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In Utero Proximity of Female Mouse Fetuses to Males: Effect on Reproductive Performance during Later Life

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ABSTRACT

Female mice were delivered by Caesearian section, with females between 2 male fetuses in the uterus (2M females) and females not contiguous to males in the uterus (0M females) being compared morphologically, physiologically and behaviorally during later life. The results reveal that 2M females are masculinized morphologically and behaviorally relative to 0M females; 2M females have a larger anogenital space, are more aggressive in a variety of different situations, are less effective at inhibiting male-induced puberty when group housed and urine mark their environment at higher rates (all masculine traits). On the other hand, 0M females are more sexually attractive and arousing to males than are 2M females. While no differences were noted between 0M and 2M females in terms of their sensitivity to gonadal steroids or in their capacity to produce and raise healthy young in an optimal laboratory environment, the data indicate that *in utero* contiguity to male fetuses produces a masculine bias in the transmission of and response to reproductively important social cues. Such a bias could alter the probability of mating and of successfully producing healthy offspring in a natural environment.

INTRODUCTION

During mammalian fetal development, hormones act in ways that affect the later expression of sexual differentiation. In the male, testicular androgens that appear during prenatal and, in some cases, neonatal development masculinize a broad spectrum of processes ranging from specific organ differentiation to the biasing of adult social behaviors (Neumann and Elger, 1966; DeMoor et al., 1973). There has been no evidence for similar feminizing actions by the ovarian steroids. The female phenotype is assumed to result from a female genotype and the absence of androgen exposure during development (Ohno, 1976). Two aspects of the developmental influence of gonadal steroids have dominated past studies with female mammals: 1) the experimental induction of masculinization via exogenous androgen administration, often used as a model for studying the development of the male phenotype and 2) the abnormality of masculinization in females due to developmental errors. Of the latter type is the classic study of the freemartin in cattle by Lillie (1916), which showed that fusion of the placental chorions of fraternal twins of opposite sexes leads to masculinization of the female twin (c.f. Jost, 1972).

Recently, however, two studies have suggested that some degree of masculinization of females may be a normal consequence of multiple uterine residence, at least in rodents. Clemens (1974), working with rats, and vom Saal (1976), working with mice, reported a marked relationship between the anogenital distance of a female at birth and her position in the uterus relative to male fetuses. Specifically, the tissue between the anus and genital papilla, which elongates in response to androgen exposure during gestation, was increased (masculinized) in females residing between two male fetuses relative to that of female fetuses not contiguous to males. Females residing between one male and female fetus in the uterus were intermediate in this regard. Both authors also reported an enhanced tendency for masculine behavior of females residing between male fetuses if such animals were administered androgen and tested as adults; sexual behavior was examined by Clemens (1974) and aggres-

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sive behavior was examined by vom Saal (1976).

The objective of the present study was to explore more broadly the possibility that female mice residing in the uterus between males might be somewhat masculinized as a consequence. Therefore, females between two male fetuses were compared with females residing in the uterus next to other females in a variety of test situations, all within the general framework of relative reproductive performance. Three general categories of comparisons of these two types of females were made: 1) the basic capacity to reproduce, including relative age at puberty and the number and weight of young produced and weaned as adults; 2) performance on a variety of behavioral measures including relative attractiveness to males, ability to interfere with the onset of puberty in other females via known pheromonal pathways and relative aggressiveness and 3) relative sensitivity to steroids, as assessed by both behavioral and physiological measures.

The results suggest that uterine position can bias a host of developmental and adult characteristics which could affect reproductive performance. In particular, such biases are apparent in a female mouse's capacity to transmit and respond to social cues, but not in the physiological capacity to become pregnant and bear healthy young.

MATERIALS AND METHODS

A total of 250 CF-1 females (60-days-old) was housed with adult males until insemination was verified by the presence of a vaginal plug, after which the males were removed. On the afternoon of Day 18 of gestation (shortly before parturition), each mother was killed by cervical dislocation and the uterine horns were exposed via a ventral incision. The fetuses were carefully removed from the uterine horns and the relative position and sex of each pup were recorded. After being cleaned, the pups were placed under heat lamps. All experimental comparisons discussed in this paper involved only the two types of females previously noted: 1) females residing between two male fetuses, referred to as "2M" females and 2) females not located next to a male, referred to as "0M" females. Each such experimental animal was marked as a pup for individual identification using a toe-clipping pattern. In addition, anogenital distance was measured under a dissecting microscope and body weight was recorded by an experimenter who was unaware of the group to which each animal belonged. All such experimental females then were fostered to mothers that had delivered within the previous 24 h period. Each foster mother received 4–6 pups from one group. All litters were housed in a male-free room and weaned at 21 days of age. After weaning, all animals were left in foster-litter groups, 4–6/cage, until being assigned to an experiment. Animals to be used in a particular comparison were chosen from a large pool of each of the two types of females, all of which were born within a 10 day period. Data were analyzed either by analysis of variance or by chi square (χ^2). In a few cases, females were used in more than one comparison; such cases are noted in the text.

Unless otherwise indicated, housing always consisted of plastic 18X29X13 cm cages. Animal rooms were maintained at 23 ± 1°C on a 14:10 h light:dark cycle with lights on at 0600 h and had 12 air exchanges per hour. Three nearly identical animal rooms were available for the separation of experiments and/or experimental groups when necessary.

RESULTS

The results of this research will be presented in four sections, each involving one general type of comparison, complete with its own rationale and background information. The results of specific comparisons then will be presented as subsections, with pertinent procedural details being presented along with the results.

Morphological Comparisons

At birth, the anogenital distance of the 2M females (n=154; mean ± SEM = 1.00 ± 0.01 mm) utilized in the experiments was significantly longer ($P<0.001$) than that of the 0M females (n=154; mean = 0.92 ± 0.01 mm). At 60 days of age, the anogenital distance of the 2M females (n=118; mean = 5.79 ± 0.03 mm) was again significantly longer ($P<0.001$) than that of the 0M females (n=118, mean = 5.57 ± 0.04 mm). These differences in anogenital distance between 0M and 2M females were not due to differences in body size. Mean body weight of the two types of females was not significantly different at birth, at 30 or at 60 days of age, nor was anogenital distance and body weight correlated either at birth or on Day 60.

Basic Physiological Capacity to Reproduce

The most fundamental reproductive comparison between 0M and 2M females involves their relative ability to produce healthy young when individually housed as adults with a male. It should be noted that such a test eliminates many potentially confounding effects of uterine position on stimulus and/or behavioral differences which could influence mating in more socially complex situations. Therefore, at 60 days of age, 16 females of each type were individually housed with a proven stud. All females mated within 4 days. When a female

became visibly pregnant (about Day 15 of gestation), the male was removed. Cages were subsequently checked every afternoon at 1300 h for pups. The date of delivery, number, weight and sex of pups at birth were recorded. At weaning on Day 21 following birth, the pups were again counted and weighed. At this time the mother was again paired with another sexually active male and the same procedure was repeated for a second litter.

The results of these comparisons are shown in Table 1 and reveal no effect of *in utero* position on any parameter of gross reproduction as defined in this test situation.

Behavioral Comparisons

The present set of comparisons of 2M and 0M females concerns a variety of characteristics that could influence a female's reproductive success. By way of background, mice have evolved a complex olfactory (pheromonal) communication system that is important in regulating both reproductive state and behavior (Bronson, 1974). Both male and female mice deposit urinary marks that convey pheromonal information concerning the sex, sexual state and possibly the individual identity of the depositor (Desjardins et al, 1973). Pheromonal communication in mice also includes a hormone priming action of mouse urine, i.e., urine of both sexes contains chemical factors that elicit the release of gonadotropins in recipients of the opposite sex (Bronson and Desjardins, 1974a; Maruniak and Bronson, 1976). Specifically, the onset of puberty is accelerated in young females by the odor of male urine, a process which is blocked by the presence of olfactory cues emanating from female siblings (Bronson and Desjardins, 1974b; Vandenbergh, 1973). Differences between 0M and 2M females in the tendency to urine mark their environments, transmit pheromonal information and in the time of onset of puberty under different housing conditions thus could affect reproductive success.

Additional differences between 0M and 2M females in both stimulus and behavioral characteristics could also alter realized reproduction. For example, males could prefer one type of female over the other in a choice situation. Alternatively, placing the two types of females in direct competition could reveal an enhancement of aggressive behavior in one type of female, possibly decreasing the probability of mating by the defeated female as a result of being driven out of the deme. Similarly, female mice, like many mammals, show a high degree of postpartum aggressiveness, an adaptive behavior functioning to protect the offspring. Different degrees of aggressiveness on the part of the two types of females in such a situation could influence their ability to produce healthy weaned young.

In this section, then, we present the results of five specific comparisons of 0M and 2M females, each of which could alter reproductive success: 1) time of onset of puberty, 2) urine marking, 3) preference by adult males, 4) aggressiveness when in direct competition and 5) postpartum aggression.

1. Onset of puberty in individually and group housed females exposed to males. Puberty in female mice (as indicated by the first ovulation) is modified by cues emanating from other mice of both sexes. Indeed, it has been demonstrated that in the absence of cohabitation with an adult male and some degree of isolation from other females, puberty in young female mice is markedly delayed. Specifically, juvenile female mice housed individually with

TABLE 1. Mean (±SEM) number of pups and body weight per pup (in g) at birth and weaning.

	Litter 1				Litter 2			
	Birth		Weaning		Birth		Weaning	
	Total number of pups							
0M females	12.0	0.5	11.7	0.5	12.9	0.7	12.8	0.6
2M females	11.3	0.8	10.1	0.6	12.1	0.8	11.4	0.9
	Mean body weight per pup							
0M females	1.6	0.1	9.7	0.3	1.6	0.1	9.5	0.5
2M females	1.6	0.1	10.1	0.6	1.6	0.1	9.2	0.4

an adult male ovulate and mate within a few days. When a male is placed with grouped juvenile females, the time of the first ovulation is significantly delayed by a few weeks, but subsequent estrous cycles are regular. Housing female mice with other females and completely separating them from males throughout life further delays sexual maturation and estrous cycles are prolonged and often anovulatory (Bronson and Desjardins, 1974b; Stiff et al., 1974; Vandenbergh, 1973). Therefore, this experiment, in which young females were housed either individually with a male or in groups with a male, allowed a comparison of 2M and 0M females with regard to 1) the relative age at which puberty could be induced by a male in the absence of inhibitory cues from other females and 2) the relative degree to which this process could be delayed when the females were housed in groups.

Animals of each type were obtained from the available pools of 2M and 0M females at 14–15 g body weight. Body weight rather than age has been found to be the most reliable predictor of readiness to ovulate in juvenile mice (Bronson and Desjardins, 1974a). Sixteen females of each type were individually housed with a proven stud male while 20 females of each type were housed 4/cage, each cage containing only females from one uterine position and a single stud male. All females were checked daily thereafter for vaginal plugs. The presence of vaginal plugging under such conditions is correlated perfectly with the pubertal ovulation (Bronson, 1975). The experiment was terminated after 20 days with females which had not mated by this time being assigned this number.

Females of the two types did not differ in the time of their pubertal mating when individually housed with males (Table 2). As expected, analysis of variance revealed that grouping significantly retarded the time of mating in both 0M and 2M females ($P<0.001$). More importantly, grouping had a greater inhibitory effect on time of mating among 0M females than among 2M females as indicated by a significant interaction between uterine position and type of housing ($P<0.01$). Thus, the male's puberty accelerating action was inhibited among 0M females to a greater extent than among 2M females.

2. Urine marking. Mice communicate chemically by depositing small marks of urine on the ground as they locomote. This behavior can be quantified by observing filter paper flooring under ultraviolet light. Relevant to the present comparison is the fact that male mice urine mark at much higher rates than do females, particularly in response to the presence of an unfamiliar cospecific. Moreover, the frequency of urine marking in male mice is correlated with dominance status (Desjardins et al, 1973) and may provide an index of territoriality (Harrington, 1976). This experiment compares 0M and 2M females in terms of their frequency of urine marking when alone or in the presence of a male. A high rate of marking in either situation is considered a masculine behavioral trait.

Twenty females (60-day-old) from each uterine position were placed individually into a 30×30×15 cm wooden box divided in half by a wire-mesh screen so that the compartment containing the female was 30×15×15 cm. Twenty other females from each uterine position were placed in a similar box with an intact adult male on the other side of the wire-mesh partition. The females were left in the test box for 1 h. The filter paper from the cage floor then was placed under a fluorescent light and the number of individual urine marks was counted.

Analysis of variance revealed that both 2M and 0M females urine marked at higher rates in the presence of a male than in isolation ($P<0.001$). However, 2M females marked at higher rates than 0M females whether alone or in the presence of a male ($P<0.05$, see Table 3). This finding suggests that 2M females are masculinized relative to 0M females in terms of urine-marking behavior.

3. Stimulus characteristics of 0M and 2M

TABLE 2. The proportion of animals mating within 20 days and the mean number of days of male exposure prior to mating (±SEM) in individually and group housed females.

	Individually housed		Grouped 4/cage	
0M females	(16/16) 5.5	0.5	(14/20) 14.8	0.9
2M females	(16/16) 6.5	0.9	(18/20) 11.2	0.8

TABLE 3. Mean (±SEM) number of urine marks for females of each uterine history when tested in isolation as well as in response to the presence of a male.

	Isolation		Male	
0M females	35	12	139	21
2M females	56	14	200	23

females: preference by adult males. This comparison tested the relative preference for the two types of females by sexually experienced adult males. Using an apparatus (see Fig. 1) similar to that described by Moltz (1974), adult males were presented with the opportunity of descending from a platform into one of two goal boxes, one of which contained a 0M female and the other a 2M female, each covered by a wire-mesh screen.

The two types of females were used as stimulus animals in this comparison only when in diestrus as determined by vaginal lavage and verified by examining vaginal smears over two cycles. Nine such females of each type were used. Each randomly chosen pair of females (one from each group) served as the stimulus for 5 males, i.e., after every 5 sessions the stimulus females were replaced and their positions were alternated such that for one-half of the sessions the 0M females were in the left goal box and for one-half of the sessions the 0M females were in the right goal box. In addition, the test chamber was cleaned with alcohol and water after each session. The 45 males tested for preference in this comparison had been housed singly since weaning and were allowed to mate with receptive females five times over a 2 week period prior to the start of the experiment, at which time they were 90–100 days old. Testing was conducted between 1300 and 1500 h under normal lighting conditions. Sessions were a maximum of 15 min long or were terminated as soon as the male descended from the platform onto the wire-mesh covering a stimulus female. A session consisted of placing a male in the start box and recording the length of time prior to entering a goal box as well as whether the male explored both goal areas prior to entering one of them.

Of the 45 males tested, 25 males extensively explored both goal areas prior to making a choice and entering one of them (mean latency to enter a goal box = 343 ± 45 seconds). The remaining 20 males entered one or the other goal box much more rapidly (mean latency =

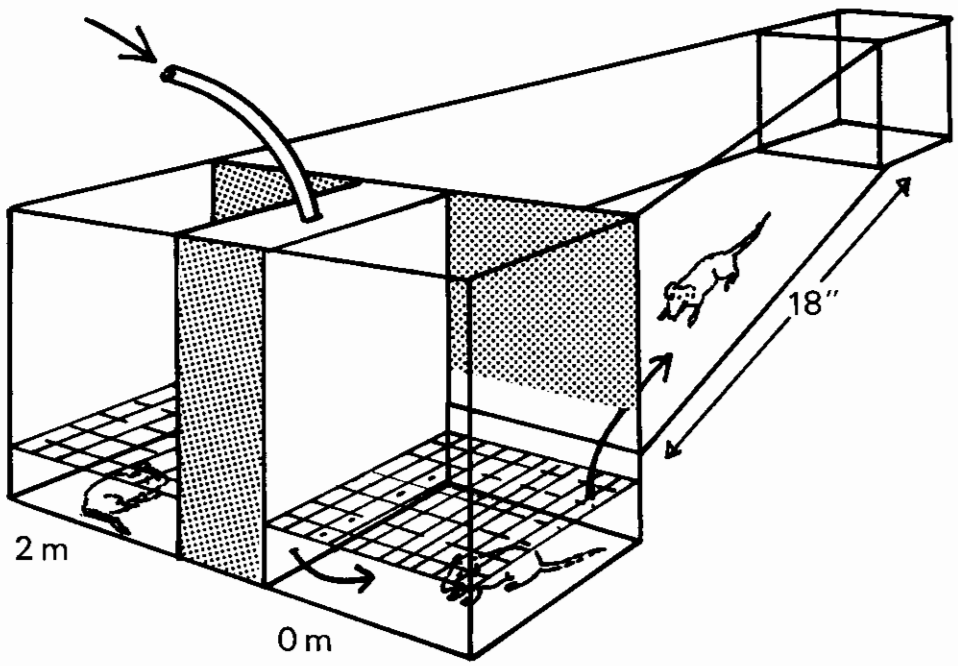

FIG 1. Apparatus used to test the relative attractiveness of 2M vs 0M females to adult males. Arrows indicate direction of airflow.

50 ± 11 seconds). These males ran down one side of the apparatus and immediately entered the goal box on that side without investigating both goal areas (10 males went to each type of female). Thus, these 20 males were not considered to have made a discrimination and were eliminated from the experiment. Significantly more of the 25 males which did explore both goal areas entered the goal box containing the 0M female (21 males chose the 0M female while only 4 males chose the 2M female; $P<0.001$, χ^2). Thus, there was a clear preference for the 0M females by sexually experienced males.

4. Direct aggressive competition between 0M and 2M females. Aggression between mice is generally considered a masculine trait except in the case of the female with litter (Scott, 1966). While nonlactating female mice do not fight with the frequency or intensity of males or of lactating females, adult CF-1 females do exhibit some spontaneous aggressive and mounting behavior toward other females. This experiment assessed the incidence of spontaneous aggression between 0M and 2M females when matched for precise age (75 days old) and weight (<0.5 g difference in all cases). Experimental pairs (1 0M and 1 2M female) were housed in 30×30 ×15 cm wooden boxes divided into two living areas by wooden partitions. Vaginal smears from all females were examined daily starting 7 days after such housing began. Testing was accomplished between Days 10 and 15 on the first day that both members of a pair exhibited a late metestrous or early diestrous vaginal smear. Testing consisted of removing the partition and observing the animals for 30 min. Incidence and duration of aggressive grooming and tail rattling (which normally precede an attack in mice), fighting (chasing and biting), as well as mounting and lordosis, were recorded.

Of the 28 pairs of 0M and 2M females tested, 14 pairs fought repeatedly, 6 pairs did not fight but exhibited rough, aggressive grooming and tail rattling, while 8 pairs exhibited no aggressive activity of any kind. Of the 14 pairs which fought, fights were initiated in 12 cases by the 2M females and in only 2 cases by the 0M females ($P<0.01$; χ^2). Aggressive grooming and tail rattling, where observed in the absence of fighting, also seemed to be exhibited typically by 2M females (5 2M females vs 1 0M female, $P<0.05$; χ^2). In virtually all cases, the animal that did not initiate aggressive behavior exhibited submissive postures or ran away from its opponent during the rest of the test period. Only 2 animals exhibited mounting, 1 0M female and 1 2M female, but in each case the female also exhibited aggression.

5. Postpartum aggression against a strange male or female. To determine if aggression during the postpartum period is also influenced by uterine position, a conspecific intruder was placed into the home cage of females of each type when these females were adult, pregnant and suckling their second litter. Actually, two experiments were conducted, each using a different type of intruder and separate sets of test females. In each case, however, the females tested for aggressiveness had been used previously for other types of comparisons. The first experiment employed the females which had been used to assess the time of onset of puberty under the conditions of group housing. Upon termination of the experiment which dealt with puberty, the 20 females of each type were housed singly and allowed to deliver the first litter. Following weaning of their young, the females were remated and after delivering the second litter, the stud was removed. One test for aggressiveness was administered on Day 7 following parturition while suckling the second litter. Testing consisted of placing an adult ovariectomized female mouse into the home cage of the lactating mother. The latency, number and cummulative duration of fights (chasing and biting) were recorded. Sessions were 10 min long, after which the intruder was removed.

The second comparison involved the 16 females of each type which were used to assess basic reproductive capacity (see Table 1). Timing and procedures for aggression testing were as described above except that the stimulus animals in this experiment were adult males that had been rendered anosmic via olfactory bulbectomy. Bulbectomized male mice elicit aggression, but do fight back when attacked (Denenberg et al., 1973). The number of young in each female's cage was counted before and after encountering the strange male. Since no evidence of damage to the pups was seen, this procedure appears not to have biased the data in Table 1. Finally, it should be noted that the frequency and intensity of postpartum aggression is independent of parity in mice and is also not correlated with litter size (Svare and Gandelman, 1973).

Almost all lactating females displayed attack behavior in these experiments regardless of their former uterine position. As shown in

TABLE 4. The proportion of lactating females from each uterine position which attacked the male or female intruder and the total duration of such attacks in seconds per 10 min test.

	Female intruder		Male intruder	
	Proportion attacking	Duration of attacks (seconds)	Proportion attacking	Duration of attacks (seconds)
0M females	14/19	16.3 3.7[a]	15/15	32.6 7.6
2M females	15/18	39.9 7.5[a]	15/15	38.7 7.0

[a] $P<0.01$; t test.

Table 4, however, the duration (intensity) of aggression displayed against the female intruder by the 2M females was significantly greater than that exhibited by the 0M females ($P<0.01$; t test). Comparisons of the number of attacks and latency to attack were not significantly different. No differences were observed on any measure of aggression between the two types of females when tested against a bulbectomized male; the duration of time spent fighting was high for both 2M and 0M females. Thus, while virtually all lactating females exhibit some aggression against intruders of both sexes, only males, which cannibalize newborn mice (Gandelman and vom Saal, 1975) and are thus a threat to the young, elicit intense aggression by both 0M and 2M females. Female intruders, on the other hand, elicit intense aggression by 2M females, but not by 0M females.

Relative Sensitivity to Steroids

An obviously important source of variation between 0M and 2M females could reside in a differential sensitivity to steroid hormones. In both males and females, sexually related behaviors are influenced by these hormones, while the potential interactions of steroids with the physiological bases of reproduction are manifold. This section presents the results of three comparisons of the relative sensitivity of 0M and 2M females to gonadal steroids. The comparisons represent a spectrum of events that are modulated either by testosterone or estradiol. Specifically, the three comparisons of 0M and 2M females are: 1) the relative sensitivity of lordosis behavior to estradiol; 2) the relative sensitivity of the negative feedback mechanism by which estradiol inhibits luteinizing hormone (LH) secretion and 3) the relative sensitivity to testosterone, as assessed by both mounting and aggressive behavior in response to a sexually primed female.

1. Relative sensitivity of lordosis to estradiol. This experiment examined the sensitivity of 0M and 2M females to estradiol as assessed by their potential for exhibiting lordosis in the presence of a male. It is important to note that the reliable occurrence of lordosis in mice, within a restricted period of time, requires estrogenic sensitization and subsequent exposure to progesterone (Ring, 1944).

Ovariectomized 0M and 2M females were exposed to a spectrum of dosages of estradiol while the subsequent dosage of progesterone was held constant. Forty females of each type, which had been tested for urine marking 7 days before, were ovariectomized and implanted with one of the following doses of estradiol (n=8/dose): 0 (blank capsule), 0.5, 1, 5 or 10 μg/capsule. Estradiol was mixed with Silastic adhesive (Type A, No. 891, Dow Corning), and this suspension was packed into a 10 mm Silastic capsule (0.04 in ID, 0.085 in OD, Dow Corning). Dilution with Silastic adhesive previously has been found to be preferable to a variety of other methods of chronically administering estradiol to mice (Bronson, 1976). Seven days after ovariectomy and capsule implantation, between 0900 and 1100 h, all animals were given s.c. injections of progesterone, 200 μg/0.02 ml oil (females bearing blank capsules were injected with oil). All females then were placed singly in a clean cage and 4 h later were given a 30 min test for sexual behavior. Males used in these tests each had a history of at least 4 prior matings. The latency, number and duration of male mounting and of female lordosis were measured. Following behavioral testing, the males remained in the female's cage overnight. Each female was examined the next morning for the presence of a vaginal plug.

Regardless of uterine position, no female that was implanted with the blank, 0.5 or 1 μg estradiol capsules exhibited lordosis during sex testing or was found to have a vaginal plug the

following morning. Almost all females carrying the 5 and 10 μg capsules exhibited lordosis and were found to have vaginal plugs. There was no difference between the two types of females on any of the specific measures of lordosis, the lordosis quotient (number of lordosis/number of mounts × 100) or in the incidence of plugging. Interestingly, the frequency with which the test females were mounted did not change appreciably with dose of estradiol. Blank implanted females of both types also elicited mounting behavior on the part of the test males. However, the test males mounted 0M females significantly more often than they did 2M females. Specifically, disregarding dose of estradiol, the 40 0M females tested in this experiment were mounted an average of 14.0 ± 2.3 times each per 30 min test, while the comparable figure for the 40 2M females was 8.3 ± 1.2 ($P<0.01$). In addition to the mounting behavior, the unreceptive females implanted with the blank, 0.5 and 1 μg estradiol capsules also were attacked by the males when their attempts at mounting were rejected. However, the test males exhibited more attacks against the unreceptive 0M females (mean = 11.1 ± 2.3) than they did against the unreceptive 2M females (mean = 6.7 ± 1.0; $P<0.05$). No aggression was observed toward the receptive 0M and 2M females implanted with the 5 and 10 μg capsules. The results are interpreted as indicating that 0M females are more sexually arousing to males than are 2M females, this phenomenon being independent of hormonal state.

2. Sensitivity of the negative feedback inhibition of LH secretion to estradiol. The secretion of LH in females is regulated in part by the ovarian steroids. Ovariectomy of an adult female results in a marked elevation in blood titers of LH; treatment with exogenous estradiol then depresses these high levels via a negative feedback mechanism (Schwartz and McCormack, 1972). In the present experiment, we examined the relative sensitivity of this mechanism in 0M and 2M females by ovariectomizing each type of female, exposing representatives of each type to progressively increasing doses of estradiol and evaluating the effects of such treatment by examining blood LH titers. Uterine weights also were obtained as a bioassay of estradiol dosage.

This experiment utilized the same animals that were tested for sexual behavior in the previously described experiment. The females remained individually housed and were reimplanted with fresh capsules of the same dosage of estradiol on the day after sexual testing; all old capsules were removed at this time. All animals were killed 7 days later (1200 h) and 100 μl serum samples were assayed for LH using the NIAMMD anti-rat radioimmunoassay kit, previously verifed for use in the mouse (Beamer et al., 1972). Results are expressed in terms of ng of the RP-1 LH preparation per ml of serum.

As shown in Fig. 2, the two types of females did not differ either in the sensitivity of their LH negative feedback mechanism to estradiol or in their uterine sensitivity to the same steroid.

3. Effects of exposure to testosterone on behavior toward a receptive female. The present experiment was conducted to determine whether the androgenic induction of male sexual (mounting) or aggressive (chasing and biting) behavior in female mice would vary as a function of *in utero* proximity to male fetuses. Twenty-five females from each uterine position were matched for both age and weight (<0.5 g difference). All females were 75—80 days old at the start of the experiment and were housed individually throughout the experiment. Each female was tested 5 times at 7 day intervals over a 28 day period. Each test consisted of a 10 min exposure to a sexually primed adult female. Testing was conducted in the home cage between 1200 and 1300 h during which both mounting and aggression (latency, number and duration of fights) were recorded. During the first test session, the 2M and 0M experimental females were intact. Immediately following this test, all females were ovariectomized and

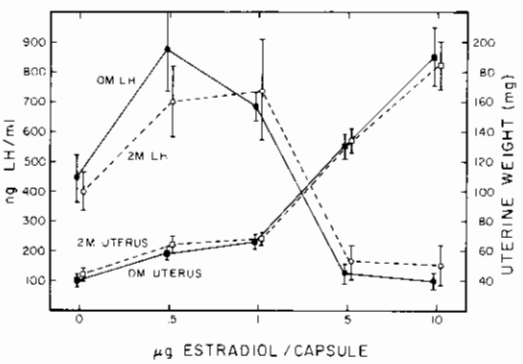

FIG. 2. Serum LH (ng/ml ± SEM) and uterine weight (mg ± SEM) in ovariectomized 0M and 2M females individually housed and implanted with 1 of 5 doses of estradiol and then killed on Day 7 following implantation of the capsules.

implanted with 40 mm Silactic capsules (0.078 in ID, 0.125 in OD, Dow Corning) containing testosterone. These capsules previously have been found to produce blood levels of testosterone similar to those of intact adult male mice (Maruniak et al., 1977).

Stimulus females used in this experiment were drawn from a large pool of ovariectomized animals, each of which was injected with 5 μg estradiol benzoate in oil 2 days before the test session. On the day of testing, the stimulus females were injected with 200 μg progesterone in oil at 0800 h. As previously noted, this sequence of steroid administration reliably induced sexual receptivity in female mice 4–5 h following administration of progesterone. Prior to testing, each stimulus female was placed with a sexually experienced male both to verify receptivity and to "behaviorally prime" the females to exhibit lordosis when mounted.

The cumulative number of 2M and 0M females which fought or mounted during the 4 weeks of testing is presented in Fig. 3. No sexual or aggressive behavior was observed during the first session. The occurrence of mounting increased with time after testosterone implantation, but no significant difference between the two types of females was observed. However, significantly more 2M females exhibited aggression toward a receptive female over the 4 weeks of testing than did 0M females ($P<0.001$; χ^2; Wilson, 1956). No difference in the latency, number or duration of attacks was found between 0M and 2M females. It should be noted that this latter finding is consistent with the finding of vom Saal (1976) that prenatal exposure to androgen influences the duration of exposure to testosterone in adulthood required to induce aggression in mice, but not the intensity of aggression exhibited.

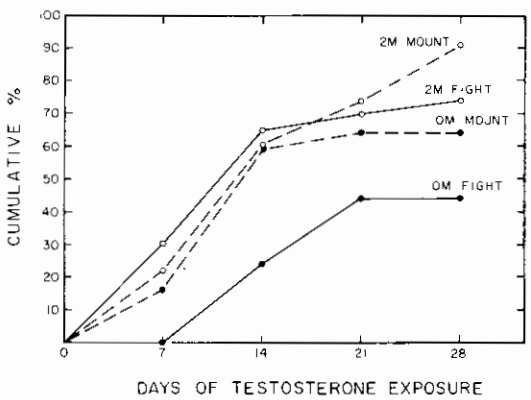

FIG. 3. The cumulative percentage of 0M and 2M females that mounted or attacked a sexually receptive stimulus female during 10 min test sessions prior to and following implantation of a capsule containing testosterone.

DISCUSSION

The present experiments confirmed that anogenital distance of female mice at birth is influenced by *in utero* proximity to male fetuses. Additionally, the present data also show that such effects of uterine position on anogenital distance persist at least until 60 days of age. Thus, the uterine position of a female mouse is an important source of neonatal and adult morphological variation. The objective of the present comparisons was to assess whether *in utero* proximity to males was also related to variation in adult reproductive performance of female mice. It is important to note that the concept of reproductive performance is interpreted broadly in the present studies. Thus, as summarized in Table 5, the two types of females (0M vs 2M) were tested not only for basic physiological capacity to reproduce, but also for a variety of behavioral qualities which could influence their propensity to mate and produce healthy weanling young. It should be noted that the sequence of comparisons has been rearranged in Table 5 to facilitate the following discussion.

Some general conclusions can be drawn from these experiments. For one, there is no indication that the two types of females differ in what can be considered to be their basic physiological capacity to reproduce. Specifically, in the simple confines of a laboratory cage containing one female and one male, neither the onset of puberty nor the ability to mate, reproduce and rear young was affected by *in utero* position. Similarly, the two types of females do not appear to differ in their sensitivity to either estradiol or testosterone, at least as assessed by several procedures that involved both physiological and behavioral responses. Three additional comments are appropriate in this regard. In the experiment that compared the behavioral sensitivity of 0M and 2M females to estradiol, 0M females were mounted more by males than were 2M females, but this enhanced state of arousal was independent of the presence, absence or dose of estrogen administered. Also, more 2M than 0M females were induced

TABLE 5. A rearranged summary of the results of experimental comparisons between 0M and 2M females.

Experimental comparison	Significant difference	Source of data
Relative stimulus quality of female:		
Antagonism of male-induced puberty	0M>2M	Table 2
Preference by males	0M>2M	Text
Mounting by males, independent of estrogen levels	0M>2M	Text
Masculinization of behavior of female:		
Aggressiveness: direct competition	2M>0M	Text
Aggressiveness: postpartum	2M>0M	Table 4
Urine marking	2M>0M	Table 3
Basic physiological capacity to reproduce:		
Puberty induction by male when individually housed	None	Table 2
Number and weight of young produced over 2 litters	None	Table 1
Sensitivity to steroids:		
Sensitivity to estradiol:		
Lordosis	None	Text
LH feedback	None	Fig. 2
Uterine weight	None	Fig. 2
Sensitivity to testosterone:		
Induction of mounting	None	Fig. 3
Induction of aggression	2M>0M	Fig. 3

to be aggressive toward other females when exposed to androgen. However, 2M females were also more aggressive toward other females under all other conditions that were examined in the absence of androgen exposure, except when tested against receptive females (see Fig. 3; Day 0). Finally, in the experiment that utilized intact 0M and 2M females as stimulus objects for male preference testing, these females always were tested when in metestrus or diestrus, the quiescent phases of the estrous cycle. Thus, the results of these comparisons seem consistently unrelated to any difference between 0M and 2M females in their sensitivity to gonadal steroids.

In the process of interacting socially, an individual is both a stimulus and a responder to other animals. In the case of the observed differences between the social behavior of 0M and 2M females, it is of interest to determine whether such differences are traceable either to variation in stimulus quality or to a differential tendency to respond to environmental and social cues. When viewed *in toto* (see Table 5), the data indicate that both factors probably are involved. A clear case of different stimulus qualities of the two types of females seems involved in the 80% preference for the 0M females by sexually experienced males. Likewise, males spent more time mounting 0M females than they did 2M females, again indicating that 0M females are more sexually arousing. On the other hand, the higher rate of urine marking that was characteristic of 2M females clearly seem traceable to a difference in behavioral responsiveness to environmental cues. Additionally, since all tests of aggressiveness showed 2M females to be more aggressive than 0M females, this difference is most likely traceable to a general enhancement of aggressive behavior in 2M females, particularly toward other females. Finally, however, in the comparison which concerned the male-induced acceleration of puberty among group housed females, the observed difference in the time of onset of puberty could be accounted for by either (or both) a basic difference in the transmission of or response to puberty inhibiting cues. Thus, when all of the data are considered, they support a generality that uterine position near male fetuses yields biases in both the transmission of and responsiveness to social cues, such biases showing up in both a sexual and aggressive context.

The source of the observed variation between 0M and 2M females is of obvious interest. It is important to note, however, that the nature of the present comparisons actually does not allow a clear separation of the two most obvious possibilities; a masculinizing action via proximity

to male fetuses vs a feminizing action as a consequence of proximity to female fetuses. Nevertheless, support for a masculinizing action is provided by an extensive literature and by the present anogenital assessment, a parameter of long standing utility for the bioassay of androgenic activity. Therefore, in the absence of any information in the literature which would lead one to suspect a feminizing action by the prenatal ovary, it seems reasonable to presume that the differences observed between 2M and 0M females result from the 2M females being exposed to androgen produced by contiguous male fetuses (Black et al., 1971; Clemens, 1974). With specific regard to the action of such presumed androgen exposure on neural tisues and, hence, on potential behavioral differences, it should be noted that, based on previous research with rodents, the action of early androgen exposure has been interpreted as "sensitizing" neural mechanisms to the same hormone later in life (Beach, 1945; Eaton et al., 1973; Gandelman et al., 1977; vom Saal et al., 1976). Importantly, the present data demonstrate that the *in utero* masculinization we observed had direct effects on stimulus and behavioral characteristics that were independent of any interaction with steroid hormones in adulthood.

The present results stimulate a final dimension of interest, namely, the meaning of the uterine related biases found in these studies to the reproductive biology of wild populations of mice. Of primary importance is the finding that the basic ability to mate and successfully produce and rear young in a small cage in a laboratory is not influenced by uterine position. While generalization from the lab to the field is a tenuous process at best, it is reasonable to suspect that the differences observed between 0M and 2M females with regard to their urine marking, onset of puberty, sexual behavior, sexual attractiveness and aggressiveness could translate into each type of female being better suited to successfully reproduce in a particular type of social environment. For example, it is possible that 0M females might have a reproductive advantage at low population densities while 2M females would be more likely to successfully reproduce at higher population densities. Specifically, 0M females are strongly preferred by males and, thus, at low densities might be most likely to mate. On the other hand, at high densities, juvenile 2M females might go into puberty sooner than 0M females. Additionally, the enhanced aggressiveness of 2M females might increase the probability of their successfully establishing and defending a nest area where considerable competition existed. Thus, female to female differences relating to uterine position could be viewed as a potentially important source of nongenetic variation and, as such, could be an important component of the mouse's total reproductive strategy. Conversely, within the evolved framework of sexual differentiation that has been established for mammals, some degree of masculinization of some female fetuses could be a penalty necessitated by the mouse's overwhelming need as a prey species for a high rate of production of young and, hence, multiple uterine residence. Whether contiguity to male fetuses will prove to be adaptive, a penalty or neutral in terms of a female mouse's reproductive success outside of the laboratory remains to be examined.

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