlooked, is the fact that the sensitivity of target tissues to steroids is modulated by the concentrations of steroids that animals are exposed to during early sensitive periods in development. In mice and rats the period in early

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life during which steroids have the greatest effect on the subsequent sensitivity of tissues to steroids is during the late prenatal and early neonatal peiods (vom Saal, 1983a). Although Mallampati and Johnson (1974) and Finch, Felicio, Flurkey, Gee, Mobbs, Nelson, and Osterburg (1980) have suggested that exposure to androgen around the time of birth may accelerate the rate of decline of reproductive function during aging in female rodents, little effort has been directed toward studying the effect of perinatal androgen exposure on feminine and masculine behavior in females as a function of aging.

Both female sexual behavior and the capacity to ovulate are influenced by the exogenous administration of androgens shortly after birth in rats and mice. For example, normal females in estrus will exhibit lordosis when mounted by a male, but females that are administered androgen shortly after birth have a reduced ability to exhibit lordosis as adults (Gorski, 1979), even if they are first primed with estradiol benzoate and progesterone (Edwards and Burge, 1971; Goy and McEwen, 1980). Exposure to high doses of androgen shortly after birth completely inhibits ovulation in female mice and rats (the androgen sterility syndrome), while exposure to low doses of androgen results in a reduced period of fertility (the delayed anovulatory syndrome; Gorski, 1979).

There are significant differences in morphology, physiology, and behavior among male and among female mice based on prior intrauterine position (vom Saal and Bronson, 1978, 1980a; vom Saal, Grant, McMullen, and Laves, 1983). Intrauterine positioning by sex is a random event. Therefore, within a given litter, 50% of the females are positioned next to one male fetus (1M females), while the proportion of females between two male fetuses (2M females) and those not next to a male fetus (0M females) varies as a function of litter size (vom Saal, 1981). During the last 4 days of gestation male mouse fetuses secrete high concentrations of testosterone (unpublished observation), and 2M female fetuses have higher amniotic fluid and blood concentrations of testosterone than do 0M females (vom Saal and Bronson, 1980a). These findings have led to the use of the intrauterine position phenomenon as a model system for studying the relationship between fetal hormones and adult phenotype. In the present studies, both young-adult and old 0M and 2M female mice were compared in terms of their neural sensitivity to gonaal steroids utilizing behavioral measures as indices of hormone sensitivity.

GENERAL METHODS

CF-1 albino mice, maintained as an outbred strain in a closed colony, were housed in rooms maintained at 23 ± 1°C on a 14:10 light:dark cycle, with lights on at 0600 hr. Nulliparous females were time mated. CF-1 mice reliably give birth 19 days after insemination (Day 0). Beginning at 08:00 hr on Day 19, pregnant females were killed by cervical dislocation,

and the fetuses were removed sequentially from the uterine horns. Fetal sex was determined by the length of the anogenital space, and the positioning of females with respect to males was recorded. All young were fostered to mothers that had delivered naturally within the preceding 24 hr. Foster litters consisted of 10 young, with 5 1M females being placed with either 5 0M or 5 2M females. A toe-clipping pattern was utilized to identify individual young. Weaning occurred on Day 23 after birth, at which time the foster-litter groups were divided, and females were housed five per cage. Statistical comparisons were made using analysis of variance or χ^2 analysis.

RESULTS

Results of the individual experiments will be presented as subsections preceded by the pertinent procedural details.

Feminine Sexual Behavior as a Function of Age

This experiment was designed to examine how aging influenced both attractiveness and sexual receptivity in 0M and 2M female mice. Two age groups (young and old) of 0M and 2M female mice were utilized. Each age group consisted of 20 0M females and 20 2M females, 1 year apart in age. Comparisons of the sensitivity of intact 0M and 2M female mice to gonadal hormones are difficult to make because of the difference in the length of estrous cycles (and quite likely also the timing of the onset of estrus) between 0M and 2M females (vom Saal and Bronson, 1980b; vom Saal, Pryor, and Bronson, 1981; vom Saal, 1981). Thus, in this study the females were gonadectomized and then administered a known amount of gonadal steroids in order to have adult gonadal hormonal concentrations held constant. The females were ovariectomized when the young females were 3 months old and the old females were 15 months old. Following surgery, the females were rehoused five per cage in the same postweaning, foster-litter groups for 2 months.

The ages at initial testing were 5 months and 17 months for the young and the old females, respectively. Two days prior to testing, each female was weighed, given a subcutaneous injection of 5 μg estradiol benzoate (EB) in 0.02 cc sesame oil, and housed individually (mean $\pm$ SEM body wt: young 0M females = 33.6 $\pm$ 0.6 g; young 2M females = 33.8 $\pm$ 0.6 g; old 0M females = 38.1 $\pm$ 0.7 g; old 2M females = 39.4 $\pm$ 0.8 g). Pairs of 0M and 2M females within each age group were created based on body wt. Weight differences for each pair were less than 1 g. Tails were marked with ink for individual identification. On the evening preceding observations, sexually experienced 5-month-old 1M males (that developed between a male and a female fetus) were placed individually in 31 $\times$ 31 $\times$ 15-cm wooden boxes with food, water, and bedding, where they remained overnight. 1M males were utilized since intrauterine position influences sexual activity in male mice and rats (vom Saal *et al.*, 1983).

At 06:00 hr on the day of behavior testing, each pair of 0M and 2M females was injected subcutaneously with 200 μg progesterone (P) in 0.02 cc sesame oil. Four hours later each pair of females was placed together with a 1M male in a wooden box. After being placed together, the animals were observed for 30 min, and behavior was recorded on an event recorder. Since most CF-1 male mice do not ejaculate within 30 min (vom Saal and Bronson, 1978; vom Saal *et al.*, 1983), the females were checked every half hour for 4 hr after the observation period ended for the presence of a vaginal plug in order to determine which of the females had been inseminated. Unlike male rats, most CF-1 male mice do not exhibit rapid series of ejaculations (Huber, Bronson, and Desjardins, 1980), but some of the males did ejaculate into both females. Data recorded for each female included the number and duration of mounts and intromissions by the male, latency to the first mount, latency to the first intromission, lordosis quotient (number of lordoses/number of mounts × 100), intensity of the lordosis response (low: standing still long enough to allow intromissions, but with little rump or head elevation; high: standing rigid with head perpendicular to the trunk and rump elevated), and whether the female was inseminated first or second (for those cases in which two ejaculations occurred). Proceptive behaviors (such as darting or ear wiggling) are not clearly identifiable in CF-1 female mice, and no assessment of proceptive behaviors was attempted.

Young females. For the young females, no significant difference in mounting or intromission rate by the males occurred between 0M and 2M females. Total time and frequency of mounting was divided equally between 0M and 2M females, as were the initial mounts and intromissions. The ejaculation data, however, revealed a significant difference: out of a total of 16 boxes in which the male ejaculated, 11 0M and 5 2M females received the first ejaculations, while 4 0M females and 9 2M females received the second ejaculation ($\chi^2(1) = 4.1$, $P < 0.05$; Fig. 1).

Old females. For the old females, males also did not exhibit a preference toward either the 0M or 2M females in terms of mounts, intromissions, or in choice of first mount or intromission. But, out of a total of 11 boxes in which ejaculations occurred, 9 of the first ejaculations were into the 0M female. Five males that had first inseminated a 0M female then inseminated the 2M female within the box, while no 0M female received the second ejaculation by a male ($\chi^2(1) = 9.3$, $P < 0.01$; Fig. 1). There was thus a slight, but not statistically significant, decrease in the proportion of males that inseminated a female as a function of the age of the females: 16 males inseminated at least one of the young females while only 11 males inseminated at least one of the old females. Also, more males placed with the pairs of young females ($N = 13$) than old females ($N = 5$) ejaculated into both the 0M and 2M female.

Old vs young females. The lordosis quotient data are also presented in Fig. 1. A two-factor ANOVA revealed a significant main effect of

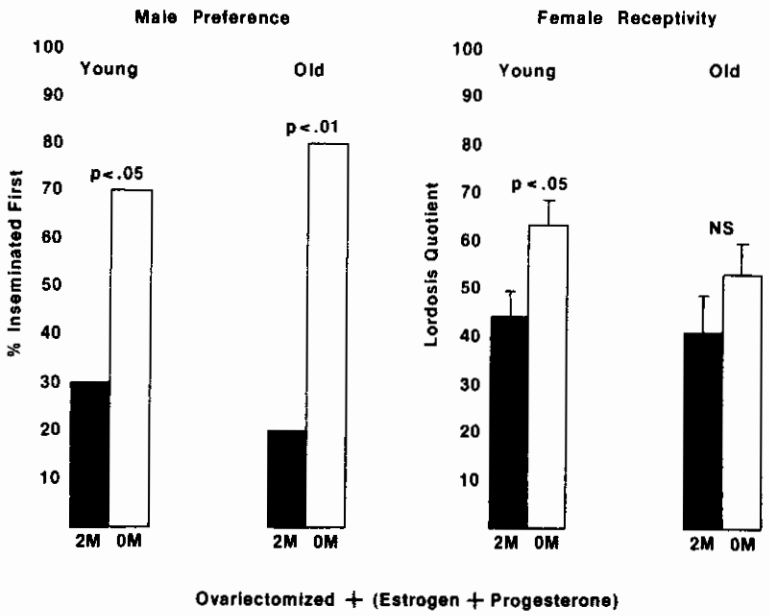

FIG. 1. *Male preference.* The percentage of ovariectomized, estradiol benzoate- and progesterone-treated young (5 month old) and old (17 month old) 0M and 2M female mice which were the first female within a pair to be inseminated by a male. A 0M and 2M female of the same age (N = 20 pairs/age group) were placed together with a male and examined for the presence of a vaginal plug every 30 min for 4 hr. The order of insemination of the 0M or 2M females was recorded. Comparisons were made by chi-square analysis on the data for the sequence of first and second ejaculations. *Female receptivity.* The mean (±SEM) lordosis quotient (number of lordoses/number of mounts × 100) for all of the young and old 0M and 2M females (regardless of whether they were eventually inseminated) during the first 30 min that they were paired with males (NS = not significant; t test).

intrauterine position on lordosis: the 0M females had significantly higher lordosis quotients than did the 2M females ($F(1, 49) = 7.5$, $P < 0.01$). Post hoc comparisons utilizing the least significant difference test (LSD; $P < 0.05$) revealed that young 0M females exhibited lordosis in response to a mount significantly more often than did young 2M females, but old 0M and 2M females did not differ significantly. A significantly greater percentage of the lordoses exhibited by the young 2M females were rated as reflecting a low level of receptivity relative to the young 0M females (mean ± SEM; 2M females = 78.3 ± 6.5% vs 0M females = 57.2 ± 7.5% of lordoses were rated as indicating low receptivity; $F(1, 28) = 4.3$, $P < 0.05$). No significant difference between the old 0M females and old 2M females on this measure was observed (mean ± SEM; 2M females = 80.9 ± 6.7% vs 0M females = 59.2 ± 11.2% of low lordosis responses; t test, $P > 0.1$). Finally, sexual receptivity (either lordosis quotient or lordosis intensity) was not found to change significantly as

a function of age, although the old females had somewhat lower lordosis quotients than did the young females.

Masculine Sexual Behavior as a Function of Age

In this experiment the sensitivity of the same young and old 0M and 2M females to testosterone was compared using behavioral measures. Each animal was administered testosterone and examined for its ability to exhibit male copulatory behavior (mounting) and for the frequency of aggressive attacks.

After completion of the prior experiment, the young 0M and 2M females and old 0M and 2M females were individually housed. When the young females were 9 months old and the old females were 21 months old, the originally matched pairs were again placed together in cages (31 × 31 × 15 cm), but the females were initially separated by a wooden barrier. The animals were provided with food and water *ad libitum*. The next day the wooden dividers were removed, and at the same time, a sexually receptive (EB- and P-treated) 9-month-old stimulus 1M female mouse (that developed between a male and a female fetus) was placed into each box. Observations continued for 10 min for each box. Attacks (biting and chasing) and mounts were recorded on an event recorder. Only the 2M females exhibited aggression and mounting during the baseline test (between 10 and 20% of the young and old 2M females exhibited aggression and/or mounting), but the differences between 0M and 2M females were not statistically significant.

Two days after the above baseline (pretestosterone treatment) observations, each 0M and 2M female was implanted subcutaneously with a 10-mm long silastic capsule (0.068 in i.d., 0.125 in o.d., Dow Corning). The capsules contained 5 mg testosterone in 0.02 cc sesame oil (Barkley and Goldman, 1977a). All operations were done under ether anesthesia. The mice were allowed 7 days for recovery, after which they were again paired with their original partner and tested as described for the baseline study once a week for 4 weeks. Tests were begun at 1000 hr.

The option presented to the 0M and 2M females was to interact with either the sexually receptive stimulus 1M female or the opponent, which was implanted with testosterone. Without regard to the possible effects of prior intrauterine position, one might expect that testosterone treatment would elicit aggression and not sexual behavior from an opponent that was also treated with testosterone (Mugford and Nowell, 1971). Estradiol and progesterone treatment does not cause females to behave aggressively or to exhibit mounting behavior, but induces female sexual behavior in mice (Simon, 1979). In contrast, animals implanted with silastic capsules containing testosterone were expected to exhibit aggression and mounting. Thus, when 0M and 2M females were placed together after being implanted with testosterone, fighting and not sexual behavior was expected to occur

between the 0M and 2M females, and the testosterone-implanted females were expected to mount the EB- and P-treated 1M females. However, in addition to exhibiting mounting behavior, testosterone-implanted CF-1 female mice were previously observed to also exhibit aggression toward EB- and P-treated females (vom Saal and Bronson, 1978; vom Saal, 1983b).

Mounting. In general, during the 4 weeks of testing, among young animals there was very little mounting observed toward the testosterone-treated opponent. None of the young 0M females exhibited mounts toward the testosterone-implanted 2M females, whereas 28% of the 2M females mounted the testosterone-treated 0M females ($\chi^2(1) = 5.8$, $P < 0.05$). Among old 0M and 2M females there was a considerable amount of mounting toward each other, although there was no difference in the amount shown by either group (0M females = 54%; 2M females = 57%). There was thus an increase in mounting toward the testosterone-treated opponent as a function of age for both the 0M and the 2M females.

Significantly more of the young 2M than young 0M females exhibited mounting behavior toward the stimulus 1M female ($\chi^2(1) = 11.1$ $P < 0.001$). In contrast, in the pairs of old 0M and 2M females, there was no significant difference on this measure (Fig. 2). In terms of mounting

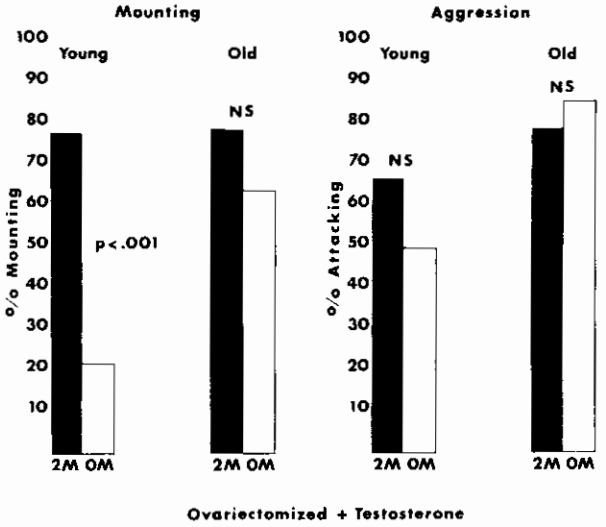

FIG. 2. *Testosterone-induced behavior*. Young (9 month old) and old (21 month old) 0M and 2M females were implanted subcutaneously with a silastic capsule containing testosterone and placed together in like-age pairs with a sexually receptive young 1M female. The proportion of 0M and 2M females which exhibited mounting of the 1M female and the proportion of 0M and 2M females that attacked the opponent testosterone-treated female were recorded. Comparisons were made by chi-square analysis.

behavior by young females toward any other animal in the box, more 2M females than 0M females mounted the 1M female and/or opponent female (0M females = 22%; 2M females = 78%; $\chi^2(1) = 11.1$, $P < 0.001$). Among old animals, there was again no significant difference in the proportion of 0M and 2M females that exhibited mounting behavior toward any other animal in the box (0M females = 86%; 2M females = 79%). In conclusion, there was a significant increase in mounting during aging in 0M females, while no significant difference between young 2M females and old 2M females was observed.

Aggression. The data presented in Fig. 2 reveal that significantly more old 0M females exhibited aggression (biting attacks) toward the old 2M opponent than did the young 0M females toward the young 2M opponent [$\chi^2(1) = 4.5$, $P < 0.05$]. Aggression by the 2M females toward the opponent 0M females did not differ significantly as a function of age. About 50% of the females, regardless of intrauterine position or age, also exhibited some aggression toward the sexually receptive 1M female. There were no significant differences between any of the groups in any quantitative measure of aggression (latency to attack, number, or duration of attacks). In summary, there was a significant increase in the incidence of aggression with age in 0M females but not in 2M females. Thus, in terms of both mounting and aggression, 0M females became more like 2M females in their behavioral response to testosterone as they aged.

DISCUSSION

The major finding of this experiment is that during aging, female mice that did not develop *in utero* next to a male fetus (0M females) became more sensitive to the activating effects of testosterone on aggression and mounting behavior. In contrast, the female mice that developed *in utero* between two male fetuses (2M females) were already sensitive to testosterone in young adulthood, and these females did not change in their sensitivity to the activating effects of testosterone on aggression and mounting during aging (i.e., most young 2M females and old 2M females exhibited mounting and aggression when administered testosterone).

It is possible that the increase in sensitivity to testosterone during aging in 0M females reflects the fact that they are exposed to androgens throughout their adult life, and these androgens are having a cumulative effect on the neural areas mediating male sexual behavior and aggression (c.f., Finch *et al.*, 1980). The fact that the old 0M and 2M female mice were left gonadally intact 1 year longer than the young females in our experiment suggests that continuous exposure of the brain to endogenous androgens (or possibly other steroids) secreted by the ovaries and/or adrenals may account for the change in sensitivity to testosterone in 0M females during aging. For example, Lu *et al.* (1979) reported that blood testosterone and androstenedione concentrations double during the

proestrus phase of the estrous cycle in female rats, and Chan and Leathem (1977) observed a marked increase in the conversion of pregnenolone to both testosterone and androstenedione between 4 and 18 months of age in the ovaries of female rats. In mice, young adult, diestrus females have serum testosterone concentrations in the range of 250–300 pg/ml (vom Saal and Bronson, 1980a).

The implication of the above hypothesis is that the brain may remain capable of responding to the sensitizing effects of steroids throughout life. The results of two previous experiments concerning testosterone-induced aggression in mice also support this hypothesis (Edwards, 1970; Barkley and Goldman, 1977b; for a review see vom Saal, 1983b). In addition, individual differences in the capacity to have such changes in neural sensitivity to testosterone occur throughout adult life are related to the concentrations of hormones that female mice are exposed to during prenatal life based on intrauterine position. Rodents have been hypothesized to pass through a period of high sensitivity to steroids around the time of birth, with the capacity to ovulate and to exhibit both female and male sexual behaviors being influenced by the hormonal milieu during this early sensitive period. By adulthood, steroids were presumed to only have transient "activational" effects on behavior (c.f., vom Saal, 1983b).

In the present study the increase in sensitivity to the activational effects of testosterone on male sexual behavior and aggression in 0M females during aging is interesting in that the hypothalamic–pituitary–gonadal axis has been reported to become less sensitive to circulating steroids as a function of age in female (but not male) rats (Gray and Wexler, 1980), and neonatal treatment with androgen accelerates this process (Mallampati and Johnson, 1974). Indeed, during aging in male CF-1 mice there is a decrease in sensitivity to the activational effects of testosterone on intermale aggression (vom Saal *et al.*, 1983). These findings thus provide further evidence in support of the hypothesis that steroids have different effects on neural areas regulating pituitary function and sex-related behaviors such as lordosis, mounting, or aggression, and that in addition, there are both sex differences and intrauterine position differences in the effects of steroids on neuroendocrine function and behavior.

Urinary pheromones which influence the rate of sexual maturation of other female mice are influenced by secretions from the adrenals rather than from the ovaries (Drickamer and McIntosh, 1980), and 0M and 2M female mice differ both in their production of and sensitivity to pheromones which influence the timing of puberty (vom Saal, 1981; vom Saal *et al.*, 1981; vom Saal, Grant, and Howard, unpublished observation). In addition, in a previous experiment, intact, diestrus 0M and 2M female mice were placed in wire chambers in a two-choice apparatus, and over 80% of males that were tested chose the 0M females (vom Saal and Bronson, 1978, 1980a). Taken together with the present results, these findings

provide evidence for a difference in the emission of cues in both young and old female mice due to intrauterine position, and such a difference is likely due to an effect of intrauterine position on the potency of pheromones produced by female mice. But, since the 2M females tested in the present study were less sexually receptive than the 0M females, it cannot be ruled out that the difference between 0M and 2M females in terms of the probability of being inseminated was due to a difference in sexual receptivity as well as a difference in the production of olfactory cues.

The finding that during fetal life 2M females are more defeminized than 0M females in terms of adult sexual receptivity was not unexpected. Gladue and Clemens (1978) had reported that the female offspring of pregnant rats treated with the antiandrogen, flutamide, had higher lordosis quotients when treated with estradiol benzoate than did control females. Since 2M and 1M females are exposed to higher concentrations of testosterone during fetal life than are 0M females, Gladue and Clemens had hypothesized that flutamide had eliminated the effect of developing next to male fetuses, and all flutamide-treated females resembled 0M females in their sexual receptivity. In a previous experiment young 0M and 2M females had been ovariectomized, implanted with a silastic capsule containing estradiol, and tested for sexual receptivity 7 days later. No difference between the 0M and 2M females in terms of lordosis quotient was observed (vom Saal and Bronson, 1978). In the present study females were ovariectomized 2 months prior to hormone administration, and a difference in lordosis quotient based on prior intrauterine position was observed.

Young, 3-month-old 0M and 2M female mice have also been administered testosterone contained in a silastic capsule and examined for their behavior when placed individually with a sexually receptive 1M female once per week for 4 weeks. More 2M females (91%) than 0M females (64%) exhibited mounting during the 4 weeks of testing, although the difference was not statistically significant (χ^2; $P = 0.08$). But, significantly more of the 2M females (74%) than 0M females (44%) exhibited biting attacks toward the sexually receptive 1M female (vom Saal and Bronson, 1978). Clemens, Gladue, and Coniglio (1978) also reported that in Holtzman rats, young 2M female exhibited more mounting than did young 0M females when they were administered testosterone and paired with a sexually receptive female. 1M females are intermediate between 0M and 2M females in every characteristic that has been examined (vom Saal, 1981, 1983b).

Studies have also been conducted comparing 0M and 2M female mice for their capacity to repeatedly produce and raise young throughout life. 2M females ceased producing live young at an earlier age and after fewer litters than did 0M females (vom Saal, Moyer, and Rines, 1982). This finding indicates that the prenatal hormonal environment of a fetus in-

fluences not only reproductive behaviors during aging but reproductive capacity as well.

Unlike rats, male mice do not normally ejaculate many times in succession. The fact that 0M females were more likely to be inseminated than 2M females in a situation in which a male had a choice of females with which to mate suggests that in a natural environment, where a male would be around 0M, 1M, and 2M females, 0M females would be the most likely to become pregnant if more than one type of female were in estrus at the same time. The intrauterine position phenomenon occurs in wild house mice (2M females have longer anogenital spaces than do 0M females; M. McCarthy and F. vom Saal, unpublished observation), and intrauterine position has been proposed to influence reproductive success in female mice in natural environments (vom Saal, 1981).

In summary, there was a significant change in the behavioral response to testosterone in 0M but not 2M female mice as a function of age. But no significant age-related change in the behavioral response to estradiol and progesterone was observed in 0M or 2M females in this experiment (0M females were more responsive than 2M females regardless of age). This latter finding concerning aging and sensitivity to estradiol in female rodents agrees with earlier reports by Peng *et al.* (1977) in rats and by Hollinka and Finch (1981) in mice.

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