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Social stress decreases marking behavior independently of testosterone in Mongolian gerbils

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Abstract

This study examined the endogenous androgen regulation of the marking behavior in Mongolian gerbils (*Meriones unguiculatus*). In the first experiment, developmental changes of fecal testosterone levels, ventral gland growth, and the marking frequency of male gerbils were investigated. From 9 weeks of age, marking frequency increased with increases in fecal testosterone levels and ventral gland size. The ventral gland size and marking frequency were significantly correlated to the fecal testosterone level. In the second experiment, we hypothesized that reduction in the marking frequency of subordinate males after social confrontations was controlled by a decrease in the circulating testosterone level, and we followed changes in marking frequency, endocrine status, and ventral gland size after social confrontations in which two adult male gerbils established their social ranks by fighting. As expected, marking frequency and ventral gland size were significantly related to social rank, that is, marking frequency was higher among dominant gerbils and lower among subordinates. In addition, fecal corticosterone levels among subordinates were higher than those of dominant animals. However, neither the fecal and plasma testosterone levels, nor testis size, differed between dominant and subordinate gerbils. These results revealed that endogenous androgen played a role in regulating marking behavior and ventral gland size during the developmental stage and that the reductions in marking frequency and ventral gland size occurring in subordinate males after social confrontations were not directly regulated by androgen changes.

Keywords: Social stress; Marking behavior; Mongolian gerbils

Introduction

The social behavior of most mammalian species depends primarily on olfactory communication. Marking is the most commonly observed behavior related to olfactory communication (Beach, 1974; Hart, 1974; Johnston, 1975a,b; Thiessen and Rice, 1976), and some mammalian species have sebaceous glands used for marking behavior. It has been suggested that male social rank influences the size of these sebaceous glands (Gustafsson et al., 1980) and testis function (Sapolsky, 1991), as well as endocrine status (Barrett et al., 2002; Coe et al., 1979; Mann et al., 1998). Gerbils have distinguishable ventral glands that they use for carrying out marking behaviors, and, similar to other species, levels of circulating testosterone have been correlated with marking behavior in gerbils (Blum and Thiessen, 1971; Thiessen et al., 1968; Turner, 1979). Marking frequency and sebaceous gland size have been noted to decrease after castration, and they can be restored by the replacement of testosterone in adult gerbils (Blum and Thiessen, 1971). The modulation of marking behavior by testosterone was mediated via the central nervous system, particularly through the medial preoptic area (MPOA) (Thiessen and Yahr, 1970; Thiessen et al., 1971, 1973). Social rank also exerts profound effects on reproductive endocrine function, including the induction of decreases in serum testosterone levels following social defeat. Moreover, the frequency of marking behaviors among Mongolian gerbils decreases after a social defeat or an event determi-

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native of subordination (Nyby et al., 1970; Yahr, 1977); such behavioral changes might be mediated by testosterone as well. However, there also remains the possibility that marking frequency decreases independently of testosterone levels, as suggested by Yahr (1977); in that study, marking behavior among subordinate gerbils was found to rapidly decrease after social defeat, and this change occurred more rapidly than those associated with decreased levels of testosterone after castration. However, the relationship between testosterone levels and marking frequencies among subordinates is still unknown. Based upon the above-mentioned previous findings, we studied the relationship between testosterone levels and marking frequency. Here, we conducted a fecal steroid analysis, which has the advantages of being noninvasive and much less stressful than serial blood sampling, and of buffering the pulsatile secretion of testosterone. First, we confirmed the correlation between the frequency of marking behavior and the fecal testosterone level during the developmental period. Second, we followed changes in marking frequency, endocrine status, and ventral gland size after social confrontations in which two adult male gerbils established their social ranks by agonistic behaviors.

Materials and methods

Animals

Male Mongolian gerbils (MGS/Sea), 4 weeks of age, were obtained from Seack Yoshitomi (Fukuoka, Japan). The animals were housed singly in acrylic cages (215W × 320D × 140H mm) with free access to water and food (F1: Funabashi Farm, Chiba, Japan). The vivarium was maintained under a 12-h light/dark cycle with lights on at 8:00 A.M. and at a constant temperature of 23 ± 0.5°C and humidity of 50 ± 5%. All experiments were conducted under the guidelines of the "Policies Governing the Use of Live Vertebrate Animals" of the University of Tokyo, "The Public Health Service Policy on Humane Care and Use of Laboratory Animals" of the Awardee Institution (revised in May 1985), and "The National Institutes of Health Guide for the Care and Use of Laboratory Animals" (revised in 1985).

Measurement of marking behavior and gland size

Marking behavior was observed in an open-field apparatus of gray Plexiglas (60W × 60D × 46H cm), with a clear side panel, which allowed for observations from a lateral view. Six Plexiglas pegs (1.2 W × 2.5 D × 0.5 H cm) were attached to the floor at regular distances. The open field was illuminated by an incandescent bulb (15 lx at the center of the field). All gerbils were acclimated to the open field 1 week prior to the experiment, and on the test day, the animals were habituated to the observation room for at least 2 h before the test. Each animal was placed in the center of the open field apparatus, and behavior was video-recorded for 15 min for later analysis. The frequency of marking behavior was scored using a computer-based event recorder system (Toyo-Sangyo, Toyama, Japan). After each test, the floor and pegs were removed and wiped with 70% alcohol. All of the tests were carried out between 12:00 A.M. and 2:00 P.M. After the tests, the ventral gland size was measured on the same day using an electronic micrometer caliper.

Extraction of steroid hormones

Feces were dried at 100°C for 1.5 h in an oven and thoroughly crushed with a mortar and pestle. A portion (0.1 g) of crushed feces was placed in a glass tube with a Teflon-sealed cap. Thereafter, 1.5 ml of distilled water and 5 ml of diethyl-ether were added, and the tubes were shaken vigorously for 10 min. The ether layer was recovered in another tube by decantation after snap-freezing (−80°C) and was evaporated in a bath kept at a constant temperature of 42°C. The residue was re-dissolved in 2 ml of phosphate-buffered saline with bovine serum albumin (PBS–BSA) by vortex mixing for 10 min. These samples were diluted 5- to 40-fold for the testosterone analysis and 10-fold for the corticosterone analysis. The extracts were frozen at −20°C until used for the assay.

Fifty microliters of plasma was placed in a 5-ml culture tube. Two milliliters of diethyl-ether was added, and the steroids were extracted for 10 min with a vortex mixer. The ether layer was recovered in another tube by decantation after snap-freezing (−80°C), and the layer was then evaporated in a bath kept at a constant temperature of 42°C. The residue was re-dissolved in 100 μl of PBS–BSA by vortex mixing for 10 min.

Enzyme immunoassay

Testosterone and corticosterone levels were measured by specific enzyme immunoassays (EIA). Ninety-six-well flat-bottomed polystyrene microtiter plates (Cornig; ELIZA plates 25801) were coated with 200 μl/well of secondary antibody solution (anti-rabbit γ-globulin serum raised in goats, 5 μg/200 μl, Seikagaku Co.; Tokyo, Japan) and the samples were incubated overnight at 4°C. Afterwards, the non-bound antibodies were removed from the wells by emptying and washing the wells four times using a plate washer (BioRad; IMMUNOWASH MODEL 1250); the plates were then left inverted to dry. Standard testosterone (Sigma-Aldrich, St. Louis, MO) or corticosterone (Wako Chemicals, Osaka, Japan) was diluted in an assay buffer (sodium phosphate buffer of pH 7.4 containing bovine serum albumin at 1 g/l). Standard curves were constructed by using 12 standard solutions, namely, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, and 0 ng/well for corticosterone, and 10, 5,

2.5, 1.25, 0.625, 0.3125, 0.1563, 0.0781, 0.0390, 0.0195, and 0.0098 ng/well for testosterone. Standards and samples were analyzed in duplicate. Twenty-five-microliter aliquots of standard and sample solution, 100-μl aliquots of anti-serum solution, and then 100-μl aliquots of labeled steroid hormones were sequentially pipetted into each well. The anti-testosterone serum (first antibody raised in rabbits; FKA102; COSMO Bio, Tokyo) was diluted 500,000-fold with assay buffer and the anti-corticosterone serum (first antibody raised in rabbits, FKA420; COSMO Bio, Tokyo) was diluted 100,000-fold with assay buffer. As regards the labeled steroids, horseradish-peroxidase (HRP)-labeled testosterone was generously provided by Dr. Miyamoto (Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan), and HRP-labeled corticosterone (FKA419) was obtained from COSMO Bio (Tokyo, Japan). The plates were covered and incubated overnight at 4°C. Then, non-bound ligands were removed by emptying the plates and washing them four times with a plate washer. In order to measure the amount of labeled steroid bound to the antibody, 150-μl aliquots of substrate solution for HRP were added to all wells, and the wells were then incubated for an additional 40 min at room temperature until the reaction was stopped by the addition of 50 μl 4N-H_2SO_4. The absorbance at 450 nm was recorded with an automatic microplate reader (BioRad; MODEL 550) for each well containing standard or sample solution. Fecal testosterone and corticosterone concentrations were expressed as ng/g (dried feces) and plasma testosterone concentrations were expressed as ng/ml (plasma). The minimum detectable level of testosterone was 4.9 pg/well, and the intra- and inter-assay coefficients of variation were 11.0% and 13.7%, respectively. The minimum detectable level of corticosterone was 9.9 pg/well, and the intra- and inter-assay coefficients of variation were 3.7% and 12.8%, respectively.

Protocol for Experiment 1

Six male gerbils were singly-reared in a polycarbonate cage (215W × 320D × 140H mm) with a wire mesh top. When the gerbils were 6–18 weeks of age, marking frequency and ventral gland size were measured weekly. The bedding was changed for cleaning 24 h prior to the behavioral test, and the feces that were eliminated during this 24-h period were collected. Fecal steroid was extracted immediately after sample collection. At 18 weeks of age, all of the animals were castrated by cutting the ambilateral scrotum under conditions of anesthetization with pentobarbital sodium (50 mg/kg). After 1 week of recovery, marking behavior, ventral gland size, and fecal steroid levels were measured daily for 3 weeks, as described above. In addition, testosterone propionate injections were administered in order to confirm the modulating effects of testosterone on marking behavior and ventral gland size. Four weeks after castration, four male gerbils were given testosterone propionate (1 mg/ml/body) subcutaneously under ether anesthesia. Testosterone propionate injections were administered every 3 days for 12 days, immediately after the measurement of marking behavior and ventral gland size.

Protocol for Experiment 2

After reaching the age of 4 weeks, 16 male gerbils were singly housed in acrylic cages (215W × 320D × 140H mm). Starting when the animals reached 16 weeks of age, marking behavior, ventral gland size, and fecal steroid levels were measured as described above for Experiment 1; this was done in order to confirm the baseline values of interest. At the age of 18 weeks, 8 pairs of gerbils were selected such that both members of each pair had equivalent marking behavior frequencies. Social confrontations between the members of each pair were conducted twice, once at the age of 18 weeks, and again 4 days later. Each gerbil was placed in one of two compartments of a Plexiglas cage (280W × 440D × 180H mm) divided by a white, hard-wire mesh to prevent physical contact. After allowing the pair to communicate using auditory, visual, and chemical signals for 5 min, the wire net was removed and the pair began to initiate physical confrontations, which were videotaped for later analysis. The period of social confrontation was terminated immediately after any of the following three criteria were met: (1) one of the gerbils exhibited fleeing behavior for 5 s; (2) one of the gerbils mounted the other for 10 s; or (3) the confrontation lasted for 5 min. The social confrontation was repeated once a week for the next 2 weeks, with a total of four confrontations for each pair. For the remainder of the experiment, the pair of animals was kept in the same cage, but the two animals were separated by the wire mesh, with the exception that on the day prior to the marking behavior test, the gerbils were housed singly. The observation of marking behaviors was conducted 1 day before each social confrontation when the animals were 19–21 weeks old; in order to collect feces during this procedure, each animal was housed singly for 24 h on the day before the observation of the marking behaviors. After the last observation of marking behaviors, all animals were deeply anesthetized with pentobarbital sodium, the blood was collected by cardiac puncture, and then the ventral gland and the testes were sampled for analysis.

The video-recorded behavior was analyzed later using a computer-based event recorder. Three aggressive behaviors, i.e., biting, mounting, and the adoption of a sideways posture, and four defensive behaviors, i.e., the adoption of a supine posture, fleeing, the adoption of an upright posture, and submissive grooming, were scored. In cases in which the social confrontation was terminated by the 5-min criterion, the aggressive and defensive scores of each member of the pair were compared.

Statistical analysis

A relationship between two sets of paired numbers was evaluated by Pearson's product moment coefficient correlation with Fisher's z transformation. Between-group comparisons were performed by Dunnett's multiple t test after one-way ANOVA, whereas within-group comparisons were analyzed by Fisher's PLSD post hoc test after two-way ANOVA.

Results

Experiment 1

The present study revealed that fecal testosterone levels increased during the developmental period and castration resulted in a decrease in fecal testosterone levels; these changes were accompanied by changes in the frequency of marking behaviors. It therefore seems likely that these variations in fecal testosterone levels reflect conditions known to produce changes in T levels in the blood (Probst, 1985). During development, fecal testosterone levels increased slightly from 6 to 10 weeks of age, and then fluctuated (Fig. 1A). In parallel with the observed changes in testosterone levels, marking frequency increased among the animals from 6 to 18 weeks of age (Fig. 1B). At the age of 6 weeks, the animals' ventral glands could hardly be observed upon gross analysis, but thereafter became apparent. The size of the ventral gland increased until the animals reached approximately 11 weeks of age, and then the size became stable. There was a significant correlation between marking frequency and fecal testosterone levels during the developmental period (Figs. 1A and B; $r = 0.684$, $z = 2.09$, $P < 0.01$), and also between ventral gland size and fecal testosterone levels (Figs. 1A and C; $r = 0.649$, $z = 3.16$, $P < 0.01$). Testosterone levels declined immediately after castration at 18 weeks of age ($F(5,15) = 92.8$, $P < 0.01$), and there were also significant decreases in marking frequency ($F(5,15) = 137$, $P < 0.01$) and ventral gland size ($F(5,15) = 24.4$, $P < 0.01$). Testosterone treatment in castrated males restored marking frequency (Fig. 2A, $F(1,15) = 3.38$, $P < 0.05$) and ventral gland size (Fig. 2B, $F(1,15) = 3.77$, $p < 0.05$). Fisher's PLSD test revealed that the marking frequencies on days 9 and 12 after testosterone treatment were higher than that on day 0 before testosterone treatment (Fig. 2A). Similarly, the gland size on day 12 after testosterone treatment was larger than that on day 0 before testosterone treatment (Fig. 2B).

Experiment 2

Among a total of 32 confrontations, 21 were terminated in less than 5 min according to the criteria described above. In 10 of the remaining 11 confrontations, social rank was clearly confirmed by the scores for aggression and defense.

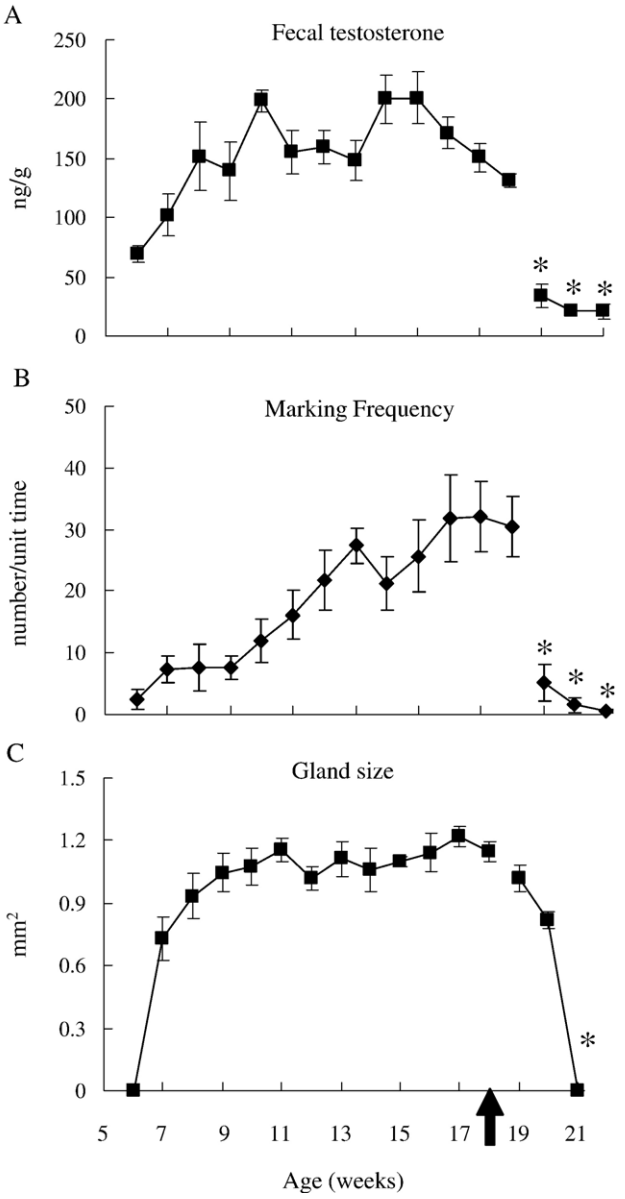

Fig. 1. Developmental changes in fecal testosterone (A), marking frequency (B), and ventral gland size (C) in male gerbils. All measurements were carried out every week in gerbils ranging from 6 to 21 weeks of age, and castration was performed when the gerbils reached 18 weeks of age (arrow). Castration led to a decrease in testosterone, marking frequency, and ventral gland size. *$P < 0.01$, as compared with the data from 18-week-old gerbils (Dunnett's t test). Mean ± SEM ($n = 6$).

In one confrontation that lasted 5 min, the scores of aggression defense were found to be equal between members of the pair, and thus the data for this pair were omitted. In most cases, the first social confrontation was terminated by the criterion of climbing behavior, and the second confrontation was terminated by the criterion of fleeing behavior (11 cases by fleeing, 10 cases by climbing, and 11 cases by aggression and defense). This result may indicate that the social ranking of dominant and subordinate was established by the first confrontation, and the subordinate gerbil tried to escape from the dominant one during

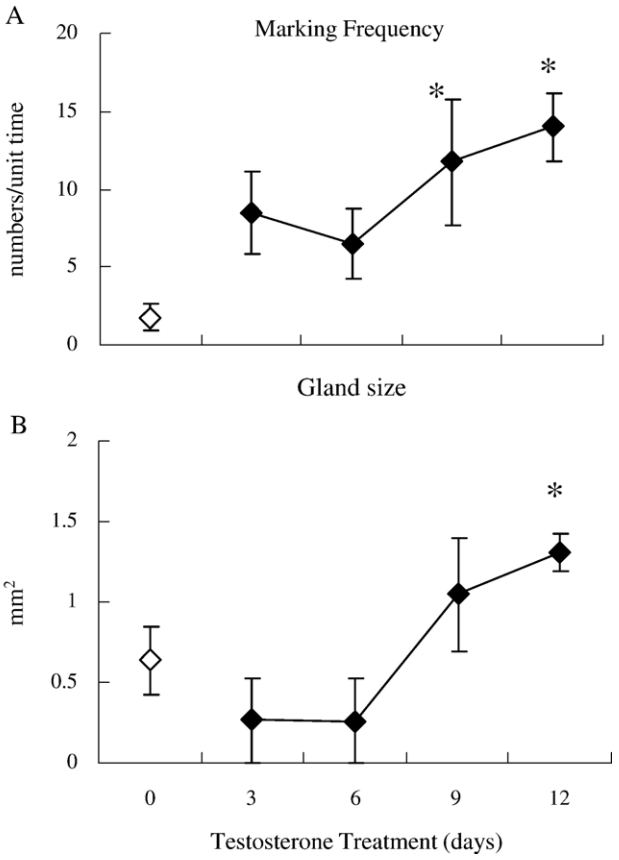

Fig. 2. Changes in marking behavior (A) and ventral gland size (B) after testosterone treatment in castrated male gerbils (n = 4). Four weeks after castration, testosterone propionate was administered subcutaneously every 3 days. *$P < 0.05$, as compared with the data before testosterone treatment (Fisher's PLSD t test). Mean ± SEM.

the subsequent confrontations. Once the hierarchy was determined, the subordinate animal performed intensive grooming of the oral and head area of the dominant animal, a behavior referred to as "submissive grooming". Once the dominant gerbils accepted this submissive grooming behavior, they no longer showed attacking behavior.

The subordinate animals showed a reduction in marking frequency over the 3 weeks following the first social confrontation (Fig. 3A, $F(7,28) = 6.112$, $P < 0.01$). Similar to marking frequency, the ventral gland size of subordinate gerbils was significantly diminished after social confrontation (Fig. 3C, $F(7,28) = 5.40$, $P < 0.01$). Fecal corticosterone levels increased in subordinates at 20 and 21 weeks (Fig. 3B, $F(7,28) = 3.41$, $P < 0.05$), while there was no change in the fecal testosterone level (Fig. 3D). On the other hand, in dominant gerbils, no changes were observed in

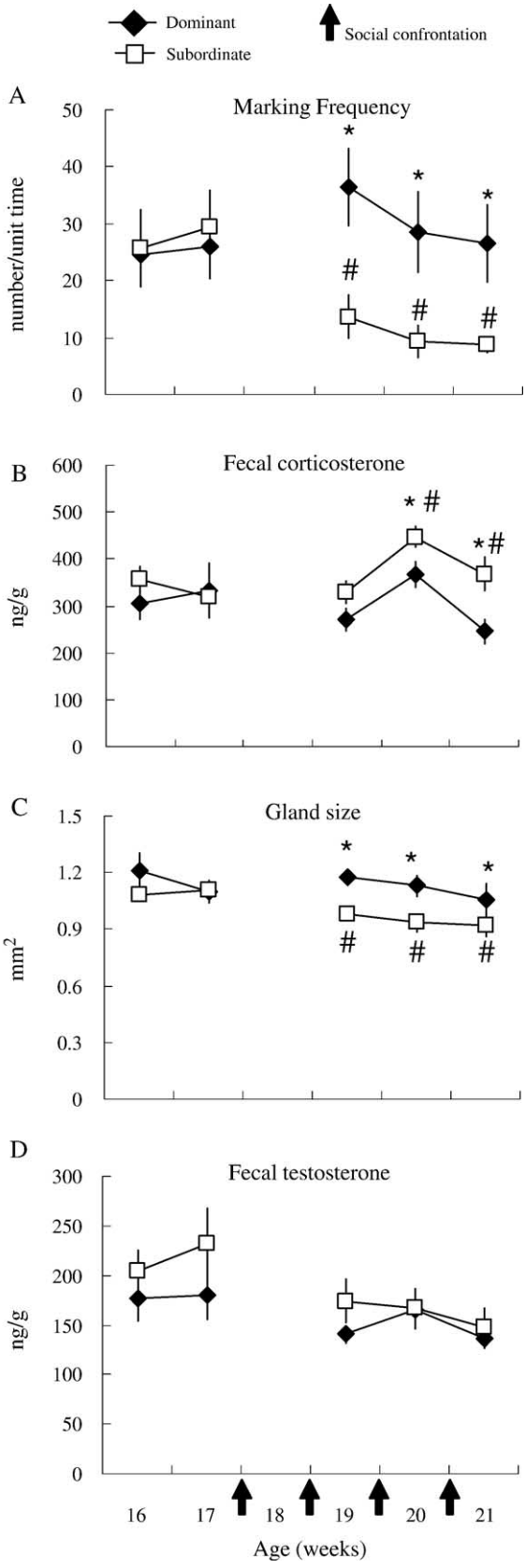

Fig. 3. Marking frequency (A), fecal corticosterone (B), gland size (C), and fecal testosterone (D) in the dominant (n = 8) and subordinate (n = 8) gerbils. Social confrontations (arrows) were conducted four times for each pair, once each at the ages of 18 weeks, 18 weeks and 4 days, 19 weeks, and 20 weeks. #$P < 0.05$ within-group comparison, using the values obtained before social confrontation. *$P < 0.05$, as compared between dominant and subordinate gerbils. Mean ± SEM.

either of these four variables before or after social confrontation. When the results obtained from dominant and subordinate gerbils were compared, the marking frequency (Fig. 3A, $F(1,54) = 3.02$, $P < 0.05$) and the ventral gland size (Fig. 3C, $F(1,54) = 2.14$, $P < 0.05$) of subordinate gerbils were significantly lower than those of the dominant animals. Fecal corticosterone levels differed between the dominant and subordinate animals at 20 and 21 weeks (Fig. 3B, $F(1,24) = 10.19$, $P < 0.05$). On the other hand, there were no differences in the following measures between the dominant and subordinate animals at the end of the experiment: fecal testosterone level (Fig. 3D), plasma testosterone level (dominant vs. subordinate: 2.85 ± 0.65 vs. 3.97 ± 1.36 (ng/ml), mean ± SEM), or testicular weight (dominant vs. subordinate: 1.05 ± 0.03 vs. 1.06 ± 0.04 (g), mean ± SEM).

Discussion

In Experiment 1 of the present study, it was revealed that marking frequency and ventral gland size of male gerbils were highly correlated with fecal testosterone levels during the developmental period. Marking frequency and ventral gland size declined after castration, and testosterone replacement restored these values to their baseline levels. Therefore, it was concluded that both marking frequency and ventral gland size were dependent on the circulating testosterone level under non-stressful conditions. This finding was consistent with those of previous reports by Probst (1985) and Turner (1979), which demonstrated correlations between marking frequencies and serum testosterone levels. In addition to marking frequency, fecal testosterone levels were found to be correlated with changes in ventral gland size during development, as well as after castration. This finding indicates that the growth of the ventral gland is also dependent on circulating testosterone levels (Blum and Thiessen, 1971), which has also been observed in other species (Thiessen and Rice, 1976).

In Experiment 2, it was found that social subordinate stress led to decreases in marking frequency and ventral gland size, whereas such stress led to an increase in corticosterone secretion. However, these changes were not accompanied by a decline in the testosterone levels measured in either the feces or the blood, and there was no difference detected in the size of the testis after the social confrontations. These results suggest that decreases in the marking frequency and the ventral gland size of subordinate males after social confrontation are not preceded by decreases in the peripheral testosterone level, and some testosterone-independent factors might modulate the decline of marking frequency under conditions involving social stress.

In some mammalian species, dominant animals have been reported to show increases in aggressive behavior (Benton and Brain, 1979; Houpt et al., 1978; Kruczek, 1997), sexual behavior (Setchell and Dixson, 2001), and marking behavior (Thiessen et al., 1971), whereas decreases in marking frequency have been observed in subordinate animals (Thiessen et al., 1971; Yahr, 1977; Yoshimura, 1981). In tree shrews (*Tupaia belangeri*), which have been shown to be vulnerable to social stress (1996), subordinate individuals exhibited less locomotor activity, marking frequency, and self-grooming, and long-term subordination caused fatal problems (Fuchs et al., 1996). In subordinate gerbils, a reduction in marking frequency has been reported to occur in an open field containing odors from dominant gerbils, but such a reduction was not observed in a neutral environment (Nyby et al., 1970). In addition, visible cues have been suggested to reduce marking frequency in socially defeated gerbils (Yahr, 1977), indicating that the context in which the subordinate animals were defeated may be one of the modulators for marking behavior among subordinates. However, in our study, subordinates showed significant decreases in marking behavior in an observation room, in which they were separated from all olfactory or environmental cues associated with the social defeat they had experienced. This may be due to the magnitude of stressors, since, in the present study, housing the pair next to each other in a cage appeared to have severely stressed the subordinate. It was observed that the dominant gerbils exhibited aggressive and threatening behavior with respect to the subordinate gerbils, and the subordinate gerbils moved actively, and neither rested nor relaxed as much as did the dominant gerbils. The reduction in marking frequency and the ventral gland size occurred independently of the circulating testosterone level, suggesting that other factors may have modulated the decline in marking frequency and ventral gland size after social confrontation. One possible explanation for this finding is that social stress directly modulates the function of MPOA (Anderson et al., 1985), and marking behavior in turn decreases. An alternative possibility would be that an increase in corticosterone secretion due to social confrontations modulates the expression of marking behavior (Sheree et al., 1999). It is already well known that social stress induces an abundant secretion of corticosterone; as a result, the basal level of circulating corticosteroids in dominant animals is lower than that in subordinates of various species, including rats (Bhatnagar and Vining, 2003), rhesus monkeys (Bercovitch, 1993), and squirrel monkeys (Mendoza et al., 1978). Similarly, the subordinate gerbils in the present study had higher levels of corticosterone than the dominant gerbils, indicating that the social defeat episodes had a great impact on both behavioral and endocrine responses. Further experiments will be needed to clarify the modulation of marking behavior after social-defeat stress in gerbils.

In conclusion, the present results suggest that endogenous androgen plays a role in regulating both the marking behavior and ventral gland size of gerbils in the developmental stage. Furthermore, the present findings indicate that reductions in marking frequency and ventral gland size that

occur in subordinate males following social confrontations are not directly regulated by changes in androgen levels.

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