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Intrauterine Positions and Testosterone Levels of Adult Male Gerbils Are Correlated

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CLARK, M. M., F. S. VOM SAAL AND B. G. GALEF, JR. *Intrauterine positions and testosterone levels of adult male gerbils are correlated.* PHYSIOL BEHAV **51**(5) 957–960, 1992.—Those male Mongolian gerbils (*Meriones unguiculatus*) that developed in intrauterine positions between two male fetuses had significantly higher levels of serum testosterone, as adults, than did those adult male gerbils that developed in intrauterine positions between two female fetuses. The endogenous testosterone levels of adult male gerbils were significantly positively correlated with both the sizes of their ventral scent glands and their frequencies of scent marking. We found no evidence of pulsatile release of testosterone in adult male gerbils.

Intrauterine position Testosterone Scent marking Mongolian gerbils

THE intrauterine position occupied by a male Mongolian gerbil (*Meriones unguiculatus*) while in its dam's uterus has observable effects on both its adult behavior and its adult morphology (5). Those male gerbils that mature in intrauterine positions between two male fetuses (2M males), as adults, are more attractive to estrus female gerbils, are more efficient copulators, and are significantly more successful in impregnating female gerbils than are those male gerbils that mature in intrauterine positions between two females fetuses (2F males) (5,6). Further, and of greater relevance to the experiment described here, adult 2M male gerbils scent mark more frequently, have greater anogenital distances, larger ventral scent glands, and heavier testes than do 2F male gerbils (5; see also 2,3).

Both frequency of scent marking by, and size of ventral scent glands of castrate male gerbils increase with exposure to increasing amounts of exogenous testosterone (1,12,13,18). We, therefore, hypothesized that adult 2M male gerbils (that both scent mark more frequently and have larger ventral scent glands than do adult 2F male gerbils) would exhibit higher circulating levels of endogenous plasma testosterone than would adult 2F male gerbils.

Probst (9) has examined the relationship between scent marking frequency and endogenous plasma testosterone levels in adult male Mongolian gerbils. Although he found a positive correlation between scent marking frequency and testosterone titer ($r = 0.29$), the correlation was not statistically significant. We were, however, encouraged to pursue the matter further for two reasons: first, the number of subjects ($n = 12$) in Probst's experiment was small, and it seemed possible that a significant correlation between testosterone level and behavior might be found simply by using a larger sample of subjects than Probst had used. Second, Probst (9, p. 365) found that, over months, "testosterone concentrations obtained by repeated blood sampling from the same individuals were more or less constant in male gerbils." This observation (9,10) suggested to us that correlations between testosterone titers and behavior or morphology in male gerbils might not be obscured by episodic release of testosterone (7,8). In males of many other mammalian species, episodic release of testosterone has made meaningless attempts to correlate frequency of occurrence of various behaviors with amounts of endogenous testosterone found in single samples of blood (8).

The present experiment was undertaken to determine: (i) whether adult 2M male gerbils do, in fact, have higher plasma levels of endogenous testosterone than do adult 2F male gerbils and (ii) whether the endogenous plasma testosterone levels of adult male gerbils correlate with the sizes of their ventral scent glands and the frequencies with which they scent mark.

METHOD

Subjects

Fifty-seven 11- to 13-month old Caesarean-delivered, male Mongolian gerbils born in the vivarium of the McMaster University Psychology Department to stock females, descendants of animals acquired from Tumblebrook Farm (Brookfield, MA), served as subjects. Twenty-six of the subjects (Sample 1: 13 2F males and 13 2M males), born between August and September of 1987 and sacrificed by anesthetic overdose and cardiac puncture in August 1988 (when 11 to 12 months of age) were used to assess both the reliability of testosterone assays carried out on adult male gerbils and the likelihood of finding effects of fetal intrauterine position on adult male testosterone levels. The remaining 31 subjects (Sample 2: 16 2F males and 15 2M males),

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born between December and March of 1989 and sacrificed by decapitation while under light ether anesthesia in February of 1991 (when 11 to 14 months of age) were the main subjects in the present experiment.

Males in Sample 2 had each been bred at least eight times before they were sacrificed, and their last pairing with females occurred 2 weeks before their sacrifice. Subjects in Sample 1 had been bred only once or twice, the last time 3 to 4 months before their sacrifice.

Procedures

Breeding and maintenance. All subjects, their dams, and foster dams were maintained throughout their lives on ad lib Purina Rodent Laboratory Chow #5001 and water in a temperature- and humidity-controlled colony room illuminated on a 12:12 h light:dark schedule (light onset at 0500 h).

When 90 to 100 days of age, virgin female gerbils were weighed and each placed with a proven male in a 35 × 30 × 15 cm polypropylene shoe-box cage. The date on which each breeding pair first mated was determined using time-lapse video recording, and pair members were separated 2 weeks after mating, when females were conspicuously pregnant.

Surgery and foster rearing. Twenty-four days after 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 of observed copulation was anesthetized by ether inhalation; her abdomen was opened, her uterus was externalized, and the fetuses removed. The gender of each fetus was determined on the basis of its anogenital distance (11) and its position in its dam's uterus was recorded (15). Each infant was toe-clipped for permanent identification and was then placed in a chamber of an ice-cube tray floating in a constant temperature bath maintained at 31°C. After all fetuses had been removed from a dam, she was euthanized by anesthetic overdose.

Fostering Caesarean-delivered pups. Recently parturient Mongolian gerbils do not readily accept Caesarean-delivered (C-d) foster young and will usually neglect, attack, or cannibalize C-d pups placed in their nests. The procedures we used to successfully foster C-d gerbil pups were developed by trial and error over a period of months.

Following delivery, each pup in a C-d litter was maintained in an individual compartment in the constant-temperature bath for 3–4 hr. Each was then rubbed with a piece of cotton batting that had first been rubbed on the ventral scent gland of the female to which it was to be fostered. All foster mothers had vaginally delivered a litter within 24 h of the time of Caesarian delivery of the litter which they were to rear.

Classification of uterine positions. We classified those male fetuses located between two female fetuses as 2F males and those male fetuses located in uterine positions between two male fetuses as 2M males.

Testing scent marking. Scent-marking frequency was assessed in the 31 subjects in Sample 2 using a modification of the procedures developed by Thiessen, Friend, and Lindzey (12). Each male was observed for 5 min per day, for 4 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 into 16 squares (23 × 23 cm) by black lines painted on the floor surface. A black, 0.5-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 (where subjects had been maintained since birth) and placed in the room containing the test arena. Two hours later, the subject was removed from its cage and placed facing the arena center in a corner of the arena. During the next 5 min, an observer, unaware of the intrauterine position that a subject had occupied as a fetus, recorded the number of instances of scent marking exhibited by the subject. Scent marking was defined as an active lowering of the belly and dragging of the ventral gland pad across a peg or the floor of the arena (12,14). Scent marking was easily discriminated from both normal locomotion and perineal dragging (14).

At the end of the 5-min test session, the 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. Each subject was assigned a single scent-marking score equal to the mean number of scent marks it deposited per 5-min session in the test arena.

Physical measurements. Five days after each subject's scent-marking test was completed, it was weighed and lightly anesthetized with ether; its ventral surface was shaved and the maximum length and width of its ventral scent gland was measured by an observer who was unaware of its fetal intrauterine position. Twenty-four hours later, each subject was again lightly anesthetized with ether and decapitated; its blood was collected for assay, and its testes were removed and weighed.

Testosterone radioimmunoassay. All blood samples were analyzed using the radioimmunological technique described in detail in (17). 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 50 μl of methanol, and 10 μl was transferred to a second test tube, after which the tubes were dried under nitrogen. Each sample was thus assayed in duplicate tubes representing 10 μl and 40 μl of serum, due to the expectation of individual variability in testosterone concentrations.

The first antibody [rabbit anti-testosterone; Radioassay Systems Laboratory, Carson, CA (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 the tubes were incubated for an additional 4 h at 25°C. The 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 (PBS, pH 7.2–7.4, 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 2–128 pg/tube, and the sensitivity of the assay was 4 pg/tube. Concentrations of testosterone in different volumes (5, 25, and 50 μl) of pooled serum collected from intact male mice were determined in each assay to examine volume effects, and to 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 5, 25, and 50 ml, respectively). Binding in blank tubes and in serum collected from gonadectomized male mice was indistinguishable from baseline. Intra- and interassay coefficients of variation were 3% and 10%, respectively. The only significant crossreactivity of the antisera (as reported by RSL) was with 5α-dihydrotestosterone (DHT). We independently determined crossreactivity with DHT to be 7%.

RESULTS

Testosterone Levels of 2M and 2F Male Gerbils

As can be seen in Fig. 1, 2M subjects in both Samples 1 and 2 had significantly higher levels of serum testosterone than did 2F subjects. These differences in the serum testosterone levels

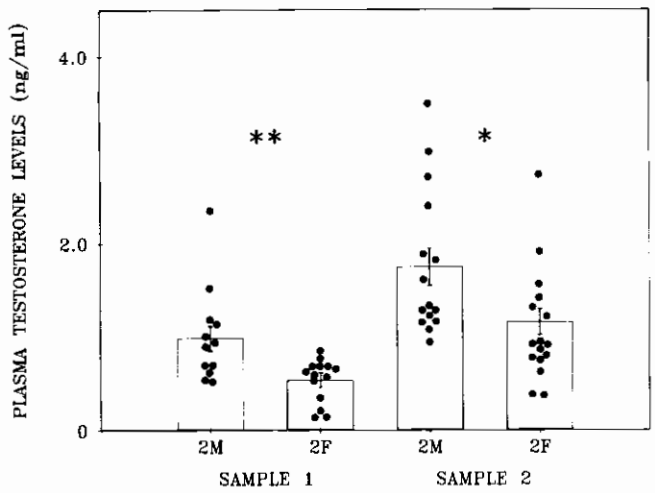

FIG. 1. Mean serum testosterone levels (ng/ml) of male Mongolian gerbils in Sample 1 (sacrificed in August, 1988) and Sample 2 (sacrificed in February, 1991) from 2M and 2F intrauterine positions. Flags indicate ±1 SE; *, $p < 0.02$; **, $p < 0.01$. Individual data points are indicated by closed circles.

of 2M and 2F subjects were observed in both samples of subjects even though Samples 1 and 2 differed significantly in their mean levels of circulating testosterone (Student's t test, $t(55) = 4.10$, $p < 0.0001$). The causes of the observed difference in mean testosterone levels in Samples 1 and 2 are not known, but the differences might have resulted from differences in the season of birth or sacrifice of subjects in the two samples, or from differences in their histories of sexual activity.

Scent Marking Frequency, Ventral Gland Size, Testes Weights, and Body Weights of 2M and 2F Male Gerbils

Figure 2 shows mean frequencies of scent marking, mean ventral gland sizes, mean testes weights, and mean body weights of 2M and 2F subjects in Sample 2. As is clear from examination of Fig. 2 and in confirmation of the results of Clark et al. (5), 2M male gerbils both scent marked more frequently and had larger ventral scent glands than did 2F male gerbils. In the present study, the testes of 2M males were marginally larger than those of 2F males, $t(29) = 1.68$, $p < 0.10$, and the mean body weight of 2M and 2F males did not differ, $t(29) = 1.26$, $p = 0.22$.

Correlations Among Testosterone Levels, Behavior, and Morphology

Table 1 presents Pearson product–moment correlation coefficients among the four main dependent variables (serum testosterone level, scent marking frequency, ventral gland size, and testes weight) independent of the intrauterine position of subjects. Individual endogenous serum testosterone levels were significantly positively correlated with each of the other three dependent variables.

As we have found previously (5), scent marking frequency was significantly positively correlated with ventral gland size, and marginally positively correlated with testes weight.

DISCUSSION

Apparent Contradiction Between the Present Data and Those of Probst

Although we did not try to determine why it was that we observed significant correlations between serum testosterone levels and frequency of scent marking in our subjects, and Probst (9) failed to find a similar significant correlation in his experiments, two explanations come immediately to mind. First, our sample size ($n = 31$) was considerably larger than Probst's ($n = 12$). Second, we used as subjects only those male gerbils that had matured either in 2M or 2F intrauterine locations, while Probst used a random selection of male gerbils as subjects. Most of Probst's randomly selected male subjects would have matured in intrauterine locations adjacent to either one male and/or one female fetus (4). By choosing as subjects only 2M and 2F males, we increased both the variance in scent-marking frequencies (5) and the variance in testosterone levels of our subjects relative to Probst's. Increasing the range of phenotypes sample, like increasing sample size, should have increased our probability of detecting any existing correlations between endogenous testosterone levels and androgen-sensitive characteristics of adult male gerbils. Thus, there is no real contradiction between Probst's (9) failure to find a significant correlation between frequency of scent marking and plasma testosterone levels in adult male gerbils and our success in that enterprise.

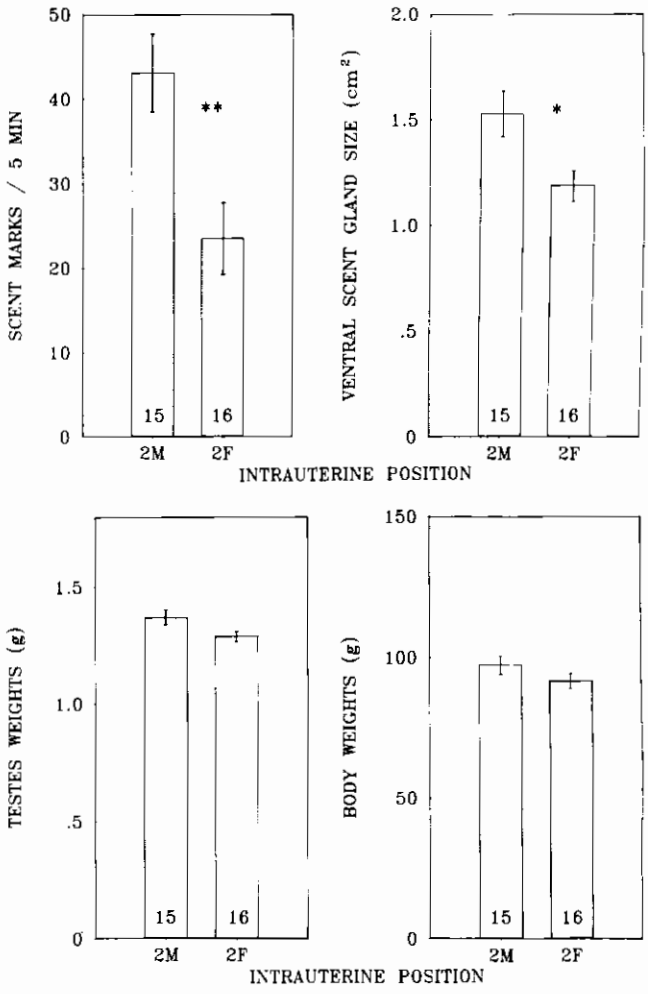

FIG. 2. Mean number of scent marks per 5-min period, mean ventral gland sizes (cm^2), mean testes weights (g), and mean body weights (g) of male Mongolian gerbils in Sample 2 that occupied 2M and 2F intrauterine positions. Flags indicate ±1 SEM; *, $p < 0.02$; **, $p < 0.01$. Numbers inside histograms = n per group.

TABLE 1

RANK ORDER CORRELATION COEFFICIENTS (PEARSON'S r) AMONG BEHAVIORAL, MORPHOLOGICAL, AND HORMONAL MEASUREMENTS OF ADULT MALE MONGOLIAN GERBILS

	Testes Weights (g)	Ventral Gland Size (cm^2)	Testosterone Levels (ng/ml)
Mean scent marks/5 min	0.09	0.62*	0.36†
Testes weights (g)		0.48†	0.53*
Ventral gland size (cm^2)			0.34†

* $p < 0.01$, two-tailed test.
† $p < 0.05$, two-tailed test.

Episodic Release of Testosterone in Male Gerbils

The data collected in the present experiment indicate that a single sample of blood from a male Mongolian gerbil can provide a reliable index of its circulating levels of endogenous testosterone. Testosterone assays of serum collected from 57 subjects (see Fig. 1) provided no indication of the extreme variations in testosterone levels observed when testosterone release is episodic, as it is in many other male mammals examined to date [see (8) for review]. Further, the standard errors of the mean of testosterone levels for both 2M and 2F males in both samples in the present experiment were similar in size to the standard errors of the mean testosterone levels of female gerbils late in gestation (5). Our results, like Probst's (9,10), provided no evidence of pulsatile release of testosterone in male gerbils.

Testosterone in Males from 2M and 2F Intrauterine Positions

The results of the present experiment provide the first direct evidence of differences in endogenous levels of testosterone in adult male mammals as a function of the intrauterine positions that they occupied as fetuses. In each of two replicates, adult 2M male gerbils had significantly higher serum testosterone levels than did adult 2F male gerbils.

Correlations Among Testosterone Levels and Other Androgen-Sensitive Phenotypic Characteristics of Male Gerbils

The finding that levels of testosterone in adult male gerbils correlated with both their frequencies of overt behavior and amounts of glandular tissue suggests that the variance in testosterone levels of adult male gerbils associated with intrauterine position is of functional significance.

In the case of male gerbils, we can, at present, conclude only that fetal intrauterine position influences blood levels of testosterone in adulthood. These differences in testosterone levels could reflect differences in rates of secretion, plasma binding, or metabolism of testosterone (16). Regardless of the cause of the difference in circulating testosterone between 2M and 2F adult male gerbils, its consequences are likely to be broad. Virtually any behavioral, physiological, or morphological measure influenced by testosterone levels in adulthood is likely to be correlated with the intrauterine positions that male gerbils occupied as fetuses.

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