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Paradoxical Effects of Maternal Stress on Fetal Steroids and Postnatal Reproductive Traits in Female Mice from Different Intrauterine Positions[1]

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ABSTRACT

We examined effects of maternal stress on prenatal serum concentrations of testosterone and estradiol and on postnatal reproductive traits in female mice from different intrauterine positions. On Day 18 of fetal life, control females positioned in utero between two male fetuses (2M females) had higher concentrations of testosterone and lower concentrations of estradiol in serum than control female fetuses located between two females (0M females). Control females positioned between a male and a female fetus (1M females) had intermediate levels of both hormones. Prior intrauterine position in control females accounted for differences in genital morphology (length of the anogenital separation) at birth and length of estrous cycles during adulthood. Maternal stress eliminated these postnatal differences due to prior intrauterine position: all 0M, 1M, and 2M female offspring of stressed mothers exhibited postnatal traits that were indistinguishable from those of control 2M females. Maternal stress resulted in an increase of over 1 ng/ml in serum testosterone in all female fetuses; the magnitude of the increase was similar for 0M, 1M, and 2M females. There was no effect of maternal stress on serum concentrations of estradiol in 0M and 2M female fetuses. Maternal stress resulted in a dramatic change in the postnatal traits of 0M females, whereas 2M females showed no change. Since the effect of maternal stress on sex steroids was similar among fetuses from different intrauterine positions but postnatal response to maternal stress varied by intrauterine position, other components of the endocrine system may mediate effects of maternal stress on these postnatal characteristics.

INTRODUCTION

Subjecting pregnant female rats and mice to stress can have dramatic effects on the characteristics of female offspring, such as fertility and fecundity [1], age at vaginal opening and length of estrous cycles [2, 3], sex ratio of offspring [4], maternal behavior [4–6], sexual behavior [7], response to pain [8], brain function [9], and pituitary function [10].

A naturally occurring cause of phenotypic variation in females in litter-bearing mammals is the in utero proximity of a female fetus to fetuses of the same or opposite sex, which is referred to as the intrauterine position phenomenon [11]. The intrauterine position of a female mouse fetus has been correlated with concentrations of testosterone in amniotic fluid and serum on Day 17 of pregnancy. Specifically, females located between two males (2M females) have greater concentrations of testosterone than do female fetuses not located next to a male fetus (0M females), although female fetuses from each intrauterine position have less serum testosterone than do males [12].

Prior intrauterine position influences many postnatal traits related to reproduction in rats, mice, gerbils, and pigs, such as genital morphology [13–17], daily activity level [18], body weight [19], attractiveness to mates [14], preputial gland enzyme activity [20], length of estrous cycles [11, 21–23], aggressiveness [14], sexual behavior [14, 24, 25], maternal behavior [14, 26, 27], age at which fertility begins [11, 14, 22, 23, 28], and the timing of reproductive senescence [29, 30].

We examined the effects of maternal stress on the relationship between plasma steroid concentrations during fetal life and two postnatal reproductive characteristics: anogenital distance at birth and length of adult estrous cycles. As discussed above, these traits have been reported to differ in comparisons of 0M and 2M females produced by nonstressed mothers. Anogenital distance was measured to examine the possibility of masculinization (induction of male-typical traits) due to maternal stress, while estrous cycles were measured to examine the possibility of defeminization (loss of female-typical traits) due to maternal stress [31].

METHODS

Animals and Housing Conditions

Adult CF-1 mice (*Mus domesticus*) were initially purchased from Charles River Breeding Laboratories (Wilmington, MA) in 1979 and have been outbred in a closed colony since that time. Mice were housed in 18 × 29 × 13-cm polypropylene cages and maintained at 25 ± 1°C on a 12L:12D cycle, with lights-on at 1200 h. All work in the animal rooms during the dark phase of the cycle was con-

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ducted with a 25-W red light. Mouse breeder chow (Ralston-Purina, St. Louis, MO) and water were available ad libitum.

Mating Procedure

Females were mated and randomly assigned to either control or maternal stress groups. For each separate experiment, the stressed and control females delivered young within a 5-day period. To obtain mouse fetuses from known intrauterine positions, adult CF-1 female mice were time-mated by being placed daily with a stud male beginning at 0800 h. Females were removed and examined for vaginal plugs 4 h later. Throughout the remainder of the paper, all times will be given relative to Hour 0 of pregnancy (at the onset of the light phase of the light:dark cycle) of the day (Day 0) on which vaginal plugs were found.

Inseminated females were housed three per cage and not disturbed until Day 10 of pregnancy, at which time the cages were changed. One day before the fetuses were to be removed from the uterus, either for fetal blood collection or for postnatal studies, pregnant females were housed individually.

Intrauterine Position Classification Scheme

The intrauterine position of each female was classified at cesarean delivery according to the sex of directly adjacent fetuses. In these experiments, 2M females developed between 2 male fetuses, 1M females developed between a male and a female fetus, and 0M females developed between 2 female fetuses in utero (see Fig. 1). Intrauterine postioning is a random developmental event [11; M. Clark, unpublished observation]; using this classification scheme we find, on average, one 0M, two 1M and one 2M female fetuses per litter. Average litter size in CF-1 mice is 12 pups; 33% of the pups (at the ends of the two uterine horns) cannot be classified using this scheme.

In prior publications [review, 32], groups of 0M females included females at the ends of a uterine horn that were not located next to a male fetus rather than just females specifically located between two other females (as in the present studies). The change in classifying 0M females was due to the observation that male fetuses located between female fetuses had greater amniotic fluid concentrations of estradiol than did male fetuses located between males. We thus predicted that female fetuses located between two other female fetuses might also have greater plasma concentrations of estradiol than 2M female fetuses. Other classification schemes have been examined in mice (such as a comparison of 1M females located on the ovarian vs. cervical side of a male fetus), but none of these other schemes have accounted for differences in postnatal characteristics [33].

Prenatal Stress Procedure

Randomly selected pregnant mice were stressed by being removed from the animal room and placed into plexiglass mouse restraining chambers (9 × 6.3 × 5 cm) under a bank of 150-W flood lights (350 fc; 38°C = temperature inside the chamber). There were two 45-min stress sessions per day at 0400 and 2100 h, beginning at 2100 h near the end of the dark phase of Day 12 of pregnancy.

Collection of Fetal Serum for Measurement of Testosterone and Estradiol

Previously, 0M and 2M female mice were compared for serum and amniotic fluid concentrations of testosterone, estradiol, and progesterone on Day 17 of pregnancy. No differences in estradiol or progesterone were found, while testosterone was significantly different (2M > 0M) in both amniotic fluid and serum [12]. In this prior study, females were classified as 0M if they did not have a male adjacent to them in utero (this included females at the end of a uterine horn or, in rare cases, alone in a uterine horn). Female fetuses not located next to a male tended to have greater serum and amniotic fluid concentrations of estradiol than did females located between two male fetuses, although the differences were not statistically significant [12]. We subsequently reported that females had greater amniotic fluid concentrations of estradiol than males, and that males located in utero between two female fetuses had greater concentrations of estradiol in amniotic fluid than did males located between two male fetuses on Day 17 of pregnancy [34]. We proposed that differences in serum concentrations of estradiol due to intrauterine position were not previously found in female fetuses because females located between females had not been compared with females located between males in utero. We thus reexamined the possibility that intrauterine position would influence serum concentrations of estradiol during fetal life by comparing females that developed in utero between two female fetuses with females that developed in utero between two male fetuses (as depicted in Fig. 1).

Serum concentrations of testosterone and estradiol were examined in fetuses on Day 18. Considerably more blood can be collected on Day 18 than on Day 17 of fetal life, and preliminary studies had revealed sex differences in testosterone (male > females) as well as estradiol (females > males). We also previously observed an increase in serum testosterone in female fetuses due to maternal stress on both Day 17.5 and Day 18.5 of pregnancy (F. vom Saal, unpublished observation).

Females were killed by decapitation at the beginning of the light phase of the light:dark cycle between 0030 and 0200 h on Day 18 of pregnancy. The last stress session for females assigned to the stress condition began at 2100 h on Day 17 of pregnancy (3.5–5 h before blood collection). A pregnant female was decapitated, and blood was collected in test tubes within 15 s of a female's cage being touched. All fetuses were removed from the uterine horns (which were left in the mother during removal of the fetuses) and decapitated. Fetal blood was collected in hepa-

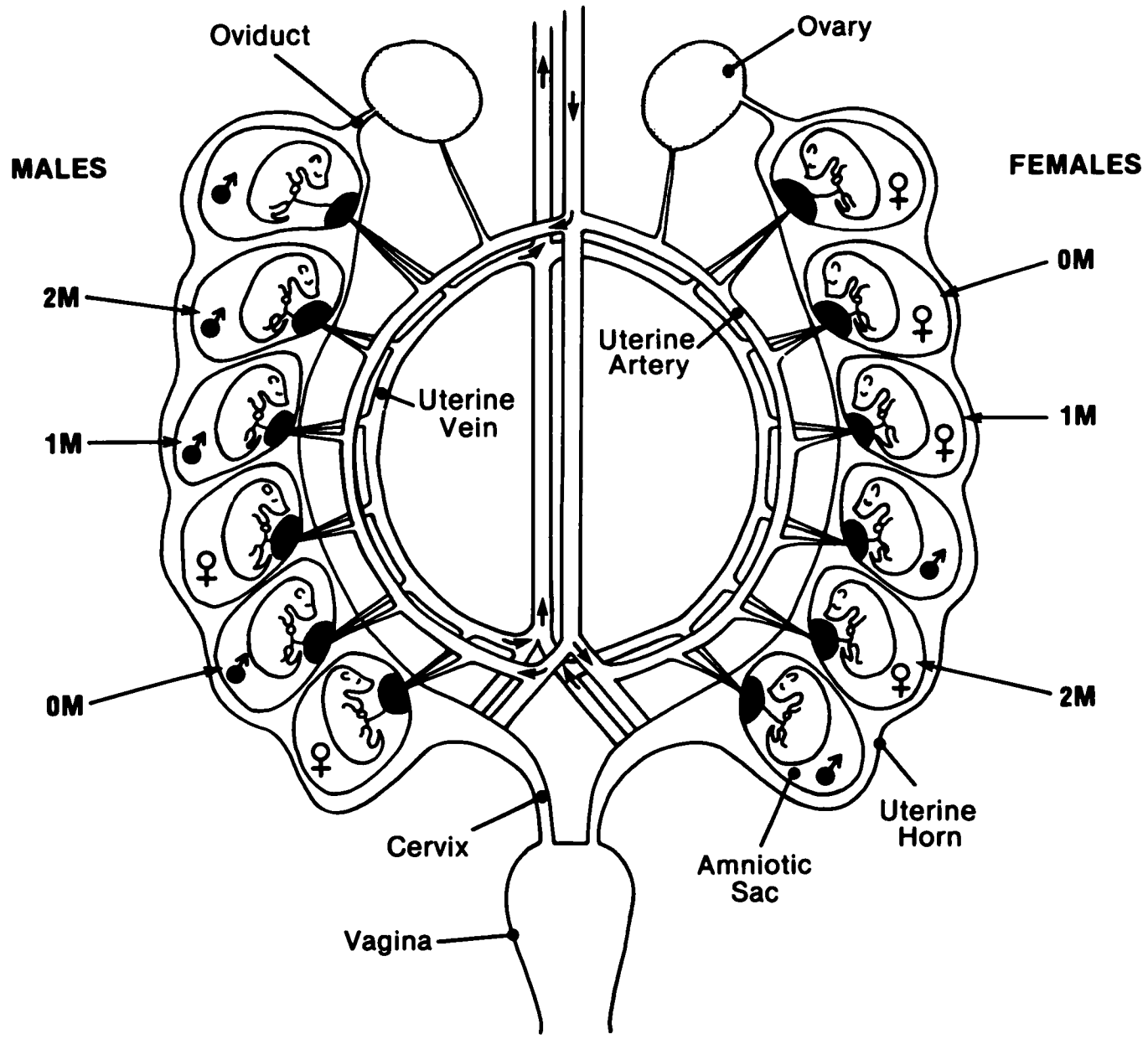

FIG. 1. The uterine horns and uterine loop arteries and veins of a pregnant mouse at term. Intrauterine position of fetuses was determined at cesarean delivery. The labels 0M, 1M, and 2M refer to the number of male fetuses to which an individual was contiguous (2M = between 2 males, 1M = between a male and a female, and 0M = between 2 females). The same classification scheme was used for both males and females. Arrows within the loop artery and vein feeding each uterine horn indicate the direction of blood flow as revealed by injecting carbon dye into the maternal heart (for arterial flow) and into individual placentae (for venus flow).

rinized micropipettes by aspiration within 4 min of maternal decapitation. The sex and position of each fetus within the two separate uterine horns were recorded. Fetal sex was determined by examining the length of the anogenital space (on Day 18 of pregnancy, this space is twice as long in male fetuses as it is in female fetuses).

Blood from six fetuses from the same intrauterine position was pooled and used to measure either estradiol (from

one set of pools) or testosterone (from another set of pools). We will report elsewhere studies showing the pattern of corticosterone in control and stressed fetuses and pregnant females during this time of pregnancy in mice (M. Montano, unpublished observation).

Delivery of Fetuses and Methods for Postnatal Studies

Pregnant females were killed by cervical dislocation beginning at 2200 h on Day 18 of pregnancy. Females in the stress group received the last stress session at 0400 h on Day 18. The mean time of parturition is 0230 h at the beginning of Day 19 of pregnancy for nonstressed CF-1 mice on this light:dark cycle.

Anogenital distance at birth. The tissue separating the anus and genital papilla at birth differentiates into the scrotum in males under the influence of testosterone (after it is metabolized to 5α-dihydrotestosterone [DHT] by 5α-reductase). In this experiment, we measured the length of this space in female mice at birth as a bioassay for masculinization as a result of differential exposure to testosterone due to intrauterine position and maternal stress [5].

Anogenital distance and body weight were determined for each pup at delivery. Anogenital distance was measured by use of a dissecting microscope with a micrometer disc by an investigator who was unaware of the intrauterine position of the pups.

Randomly selected females were raised by foster mothers that had delivered normally within the preceding 24 h in groups consisting of five 0M and five 1M females or five 2M and five 1M females from the same prenatal treatment group (animals were identified by a toe-clipping pattern). The animals were weaned, and body weights were recorded at 24 days of age; animals were then housed with other foster-littermates from the same intrauterine position (4–5 females per cage) for a subsequent study of the length of adult estrous cycles.

Length of adult estrous cycles. Previous studies have shown that the length of estrous cycles in female mice is increased by prenatal stress [3]. The length of estrous cycles during puberty and early adolescence [22, 23] and in adulthood [21, 23] also differs as a function of prior intrauterine position in mice. Specifically, adult 0M females have shorter estrous cycles (4–5 days long) than those exhibited by 2M females (5–7 days long). In this study we examined whether maternal stress had a similar effect on the length of estrous cycles in 0M and 2M females; 1M females were not examined.

At 110 days of age (full adulthood), vaginal smears were examined daily for three weeks using 28 females from each of four groups: prenatally stressed and control, 0M females and 2M females. Vaginal smears were obtained by gently irrigating the vaginal orifice with physiological saline, and the tissue was examined under a microscope. The first smear with predominantly cornified cells (referred to as vaginal estrus), which followed a vaginal smear indicative of proestrus, was labeled as the first day of a cycle. If the length of time between proestrus-estrus smears exceeded 10 days, the animal was considered to be pseudopregnant (although a decidual test for pseudopregnancy was not conducted [35], and this "cycle" was not included in the data analysis. The mean length of all cycles (less than or equal to 10 days in length) exhibited by a female during the 3 weeks of vaginal smearing was calculated before comparing females from different groups.

Estradiol Radioimmunoassay

Serum (30 μl for fetuses and 60 μl for pregnant females) was extracted twice with 2 ml of a fresh (80:20) mixture of ethylacetate:chloroform. Solvent was also added to standard curve tubes. Tubes were dried under nitrogen, and 100 μl of a 1:300 000 dilution of sheep anti-estradiol-17β serum [36] and 100 μl of PBS containing 1% BSA were added to each tube. Tubes were incubated at 37°C for 5 min and then at 4°C for 1 h. Tubes were incubated an additional 15 h at 4°C with 100 μl of ^{125}I-estradiol-17β (diluted 1:40; 1.5–2.0 mCi/mg; Radioassay Systems Laboratories [RSL], Carson, CA). Bound and free steroids were separated by addition of a charcoal-dextran solution (0.8 ml) for 10 min, after which the tubes were centrifuged at 1 000 × *g* for 10 min. The supernatant was decanted and counted in a Micromedic 2/200 Gamma counter. The range of the standard curve was 0.25–20 pg/tube, and sensitivity of the assay was 0.5 pg/tube. The *y* intercept, slope, and correlation coefficients averaged 1.5, −2.0, and −.98, respectively.

Concentrations of estradiol in different volumes (12.5, 25, and 50 μl) of pooled serum from female mice on Day 18 of pregnancy were determined in each assay to examine the effect of serum volume and calculate intra- and interassay coefficients of variation. Assay of serial dilutions of sera yielded values parallel to the standard curve. Binding in blank tubes and in serum from ovariectomized females was indistinguishable from baseline binding. The intraassay and interassay coefficients of variation were both 7%. Cross-reactivity of the antibody with estrone was 12% [36].

Chromatography of samples was not required, since validation procedures yielded virtually identical values with and without purification by LH-20 chromatography. More specifically, serum collected from female mice on Day 18 of pregnancy was pooled, and duplicate aliquots of serum (20, 50, and 100 μl) were extracted as described. Before the assay for estradiol, half of the samples were resuspended in a mixture of isooctane:toluene:methanol (62:20:18, v:v:v) and transferred onto LH-20 columns following standard procedures recommended by Isolab, Inc. (Akron, OH). Concentrations of estradiol in chromatographed and nonchromatographed samples were compared. After correction for efficiency of extraction, column transfer, and fraction collection losses (as assessed by recovery of ^{3}H-estradiol in serum from gonadectomized mice), no difference was found in concentrations of estradiol with

(144 ± 24, 112 ± 10, and 112 ± 10 pg/ml for 20, 50, and 100 μl, respectively) and without (125 ± 9, 127 ± 7, and 116 ± 10 pg/ml for 20, 50, and 100 μl, respectively) chromatography (r = 0.99). Specifically, the y intercept, slope (log/logit), and correlation coefficients for the aliquots of extracted samples eluted off the LH-20 columns and assayed for estradiol were 3.4, −1.9, and −0.97, respectively. For the aliquots extracted and assayed without chromatography, corresponding values were 3.2, −2.0, and −0.98.

Testosterone Radioimmunoassay

Testosterone was extracted from serum (5–10 μl for fetuses and pregnant females) using the methods described for estradiol. First antibody (rabbit anti-testosterone; RSL) was diluted (1:225), and 100 μl was added to each tube, which was then incubated for 15 h at 4°C. ^{125}I-Testosterone (100 μl, diluted 1:80; 2–3 mCi/mg; RSL) was then added, and tubes were incubated for an additional 4 h at 25°C. Second antibody (goat anti-rabbit, 100 μl, diluted 1:11; RSL) was then added, and the tubes were incubated at 37°C for 2 h. Buffer (3 ml) was added to the tubes, which were centrifuged at 1 000 × g for 1 h. The supernatant was poured off, and the pellet was counted. The range of the standard curve was 2–128 pg/tube. Sensitivity of the assay was 4 pg/tube. Concentrations of testosterone in different volumes (1.25, 2.5, and 5.0 μl) of pooled serum collected from intact male mice were determined in each assay to examine volume effects and calculate intra- and interassay coefficients of variation. Assay of these different volumes of serum yielded values that were parallel to the standard curve (mean ± SEM; 5.7 ± 0.09, 5.5 ± 0.04, 5.7 ± 0.03 ng/ml for 1.25, 2.5, and 5.0 μl, respectively). Binding in blank tubes and in serum collected from gonadectomized male mice was indistinguishable from baseline binding. Intra- and interassay coefficients of variation were 3% and 10%, respectively. The only significant cross-reactivity of the antisera (as reported by RSL) was with DHT. We independently determined cross-reactivity with DHT to be 7%. It has previously been reported that chromatography is not required in assays of testosterone in mouse serum, even when antisera used have >70% cross-reactivity with DHT, due to low circulating levels of DHT in adult serum [37] and fetal serum [38].

Data Analysis

Data were analyzed by analysis of variance using the Statistical Analysis System (SAS), General Linear Model procedure. Comparisons of differences between group means were made using the LS means test in SAS. In all experiments, control and prenatally stressed females were examined at the same time, and an overall ANOVA was conducted using the data from all control and prenatally stressed animals to determine whether post hoc comparisons were warranted. The criterion for rejecting the null hypothesis was $p < 0.05$; rejection at greater levels of confidence is indicated.

RESULTS

Anogenital Distance and Body Weight at Birth

Overall ANOVA An interaction ($p < 0.05$) between maternal condition (stressed vs. control) and intrauterine position on anogenital distance at birth was observed among control and stressed 0M, 1M, and 2M females (Fig. 2). An analysis of covariance conducted on anogenital distance with body weight as the covariate showed that body weight did not account for a significant component of the variance in anogenital distance ($p > 0.1$), and there was still a significant interaction between intrauterine position and maternal stress ($p < 0.01$). Post hoc comparisons of the group means for anogenital distance of prenatally stressed vs. control females from each separate intrauterine position (for example, 2M control vs. 2M stressed females) revealed that only control 0M females and prenatally stressed 0M females differed significantly from each other ($p < 0.001$).

There was a 9.5% decrease ($p < 0.001$) in body weight at birth in all prenatally stressed female pups (overall mean

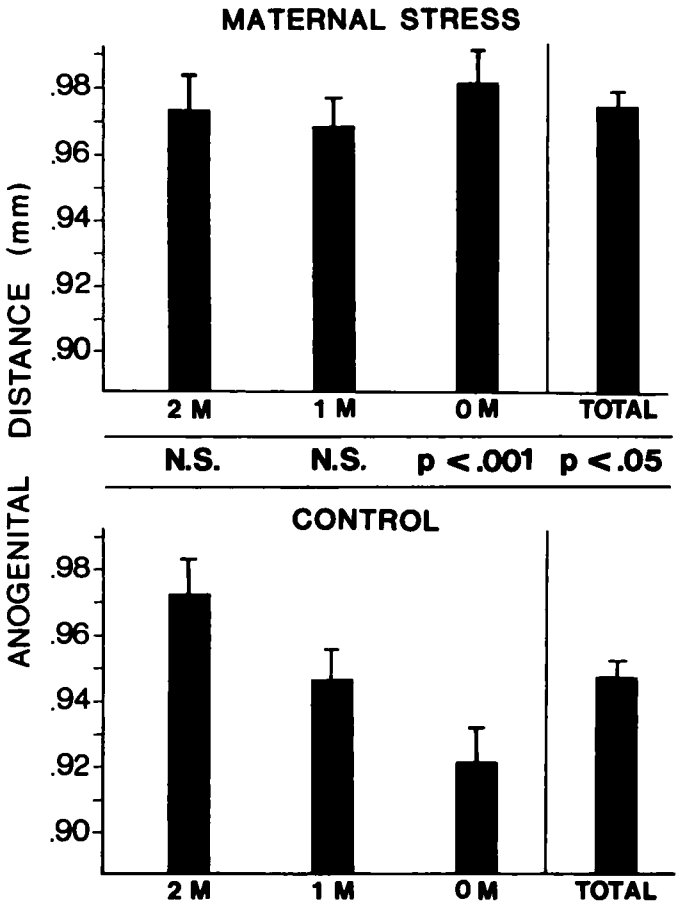

FIG. 2. Mean (+SEM) length of the space between the anus and the posterior aspect of the genital papilla at birth in 0M, 1M, and 2M female offspring of stressed and control mice. TOTAL refers to the combined mean for all 0M, 1M, and 2M females measured. Significance levels are for post hoc comparisons of prenatally stressed vs. control females from each intrauterine position (N.S. = not significantly different).

= 1.23 g) relative to controls (overall mean = 1.36 g) regardless of intrauterine position (Table 1). At 24 days of age, the mean body weight for prenatally stressed females (overall mean: 11.6 g) was slightly less than that for controls (overall mean: 11.9 g; $p = 0.07$). There was no effect ($p > 0.1$) of intrauterine position on body weight at 24 days of age for either prenatally stressed or control females (Table 1).

Control condition. The offspring produced in 44 control litters resulted in forty-three 0M females and forty-one 2M females. Although many more 1M females were delivered, forty-three 1M females were selected randomly for measurement of anogenital distance and body weight.

An ANOVA was conducted using data from only control females to examine any potential relationships between intrauterine position, body weight, and anogenital distance without the involvement of maternal stress. The analysis revealed an effect ($p < 0.01$) of intrauterine position on anogenital distance. Post hoc analysis revealed that the means for 0M and 2M females differed ($p < 0.01$), but neither group differed from 1M females. Body weight at birth for control females varied as a function of intrauterine position ($p < 0.01$). Post hoc analysis revealed no difference between 0M and 2M females in body weight at birth, whereas the 1M females randomly selected for inclusion in this study weighed less ($p < 0.01$) than either the 0M or 2M females (Table 1). Since body weight varied as a function of intrauterine position for control females, the anogenital distance data were subjected to analysis of covariance with body weight as the covariate. Body weight did not account for a significant component of the variance in anogenital distance ($p > 0.1$), and anogenital distance still varied as a function of intrauterine position ($p < 0.01$).

Maternal stress. Female offspring from 50 pregnant mice that had been stressed were examined. Anogenital distance at birth was determined for all 0M females (n = 37) and 2M females (n = 44), as well as for 44 randomly chosen 1M females. Analysis of variance on the anogenital distance data for prenatally stressed females revealed no effect of intrauterine position ($p < 0.1$); all female offspring from stressed mothers (0M, 1M, and 2M) were similar to control 2M females. Body weight did not differ as a function of intrauterine position in prenatally stressed females ($p > 0.1$).

TABLE 1. Body weight of control and prenatally stressed 0M, 1M, and 2M female mice at the time of cesarean delivery and at 24 days of age.

Group	Body weight (g)	
	Day of birth[ac]	Weaning[b]
Control		
0M	1.39 ± 0.02	12.0 ± 0.2
1M	1.31 ± 0.02	12.0 ± 0.2
2M	1.37 ± 0.02	11.8 ± 0.2
Stressed		
0M	1.22 ± 0.02	11.4 ± 0.2
1M	1.24 ± 0.02	11.8 ± 0.2
2M	1.24 ± 0.02	11.7 ± 0.2

[a]Control vs. Stressed: $F(1\ 246) = 76$; $p < 0.001$.
[b]Control vs. Stressed: $F(1\ 200) = 3.3$; $p = 0.07$.
[c]Control females (0M, 1M, and 2M): $F(2\ 124) = 4.7$; $p < 0.01$.

Length of Adult Estrous Cycles

Control condition. All control 0M and 2M females exhibited one to three complete cycles during the 21 days of the experiment. Females with only one complete cycle also exhibited one "cycle" longer than 10 days (this occurred for three 0M and six 2M females). The length of adult estrous cycles was shorter ($p < 0.01$) for control 0M females than for control 2M females (Fig. 3).

The 5-day estrous cycle of control 2M females was characterized by the vaginal smear sequence: proestrus, estrus, diestrus-1, diestrus-2, diestrus-3, proestrus, estrus. Cycles longer than 5 days typically had an additional day of diestrus; less frequently, cycles longer than 5 days were characterized by an additional day of cornified epithelial smears indicative of estrus. The 4-day cycle, which was shown predominantly by 0M females, was characterized by the sequence: proestrus, estrus to diestrus-1 transition, diestrus-1, diestrus-2, proestrus, estrus to diestrus-1 transition. Cycles exhibited by 0M females that were 5 days long showed the same vaginal smear sequence as 5-day cycles in 2M females.

Maternal stress. Four prenatally stressed 0M females and eleven prenatally stressed 2M females each exhibited one "cycle" longer than 10 days, which was not included in the

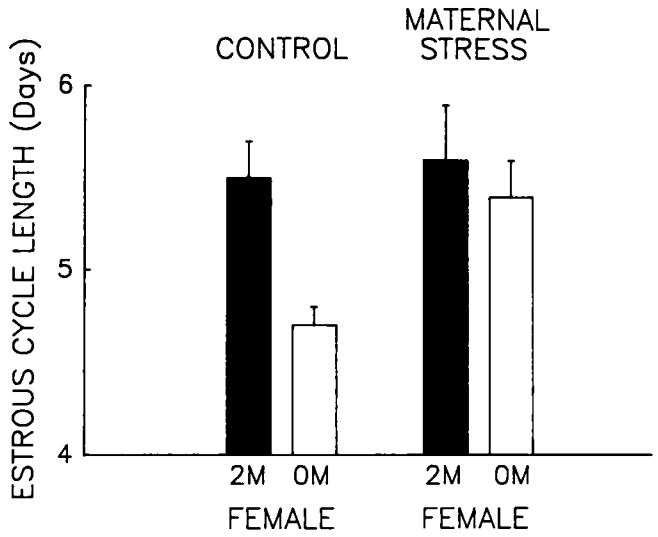

FIG. 3. Mean (+SEM) length (in days) of adult estrous cycles in 0M and 2M female mice delivered by cesarean section from control and stressed mothers. Vaginal smears were obtained daily for 3 weeks beginning at 4 mo of age; the day on which most cells were cornified was labeled as the first day of a cycle. The mean cycle length for each female during the 3-w period was used to calculate the group mean. 0M control females differed significantly from all other groups.

average cycle length for that female. The difference in the proportion of 0M vs. 2M prenatally stressed females exhibiting a cycle longer than 10 days was not statistically significant [$X^2(1) = 2.76, p = 0.1$].

Prenatal stress eliminated differences due to intrauterine position in the length of estrous cycles (Fig. 3). Both prenatally stressed 0M and 2M females resembled control 2M females in the length of estrous cycles in adulthood. The sequence of vaginal smear stages shown by both prenatally stressed 0M and 2M females was typical of the sequence of stages observed for control 2M females, namely, an extended diestrous phase of the cycle. All three groups (prenatally stressed 0M females, prenatally stressed 2M females, and control 2M females) differed ($p < 0.05$) from control 0M females. The interaction between maternal condition and intrauterine position was not significant ($p = 0.1$).

Serum Testosterone and Estradiol on Day 18 of Pregnancy

Results: overall ANOVA. An overall ANOVA was conducted on both serum testosterone and estradiol data from all control and prenatally stressed females, and both analyses revealed significant differences due to intrauterine position. Specifically, 2M female fetuses had greater ($p < 0.05$) serum concentrations of testosterone than did either 1M or 0M females, which did not differ from each other. In contrast, 0M females fetuses had greater ($p < 0.01$) serum concentrations of estradiol than did either 1M or 2M females, which did not differ from each other.

Control condition. Serum concentrations of estradiol in control pregnant dams (mean ± SEM; n = 37; 39.7 ± 2.0 pg/ml) were considerably lower than serum concentrations of estradiol in either male or female fetuses, reflecting the fetal source of estrogen binding protein (alphafetoprotein) and thus the higher concentrations of bound estrogen in fetuses [39]. In contrast, serum testosterone concentrations in control pregnant dams (1.8 ± 0.2 ng/ml) were similar to those in control female fetuses.

In control animals, there were sex differences in testosterone (combined mean ± SEM for 18 male serum pools = 4.8 ± 0.3 ng/ml vs. all 16 [0M, 1M, and 2M] female serum pools = 2.1 ± 0.1 ng/ml; $p < 0.001$) and estradiol (combined mean ± SEM for all 50 male serum pools = 90 ± 4 pg/ml vs. all 39 female serum pools = 113 ± 7 pg/ml; $p < 0.001$). Results for intrauterine position and maternal stress effects in male fetuses from these studies will be reported elsewhere.

The effect of intrauterine position on serum concentrations of both testosterone and estradiol in control female fetuses is shown in Figure 4. Serum concentrations of testosterone in control 2M female fetuses were greater ($p = 0.07$) than in 0M female fetuses, while concentrations in 1M female fetuses did not differ from either 0M or 2M females. Serum concentrations of estradiol in control 2M and 1M female fetuses did not differ, but both had lower ($p < 0.05$) serum concentrations of estradiol than did 0M female fetuses.

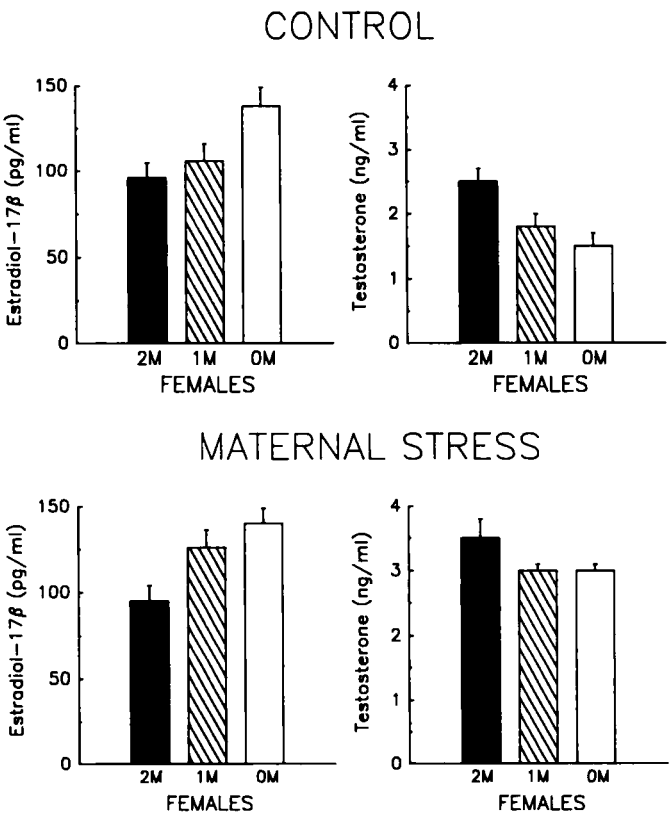

FIG. 4. Mean (+SEM) serum concentrations of estradiol-17β and testosterone in control and stressed 0M, 1M, and 2M female fetuses on Day 18 of pregnancy (Day 0 = mating).

These findings support the previous report by vom Saal and Bronson [12] that serum testosterone varies as a function of intrauterine position, with 2M female fetuses having greater concentrations than 0M female fetuses. Circulating estradiol differed as a function of intrauterine position, but the direction of the difference (0M female fetuses > 2M female fetuses) was opposite to that observed for testosterone.

The above results suggest that the intrauterine position phenomenon may be mediated by an interaction between testosterone and estradiol rather than by testosterone alone [12]. The ratio of testosterone to estradiol was more than two times greater in control 2M female fetuses than in control 0M female fetuses (Fig. 5).

Maternal stress. Maternal stress resulted in an increase ($p < 0.001$) in serum concentrations of testosterone in all females (Fig. 4). Post hoc comparisons revealed that testosterone increased ($p < 0.01$) in stressed vs. control females from each of the three intrauterine positions. Although serum concentrations of testosterone in 0M, 1M, and 2M stressed female fetuses did not differ significantly, the mean concentration for stressed 2M female fetuses was greater than the means for stressed 1M or 0M female fe-

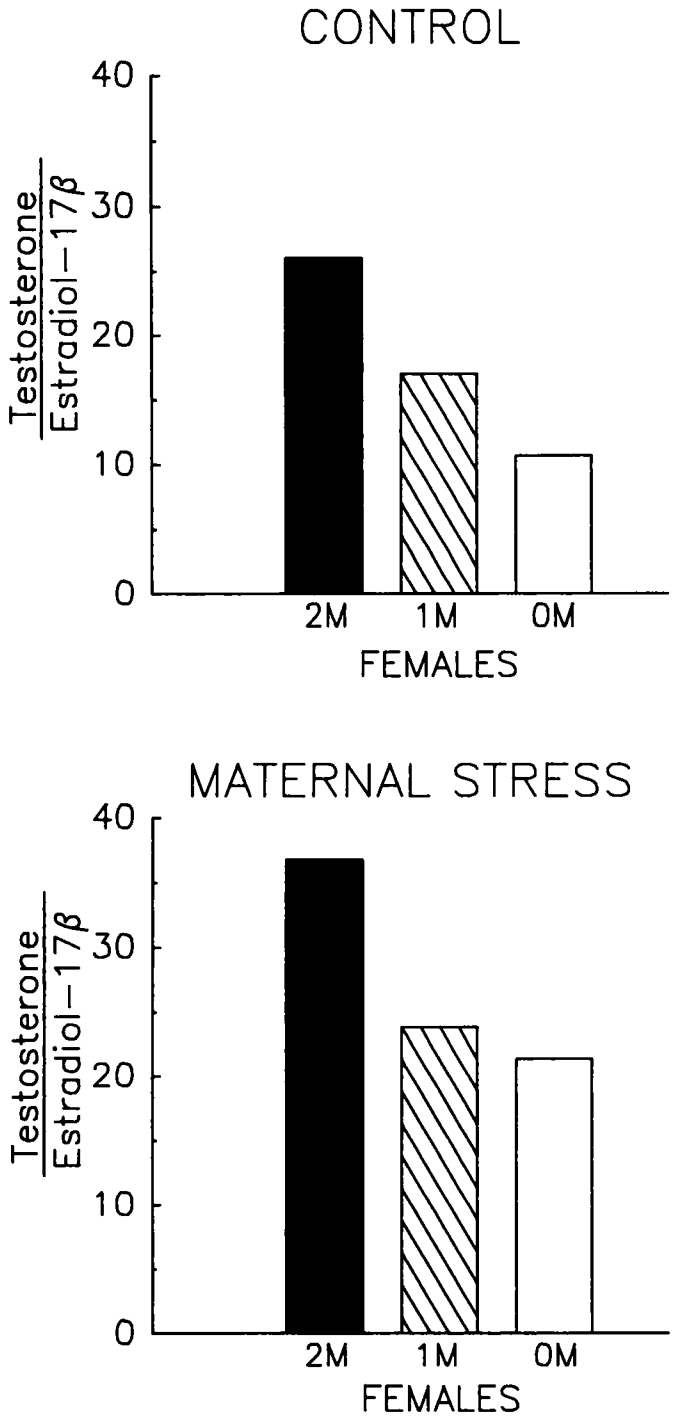

FIG. 5. Ratio of testosterone to estradiol in serum collected from control and stressed 0M, 1M, and 2M female fetuses on Day 18 of pregnancy. Ratios were calculated from the data in *Figure 4*.

tuses. Prenatally stressed males (without regard to intrauterine position) had greater ($p < 0.001$) serum concentrations of testosterone (n = 23; mean ± SEM = 5.6 ± 0.35 ng/ml) than prenatally stressed females (combined mean ± SEM for all 21 [0M, 1M, and 2M] female serum pools = 3.1 ± 0.14 ng/ml).

There was no effect ($p > 0.1$) of maternal stress on serum concentrations of estradiol in female fetuses (Fig. 4). As in control female fetuses, serum estradiol in stressed female fetuses varied as a function of intrauterine position ($p < 0.05$), with stressed 0M female fetuses having the greatest concentrations and stressed 2M female fetuses having the lowest concentrations of estradiol ($p < 0.01$). Concentrations of estradiol in 1M female fetuses were also greater ($p < 0.05$) than those in 2M female fetuses. Maternal stress eliminated the sex difference observed in serum estradiol in control fetuses, with all prenatally stressed males (n = 35; 118.8 ± 4.9 pg/ml) having similar concentrations ($p > 0.1$) to those observed in prenatally stressed females (combined mean ± SEM for 33 serum pools from prenatally stressed 0M, 1M, and 2M females = 124.1 ± 6.3 pg/ml).

The ratio of testosterone to estradiol was greater for all stressed female fetuses than for control female fetuses (Fig. 5), reflecting the fact that maternal stress increased circulating testosterone but not estradiol in females from all intrauterine positions. The ratio of testosterone to estradiol in serum from stressed 2M female fetuses was almost twice that in stressed 0M female fetuses.

Serum concentrations of estradiol were greater ($p < 0.05$) in pregnant females subjected to stress (n = 36; 46.1 ± 2.1 pg/ml) than in control pregnant females (n = 37; 39.7 ± 2.0 pg/ml). In contrast, the mean serum concentrations of testosterone in stressed pregnant females (1.8 ± 0.2 ng/ml) were identical to concentrations in control pregnant females (1.8 ± 0.2 ng/ml).

DISCUSSION

Maternal stress resulted in changes in postnatal reproductive traits (anogenital distance at birth and length of adult estrous cycles) in females positioned between two other female fetuses (0M females) in utero, whereas females positioned between two males (2M females) appeared unaffected by maternal stress. This unexpected finding led to the prediction that maternal stress would not have any effect on serum concentrations of testosterone in 2M female fetuses but would result in an increase in serum concentrations of testosterone in 0M female fetuses, which would explain the increased anogenital distance at birth and prolonged estrous cycles in prenatally stressed 0M females. All prenatally stressed female fetuses were thus predicted to be similar to control 2M females in their serum testosterone concentrations. In contrast to this prediction, 0M, 1M, and 2M female fetuses all experienced a significant increase of similar magnitude in serum concentrations of testosterone due to maternal stress. Furthermore, prenatally stressed 2M females exhibited an increase in serum concentrations of testosterone (as well as the ratio of testosterone to estradiol in serum) to concentrations that were similar to those observed in some control male fetuses, yet 2M females

showed no detectable change in the postnatal traits examined.

Being positioned between two female fetuses resulted in greater serum concentrations of estradiol in 0M female fetuses than in 2M female fetuses. It has been suggested that circulating estradiol may play a role in mediating some aspects of sexual differentiation [32, 40, 41]. Circulating estradiol in stressed pregnant females was increased by 15% relative to concentrations in control pregnant females. However, maternal stress had no effect on serum estradiol in female fetuses from any intrauterine position, and, as was the case for control 0M and 2M females, stressed 0M female fetuses had higher serum estradiol concentrations than did stressed 2M female fetuses. Thus, the masculinizing effect of maternal stress on the external genitalia and the defeminizing effect of maternal stress on the brain-pituitary-ovarian axis (estrous-cycle length) of 0M females but not 2M females could not be explained by changes in circulating testosterone or estradiol during fetal life, at least at the one time in pregnancy that these were examined in this study.

We have determined the effects of maternal stress on circulating testosterone during the last 4 days of fetal life in mice (F. vom Saal, unpublished observation). In these studies, female fetuses whose mothers were stressed by means of procedures similar to those described here experienced an increase in serum concentrations of testosterone on Days 17 and 18 of pregnancy, but not on Days 16 or 19 of pregnancy. These findings, which did not involve identifying the intrauterine position of fetuses, suggest that if maternal stress were acting on 0M vs. 2M female fetuses via differential changes in gonadal steroids, we should have been able to detect the differences at the beginning of Day 18 of pregnancy.

Other studies in which the anogenital distance of all female mice was increased to that characteristic of normal males by treatment of pregnant females with testosterone have shown that all female fetuses (including 2M females) can respond to elevated testosterone by exhibiting an increase in anogenital distance [42, 43]. Since both 0M and 2M female fetuses showed a similar increase in circulating testosterone due to maternal stress, the increase in anogenital distance at birth due to maternal stress in 0M females but not 2M females could be due to lower sensitivity of this tissue to testosterone in 2M females. One possibility is that the perineal tissue in 2M females has a lower activity of 5α-reductase. The reduction of testosterone to DHT is required for normal differentiation of the external genitalia. However, this hypothesis is contrary to findings suggesting that 2M females should have higher 5α-reductase activity, since 5α-reductase is positively correlated with circulating testosterone levels in fetuses [44]. The prediction of a positive correlation between serum concentrations of testosterone during fetal life, which are significantly higher in 2M than in 0M male mouse fetuses, and 5α-reductase activity during later life was confirmed by the finding that in both prostate and seminal vesicles, 5α-reductase activity was higher in 2M than in 0M adult male CF-1 mice [32; D. Nonneman, unpublished observation].

We confirmed findings from rats [2] and mice [3] that the length of estrous cycles in adulthood is increased in prenatally stressed females. However, we found no difference in the length of estrous cycles in prenatally stressed 2M females relative to control 2M females, whereas prenatally stressed 0M females showed cycles that were prolonged relative to control 0M females.

Our finding of an increase in serum testosterone in stressed female as well as male fetuses on Day 18 of pregnancy is consistent with other reports. Both maternal stress and administration of ACTH (to elevate maternal corticosterone secretion, similar to that induced by maternal stress) to pregnant rats result in an increase in circulating androstenedione (testosterone was not measured) in mothers and in male and female fetuses [45]. Ward and Weisz [46] reported that maternal stress results in a significant increase in serum concentrations of testosterone on Day 17 of pregnancy in male rat fetuses; however, maternal stress also resulted in a decrease in serum concentrations of testosterone on Day 18 of pregnancy in male rat fetuses. Ward and Weisz [46] suggested that maternal stress interfers with differentiation of sexual behavior in male rat fetuses by not allowing testosterone to reach peak concentrations on Day 18, which they proposed to be the critical day in prenatal development for the masculinization of male sexual behavior by testosterone.

An important aspect of our findings that needs to be considered when interpreting the results of prior studies, such as that of Ward and Weisz [46], is that if we had not conducted these experiments with females from different intrauterine positions, the erroneous conclusion that changes in reproductive traits in female mice due to maternal stress were predicted by changes in serum testosterone concentrations during fetal life would have been drawn. For example, maternal stress resulted in a significant increase in serum testosterone concentrations in all female fetuses on Day 18 of pregnancy, and even if we had not known the intrauterine position of females, we would have found a significant increase in anogenital distance at birth, indicative of masculinization (Fig. 2; see Total for maternal stress vs. control females), and a lengthening of adult estrous cycles, indicative of defeminization (Fig. 3). The obvious hypothesis would have been that changes in masculinization and defeminization were due to changes in serum testosterone concentrations during fetal life. By examining 0M, 1M, and 2M females, we observed that the effects of maternal stress on postnatal reproductive traits were not predicted by changes in serum testosterone on Day 18 of fetal life: testosterone levels increased in all female fetuses, but only 0M females exhibited postnatal changes in reproductive traits.

There are also findings in addition to ours that show that female rat and mouse fetuses are masculinized and defem-

inized as a result of maternal stress, although this appears to depend on genotype in mice [5]. Specifically, treatment of pregnant rats with ACTH results in an increase (relative to controls) in anogenital distance at birth in female offspring [47]. In another study, intruder males and females were added to cages containing pregnant wild mice, which resulted in fighting (a natural form of stress) during the time of prenatal sexual differentiation. Anogenital distance at birth was longer in female offspring from dams that fought than in female offspring from dams that did not fight during pregnancy [48]. Kinsley and Bridges [6] reported that the latency to exhibit maternal behavior when exposed to pups is longer (indicative of defeminization) in prenatally stressed female rats than in control females, and prenatally stressed females are similar to control males in their behavior. In rats, males have a lower threshold to pain than females, and prenatally stressed females exhibit a lower pain threshold (again, similar to that of control males) than do control females; prenatally stressed female rats are also similar to control males in that they exhibit greater analgesia after treatment with morphine than do control females [8]. In both of these studies by Kinsley and colleagues, prenatally stressed females resemble control males but differ markedly from control females for the traits examined.

A shift as a result of maternal stress in the reproductive characteristics of 0M and 1M prenatally stressed females toward traits normally shown by control 2M females is consistent with our finding of a general shift due to maternal stress toward traits characteristic of males and away from traits characteristic of females. What remains unexplained is the basis of the variation based on intrauterine position in the phenotypic response of female fetuses to maternal stress and the absence of a correlation between changes in circulating gonadal steroid concentrations during fetal life and changes in postnatal reproductive traits due to maternal stress. Why an increase in both the length of the tissue separating the anus and genital papilla at birth and the length of adult estrous cycles occurred in prenatally stressed 0M female mice but not 2M females is thus unknown, but these two findings may be mediated by different mechanisms. For example, there is evidence linking the endogenous brain opioid system to sex differences in neuroendocrine function and behavior, as well as effects of maternal stress [8, 49–52]. Elongation of the tissue between the anus and genital papilla in mice is influenced by changes in prostaglandin metabolism [53]. Changes other than those in gonadal steroid concentrations thus appear to be involved in mediating the different effects of maternal stress on 0M vs. 2M female fetuses.

The absence of the expected relationship between changes in either serum testosterone or estradiol concentrations during fetal life and changes in any postnatal reproductive trait in prenatally stressed 0M, 1M, and 2M female mice does not rule out some involvement in these steroids in mediating effects of maternal stress in females. It is possible that the high serum concentrations of testosterone and low concentrations of estradiol observed in control 2M females in some manner serve to attenuate the response of a variety of systems to maternal stress in female fetuses. If this is true, these findings may have important implications not only for litter-bearing species but also for single-birth species, such as humans, where there is considerable variation of unknown origin in circulating levels of gonadal steroids among female fetuses [54]. Even in single-birth species, female fetuses with the lowest serum concentrations of testosterone and the highest serum concentrations of estradiol might be the most likely to show changes in morphological, physiological, and behavioral traits as a result of maternal stress, similar to 0M female mice.

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