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Responsiveness to Testosterone of Male Gerbils From Known Intrauterine Positions

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CLARK, M. M., A. M. BISHOP, F. S. VOM SAAL AND B. G. GALEF, JR. *Responsiveness to testosterone of male gerbils from known intrauterine positions.* PHYSIOL BEHAV **53**(6) 1183–1187, 1993.—Following either a) castration or b) both castration and implantation with capsules releasing a constant, physiological dose of testosterone, adult male Mongolian gerbils that had matured in intrauterine positions between two male fetuses still scent marked with greater frequency than did male gerbils that had matured in intrauterine positions between two female fetuses. We also found significant positive correlations between the relative frequency of scent marking exhibited by individual male gerbils when intact, after castration and after both castration and implantation with capsules releasing testosterone. Each of these findings is consistent with the view that differential exposure to testosterone, as a consequence of fetal intrauterine position, has lasting effects on the organization of scent-marking by male gerbils.

Intrauterine position Testosterone Scent marking Mongolian gerbils

THE intrauterine position occupied by a fetal male Mongolian gerbils (*Meriones unguiculatus*) influences its plasma testosterone levels both in infancy and in adulthood. Those male gerbils (2M males) that, as infants, resided in intrauterine positions between two male fetuses have significantly higher levels of plasma testosterone than do those male gerbils (2F males) that resided in intrauterine positions between two female fetuses (2,4).

Some patterns of behavior, like some morphological features of adult male gerbils, vary as a function of circulating testosterone levels (8,10). As one might expect, given both the relatively elevated levels of testosterone found in adult 2M as compared with adult 2F male gerbils (4) and the sensitivity of scent-marking frequency to testosterone (8), 2M males scent mark more frequently than do 2F males. However, the different levels of plasma testosterone found in adult 2M and 2F male gerbils may not be the sole cause of this difference in frequency of scent marking. Fetal intrauterine position might have effects on development of response to testosterone that could act in concert with differences in adult hormone levels to affect androgen-sensitive aspects of adult behavioral phenotypes.

In a series of experiments analyzing causes of variability in scent-marking frequencies exhibited by male Mongolian gerbils, Turner (11) found that varying levels of perinatal exposure to testosterone propionate (TP) resulted in different levels of responsiveness to TP in adulthood. Male gerbils that had received an injection of 100 μg of TP as infants scent marked at frequencies that were both similar to those exhibited by intact control males and significantly higher than those exhibited by males that, as infants, had received each of four lower doses of TP.

It seemed reasonable to hypothesize that the different amounts of natural perinatal exposure to testosterone experienced by 2M and 2F male gerbils would produce differences in their responses to testosterone when adult similar to those that Turner induced using experimental manipulations of perinatal exposure to testosterone.

In the present experiment, designed to parallel Turner's 1984 study, we determined whether 2M male gerbils would continue to exhibit higher frequencies of scent marking than would 2F male gerbils:

1. when both 2M and 2F males were castrated and, thus, were no longer exposed to significant amounts of endogenous testosterone and
2. after castrated 2M and 2F males were implanted with identical Silastic capsules releasing physiological levels of testosterone.

METHOD

Subjects

Forty-six male Mongolian gerbils (*Meriones unguiculatus*), caesarian delivered from, and foster reared by, females born and raised in the vivarium of the McMaster University Psychology Department (Hamilton, Ontario), served as subjects. All subjects were third or fourth generation descendants of breeding stock

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acquired from Tumblebrook Farms (Brookfield, MA). All were maintained throughout life with ad lib access to Purina Rodent Laboratory Chow #5001 and water.

Procedure

Breeding and maintenance. When 90 to 100 days of age, virgin female gerbils were first weighed, then placed individually in the home cages of reproductively proven males. Breeding pairs were maintained in polypropylene cages (35 × 30 × 15 cm) housed in a temperature- and humidity-controlled colony room that was illuminated on a 12-h light/dark schedule (light onset at 0500 h).

The day on which each breeding pair first mated was determined by observation (pairs always began mating between 1300 and 1500 h), and pair members were separated 2 weeks later, when females were conspicuously pregnant.

Caesarian delivery and foster rearing. Twenty-four days after an observed copulation (i.e., 1 day before anticipated vaginal delivery), each female that had gained weight at a rate consistent with her impregnation on the day that she was seen mating was anesthetized by ether inhalation, her abdomen opened, her uterus externalized, and her fetuses removed one at a time.

The gender of each fetus was determined on the basis of its anogenital distance (1,7), and the position of each fetus in its dam's uterus was recorded (14). We classified those male fetuses located in intrauterine positions between two male fetuses as 2M males and those male fetuses located in intrauterine positions between two female fetuses as 2F males (3).

Once all fetuses had been removed from a dam, and the gender and intrauterine position of each had been recorded, the dam was euthanized by anesthetic overdose.

We toe clipped each infant shortly after delivery for permanent identification and then, using the procedures of Clark and Galef (1), fostered each infant to a gerbil dam that had delivered a litter vaginally on the day of caesarian delivery of the pups she was to foster rear.

Pups were weaned on day 30 postpartum (day of birth = day 1) and were left undisturbed in same-sex groups of four to six until they were 90 days of age when each male was transferred to an individual cage. Subjects remained in isolation from the time they were 90 days old until the end of the experiment.

Experimental treatments. Each subject (20 2M males and 26 2F males) was anesthetized twice: first, when 120 to 130 days old and again 40 days later. On both occasions, anesthesia was accomplished by intraperitoneal injection of sodium pentobarbitol (30 mg/kg), supplemented, when necessary, with ether.

During the first period of anesthesia, all 46 subjects were castrated, and immediately after castration, 30 subjects (12 2M males and 18 2F males) were each implanted subcutaneously, at the base of its neck, with a single, 7 mm long Silastic capsule (Dow–Corning Silastic tubing: 0.125 cm outside diameter, 0.06 cm inside diameter, Catalogue # Dow 602-285), capped at each end with Dow–Corning Silastic Type A Adhesive and containing a 5 mm long (0.01 cc) column of crystalline testosterone (Steroloids, Wilton, NH). Each implant was incubated in isotonic saline solution for at least 48 h before it was used. Sixteen subjects (eight 2M males and eight 2F males), those assigned to a sham-operated control group, were shaved, incised, and sutured, but did not receive implants.

During the second period of anesthesia, when each subject was 150 to 160 days of age, a sample of blood was taken from its intraorbital sinus. Blood samples were collected in heparinized capillary tubes, centrifuged for 20 min at −4°C, and the plasma stored at −110°C for later assay.

Testosterone radioimmunoassay. All blood samples were analyzed blind using procedures similar to those described in detail in (15). In brief, testosterone was extracted twice from 50 μl of serum with 2 ml of a fresh (80:20) mixture of ethylacetate:chloroform. Solvent was also added to standard curve tubes, which were dried under nitrogen. Extracted steroids were reconstituted in 1000 μl of methanol, and 200 μl was transferred to a second tube, after which the tubes were dried under nitrogen. Each sample was thus assayed in duplicate tubes representing 40 μl and 10 μl of serum, due to the expectation of individual variability in testosterone concentrations.

First, antibody (rabbit antitestosterone; ICN Biochemical) was added to each tube, which was then incubated for 15 h at 4-1/2°C. ^{125}I-Testosterone (2–3 mCi/mg; ICN Biochemical) was then added, and the tubes were incubated for an additional 4 h at 25-1/2°C. Second, antibody (goat antirabbit; ICN Biochemical) was then added, and the tubes were incubated at 37-1/2°C for 2 h. Buffer (3 ml) was added to the tubes, which were centrifuged at 1000 × g for 1 h. The supernatant was poured off, and the pellet was counted.

The range of the standard curve was 2 to 128 pg/tube. Sensitivity of the assay was 4 pg/tube. Concentrations of testosterone in different volumes (5, 10, and 25 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. Binding in blank tubes and in serum collected from gonadectomized male mice was indistinguishable from baseline. Intra- and interassay coefficients of variation based on these assays were 2.1% and 9.0%, respectively. The only significant crossreactivity of the antisera was with 5a-dihydrotestosterone (7.0%).

Behavioral testing. Scent marking was assessed in each of the 46 subjects three times: first, before surgical intervention, when each subject was 100 to 120 days old, and again both 24 days and 38 days after each subject had been castrated.

Scent-marking frequency was measured on each occasion using a modified version of a procedure developed by Thiessen, Friend, and Lindzey (1968) (8). Each male was observed for 5 min/day, for 5 consecutive days, in a 92 × 92 cm test arena with an opaque, white Plexiglas floor and shellacked wooden walls 62 cm high. The arena floor was divided by black lines painted on the floor surface into 16 squares (each 23 × 23 cm). A black, 1/2 cm high Plexiglas peg (1 × 2 cm) was attached to the floor at each of the nine points of intersection of the painted lines.

To begin a test session, a subject's cage was removed from the colony room and placed in the room containing the test arena. Two hours later the subject was removed from its cage and placed in a corner of the arena, facing the arena center. During the next 5 min, an observer, unaware of either the intrauterine position in which a subject had matured or its hormonal state, recorded the number of times the subject scent marked. Scent marking was defined as an active lowering of the belly and dragging of the ventral scent gland across either a peg or the floor of the arena (8).

Subjects were tested twice after surgery to be sure that their frequencies of scent marking had stabilized following surgical intervention. In fact, we found no significant differences in the scores of subjects 24 and 38 days after surgery. Consequently, we used each subject's mean score on the two tests as the measure of its frequency of scent marking.

At the end of each 5-min test session, each subject was returned to its home cage and transported back to the colony room. The arena was then cleaned with an 80% alcohol solution and rinsed with distilled water before the next subject was tested.

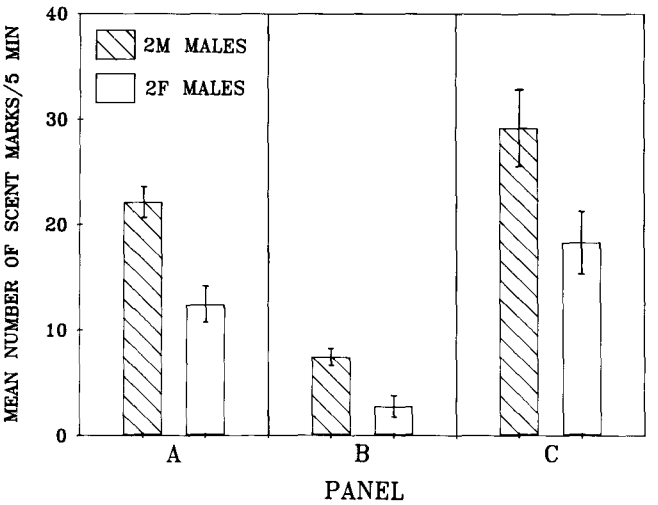

FIG. 1. Mean number of scent marks/5 min exhibited by 2M and 2F adult male gerbils: (panel A) before castration, (panel B) after castration, and (panel C) after castration and receipt of a subcutaneous testosterone implant. Flags = ±1 SEM.

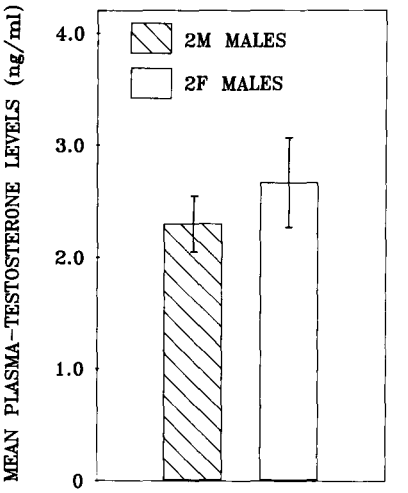

FIG. 2. Mean plasma-testosterone levels of 2F and 2M male gerbils following castration and receipt of a subcutaneous testosterone implant. Flags = ±1 SEM.

RESULTS

Scent-Marking Frequency

Figure 1 presents data describing the frequency of scent-marking behavior exhibited by subjects before (panel A) and after (panels B and C) surgical intervention. Analysis of variance revealed significant effects of both intrauterine position, $F(1, 42) = 8.51$, $p < 0.006$, and presence of capsules, $F(1, 42) = 8.79$, $p < 0.006$, on frequency of scent marking, but no significant interaction, $F(1, 42) = 0.17$, p = NS.

Inspection of panel A in Fig. 1 shows that, as we have reported previously (3,4), in the absence of surgical intervention the intrauterine positions occupied by male gerbils as fetuses affects their scent-marking behavior as adults. Intact 2M males scent marked with significantly greater frequency than did intact 2F males [Student's t test, $t(44) = 3.44$, $p < 0.002$].

The data presented in panel B indicate, as has been found before (6,8–12), that castration markedly reduced the frequency of scent marking by male Mongolian gerbils [matched t tests; $t(15) = 6.74$, $p < 0.002$] but did not totally abolish the behavior (6,8). These data show also that, even following castration, 2M males continued to scent mark with greater frequency than did 2F males [Student's t test; $t(14) = 4.21$, $p < 0.001$].

As can be seen in panel C, after castration and placement of testosterone implants, 2M males still exhibited significantly higher frequencies of scent marking than did 2F males [Student's t test, $t(28) = 2.07$, $p < 0.04$].

Plasma Testosterone Levels and Scent-Marking Frequency

The data presented in Fig. 2 show that there was no difference in the levels of testosterone found in the plasma of 2M and of 2F males 40 days after they received their implants of testosterone [Student's t test, $t(28) = 1.26$, p = NS]. The mean plasma testosterone concentration found in our 30 subjects implanted with testosterone (mean = 2.51 ± 0.25 ng/ml) was somewhat higher than mean testosterone concentrations reported for intact 2M males by Clark, vom Saal, and Galef (4), but quite similar to mean testosterone concentrations reported by Probst (6) for an unselected sample of 12, 5- to 6-month-old male gerbils.

As expected, plasma testosterone levels of castrated males that did not receive testosterone implants were all low (mean = 0.14 ± 0.03 ng/ml), and there was no difference between the mean plasma testosterone levels found in castrate 2M (mean = 0.19 ± 0.05 ng/ml) and castrate 2F (mean = 0.11 ± 0.02 ng/ml) males [Student's t test, $t(14) = 1.83$, p = NS].

To be certain that subtle differences in plasma testosterone levels of 2F and 2M gerbils implanted with testosterone capsules were not responsible for observed differences in scent-marking frequencies exhibited by 2F and 2M males, we also compared scent marking by members of seven pairs of gerbils. Each pair consisted of one 2F male and one 2M male that were matched as closely as possible for plasma-testosterone level. Our criterion for selecting pair members was severe; the members of each pair could differ in their plasma testosterone levels by no more than 0.20 ng/ml (in fact, no pair members differed by more than 0.16 ng/ml, and the mean absolute value of the difference between pair members in circulating testosterone level was 0.10 ng/ml).

As can be seen in Table 1, even with plasma-testosterone levels controlled, 2M males continued to scent mark with significantly greater frequency than did 2F males [matched t test, $t(6) = 3.66$, $p < 0.01$].

Individual Differences in Scent-Marking Frequency

Table 2 presents Pearson's product moment correlations between individual subjects' scent-marking scores before surgical

TABLE 1

MEAN NUMBER OF SCENT MARKS EXHIBITED/5 min BY SEVEN PAIRS OF MALE GERBILS MATCHED FOR PLASMA TESTOSTERONE LEVELS

	2M	2F	t
Testosterone (ng/ml)	1.90 ± 0.20	1.92 ± 0.18	0.28
Scent marks/5 min	32.8 ± 4.8	12.7 ± 4.1	3.66*

Values are mean ± SE.
* Matched t-test, $p < 0.01$.

TABLE 2

CORRELATIONS BETWEEN INDIVIDUAL SUBJECTS' SCENT MARKING SCORES BEFORE SURGICAL INTERVENTION AND EITHER AFTER CASTRATION OR AFTER BOTH CASTRATION AND RECEIPT OF A TESTOSTERONE IMPLANT

Castration	
All subjects (n = 16)	0.87*
2M subjects (n = 8)	0.74†
2F subjects (n = 8)	0.84*
Castration and implant	
All subjects (n = 30)	0.90‡
2M subjects (n = 12)	0.77*
2F subjects (n = 18)	0.94‡

* $p < 0.01$.
† $p < 0.05$.
‡ $p < 0.0001$.

intervention and following either castration or both castration and receipt of a testosterone implant.

Looking across all 30 subjects that received implants, those individuals that scent marked relatively frequently before the start of experimental manipulations continued to do so after castration and insertion of testosterone implants. This positive correlation could simply reflect the stable differences in frequencies of scent marking by 2M and 2F male gerbils that are described in the preceding section. Probst (6) reported similar stability in the relative frequency with which 12 male gerbils from unknown intrauterine positions scent marked when exposed to different numbers of testosterone implants.

More interesting is the finding, also presented in Table 2, of highly significant correlations, both within 2M males and within 2F males, between individuals' scent-marking scores before surgical intervention and after castration and receipt of a testosterone implant. There was no correlation between these individuals' relative plasma-testosterone levels and their relative frequencies of scent marking (Pearson's r: 2M males, $r = -0.16$; 2F males, $r = -0.09$; both p = NS).

Table 2 also shows correlations between scent marking scores, before and after surgical intervention, of the 16 gerbils that were castrated and did not receive testosterone implants. Once again, we found significant correlations between individuals' relative scent-marking frequencies:

1. across all 16 subjects,
2. within 2M males and
3. within 2F males.

And, once again, we found no correlation between an individual's relative plasma testosterone level and its relative frequency of scent marking (Pearson's r: 2M males, $r = 0.48$; 2F males $r = 0.01$; both p = NS).

In general, we found consistency in the relative frequencies with which individual males scent marked that was both uncorrelated with their circulating levels of testosterone at the time that measurement of scent-marking frequency took place and correlated with fetal intrauterine position. The data thus demonstrate a role of fetal intrauterine position in determining the scent-marking frequencies exhibited by male gerbils independent of any effects of intrauterine position on testosterone levels in adulthood.

DISCUSSION

The stable differences in scent-marking frequency exhibited by 2M and 2F male gerbils, both when castrated and when castrated and exposed to equivalent amounts of testosterone, provide evidence that factors other than differences in adult plasma-testosterone levels contribute to previously reported differences in scent-marking frequency exhibited by adult 2M and 2F male gerbils (4). Intrauterine position affects not only levels of exposure to testosterone (2,4), but subsequent response to testosterone as well. Perhaps perinatal exposure to testosterone acts directly on scent-marking frequency by affecting organization of tissues that mediate scent marking in adult male Mongolian gerbils (5,13). Perhaps effects of intrauterine position on scent-marking frequency are relatively indirect. Determination of the way in which intrauterine position modulates scent-marking frequency in adulthood will require technical innovations that allow gerbil fetuses to be caesarian delivered, castrated and accepted by foster dams.

Although the present experimental design does not permit precise determination of the way in which intrauterine position affects adult levels of scent marking by male gerbils, in the absence of the evidence presented here, the higher circulating levels of testosterone found in 2M than in 2F adult male Mongolian gerbils might be seen as the sole cause of the different frequencies of scent-marking exhibited by 2M and 2F male gerbils. The present results suggest, to the contrary, that effects of prior exposure to different levels of testosterone on later response to testosterone may play an important role in determining the frequencies of scent marking exhibited by adult male Mongolian gerbils from different intrauterine positions.

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