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# Vomeronasal and/or Olfactory Mediation of Ultrasonic Calling and Scent Marking by Female Golden Hamsters

ROBERT E. JOHNSTON

*Department of Psychology, Uris Hall, Cornell University, Ithaca, NY 14853*

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JOHNSTON, R. E. *Vomeronasal and/or olfactory mediation of ultrasonic calling and scent marking by female golden hamsters.* PHYSIOL BEHAV **51**(3) 437–448, 1992.—The role of the olfactory and vomeronasal systems in mediating odor-stimulated ultrasonic calling, flank marking, and vaginal marking by female hamsters was investigated by selective lesions of either system. Removal of the vomeronasal organ resulted in reduced frequencies of ultrasonic calling by estrous and nonestrous females in response to conspecific odors but it had no influence on either scent marking behavior during the same tests. When tested immediately after separation from a male, ultrasonic calling was not reduced by vomeronasal removal, indicating that such surgery does not cause deficits in calling ability and that the vomeronasal organ specifically mediates odor-stimulated calling. Zinc sulfate treatment of the olfactory mucosa led to reduction in the frequency of ultrasonic calling, flank marking, and vaginal marking in response to conspecific odors. Females' ability to discover buried food was also impaired by this treatment. Thus, the stimulation of both scent marking behaviors due to the perception of conspecific odors appears to be mediated primarily by the olfactory system, whereas stimulation of ultrasonic calling is mediated by both olfactory and vomeronasal systems.

Olfaction Vomeronasal organ Scent marking Ultrasonic communication Animal communication
Social recognition Olfactory communication Auditory communication Scent glands Hamsters

DIFFERENCES in the peripheral anatomy and neural projections of the olfactory and vomeronasal systems suggest at least some differences in function of these two systems (48), and during the past 15 years considerable progress has been made in categorizing these differences (13,20,39,53,54). One distinguishing feature is that the vomeronasal organ appears to be specialized for dealing with large, relatively nonvolatile molecules. Special mechanisms seem to have evolved to provide access of such large molecules to the vomeronasal organ (5,14,37), including vascular and muscular pump mechanisms (40). Several studies have demonstrated that large molecules are preferentially transported to the vomeronasal organ in a number of vertebrate species (14,28,55,56). The existence of such mechanisms, however, does not exclude the possibility that small, volatile molecules are also detected by the vomeronasal system or that some large molecules might be detected by the olfactory system.

There may also be higher order functional differences between the two systems. In the general realm of social behavior in mammals, scent signals that elicit hormonal changes in conspecifics seem to act preferentially through the vomeronasal system, although the olfactory system may have a parallel role in some cases (39,54). It has also been proposed that the vomeronasal system is especially important for behaviors involved in various ways with reproduction (53,54). In contrast, it has been suggested that the olfactory system may be especially important for either maintenance functions such as feeding (53), or functions that rely on pattern (or "image") recognition of complex scent mixtures, as may be involved in individual, kin and species recognition (20,23). At present all attempts to generalize are hampered by the lack of a really thorough knowledge of (i) the similarities and differences in function of the two systems in even one model species and (ii) the extent of species differences in such functions.

The present experiments are part of a continuing effort to understand the differences in function of the two systems in golden hamsters, one of the primary model species to date. The first behavioral function discovered for the vomeronasal organ in a mammal was its importance for the copulatory behavior of male hamsters (47,52). Although not essential for male copulatory behavior (20,38), the vomeronasal system does mediate sexual arousal caused by at least the large, nonvolatile molecules in vaginal secretions (4,49). The vomeronasal system is also important in mediating the increases in testosterone levels observed when males are exposed to this secretion (24,25,45). Specific functions have also been shown for the olfactory system in male hamsters. This system seems to mediate odor-stimulated flank marking by males, a behavior that depends upon perception of conspecific as opposed to heterospecific odors (21,23). The olfactory system has also been shown to mediate recognition of familiar versus unfamiliar mates (26), although in another study the vomeronasal system seemed to be involved in such recognition (50).

In contrast to what is known about the roles of these two systems in male hamsters, relatively little is known about their functions in females. Although elimination of both olfaction

and and vomeronasal function in females does not eliminate mating behavior, as it does in male hamsters (3,42), vomeronasal lesions alone can have subtle effects on the mating behavior of females (30). It is also known that vomeronasal removal does influence maternal behavior in female hamsters, whereas zinc sulfate lesions of the olfactory system have little effect (32).

In the present set of experiments, we investigated three relatively simple, discrete behaviors of female hamsters that are known to be stimulated by conspecific odors. Ultrasonic calls by both male and female hamsters are given mostly in sexual contexts. Females call most frequently when estrous, and call especially frequently in response to the presence of a male, to male odors, or to calls of other hamsters (11). Flank and vaginal scent marking both serve females as a form of individual advertisement. Vaginal marking is primarily a sexual advertisement, peaking in frequency 12–24 hours before the onset of estrus; it is especially frequent in response to males or their odors (17,19). Flank marking by females is more closely tied to agonistic behavior and is most consistently stimulated by agonistic encounters with other females or by odors of other females (17). Both scent marking behaviors are stimulated much more by the odors of conspecifics than by the odors of another species in the same genus (21). It is particularly interesting to investigate the sensory basis of flank marking since some aspects of central nervous system control have recently been uncovered (6,7).

## EXPERIMENT 1

The purpose of this experiment was to determine the role of the vomeronasal organ in mediating ultrasonic calling, flank marking, and vaginal marking by female hamsters in response to conspecific odors.

### METHOD

Twenty-three adult female hamsters (*Mesocricetus auratus*, laboratory stock derived from Charles River random bred animals) were used as subjects; 14 of these females were randomly assigned to the experimental group (vomeronasal organ removal) while the remaining nine were assigned to the control group (sham removal). Females were 4–8 months of age and all were sexually naive. An additional 10 adult females and 10 adult males served as sources for conspecific odors. All animals were housed individually in solid-bottomed polycarbonate cages. The colony was maintained on a reversed day–night cycle with 14 hours of bright and 10 hours of dim illumination. Food and water were always available in the home cages. The estrous cycle of each female was initially determined by brief daily exposures to a male and by noting the presence or absence of lordosis; subsequent brief encounters on estrous days kept track of these cycles.

All animals were tested in a repeated measures design in which (i) behavioral tests were carried out, (ii) surgery was performed and the animals allowed to recover, and (iii) behavioral tests were repeated. In the behavioral tests each female was observed for 5 min daily on 12 consecutive days while she explored a 61 × 61 cm arena that had been scented by other hamsters. This design was chosen so that we would have data for three complete estrous cycles for each female and could thus examine each of the 4 days of the cycle separately (three measures for each day of the cycle for each female). During the week following the first series of behavioral observations females were operated upon, either having their vomeronasal organs removed (VNX) or having sham removals (SHAM). After allowing three weeks for recovery from surgery, females were again observed in a series of 12 daily 5-minute trials to assess their scent marking and ultrasonic calling. Within three weeks of completion of the experiment, females were sacrificed so that the removals of the vomeronasal organs could be histologically verified. The experiment was run in two phases, approximately half of the experimentals and control animals in each phase. Females were tested in the same order on each day; within this sequence VNX and SHAM females were mixed randomly.

The arena in which females were tested was one in which the odors of both male and female hamsters were present; this arena was a 61 × 61 × 30.5 cm painted wooden box with one side of Plexiglas. Each day before trials began a male was placed into the testing arena for 30–45 minutes; after 5–6 females were tested, the male was allowed into the arena for another 30 minutes, after which the remaining 5–6 females were tested. Thus the same soiled arena was used to test all females. A different male was used every day as a source of stimulus odors. Trials were begun about 1 hour after onset of the dark phase of the light cycle in a room containing no other hamsters.

The behaviors recorded were vaginal marking, flank marking, and ultrasonic calling. Vaginal marking is a stereotyped scent marking behavior in which a female presses the genital region against the substrate under her and moves forward slowly, leaving a streak of watery, translucent fluid (17,19). Flank marking is another scent marking behavior in which a hamster arches its back and rubs its entire side, including the flank gland, against a vertical surface, often with great vigor. Each mark is discrete and easily recognized (15,17). Ultrasonic calls were detected with a QMC Model S100 bat detector and audio headphones; hamster calls are generally between 20 and 35 kHz and have a duration of about 0.1 second (9); the detector was set in the broadband mode, allowing the experimenter to hear sounds between about 15 and 160 kHz.

Surgical removal of the vomeronasal organ followed the procedures reported previously (26). After anesthetization with sodium pentobarbital (60 mg/kg) a midline incision was made in the upper palate beginning just behind the upper incisors. The hard palate was exposed and the paired, midline palatine processes were removed by drilling through them at the rostral and caudal ends. These bones were grasped with forceps and pulled out, thus bringing the attached vomeronasal capsules and organs along. The palate was sutured shut and the animals returned to their home cages. Sham surgery involved exposure of the hard palate followed by suturing the soft tissue that had been cut. All animals were allowed 3 weeks recovery before use in behavioral trials. The animals did not lose weight and all ate their normal hard pellet food the day after surgery; all females maintained regular, 4-day estrous cycles throughout the experiment.

Within 3 weeks after the end of the experiment 11 of the 14 VNX females were sacrificed. They were anesthetized with sodium pentobarbital and perfused with saline followed by 10% formalin. The snout was decalcified, embedded in parafin, sectioned at 40 microns, and stained with hematoxylin and eosin. In three cases the snouts did not completely decalcify and they could not be sectioned. In the remaining eight animals every fifth section was saved and examined for the existence of vomeronasal tissue. Because the lesions removed most of the vomeronasal capsule, and thus relevant landmarks, it was difficult to quantitatively determine the extent of damage. However, a qualitative summary seems quite sufficient, given the results.

The results were analyzed using COANOVA on the SYSTAT statistics package, with data for the trials before surgery serving as the covariant. Paired comparisons between particular conditions were made with *t*-tests.

RESULTS AND DISCUSSION

Removal of the vomeronasal organ from females decreased ultrasonic calling but did not alter the frequency of vaginal marking or flank marking (see Figs. 1–3). The results for each one of these behaviors is presented separately.

Females lacking the vomeronasal organ (VNX females) decreased the number of calls they produced to a much greater extent than sham-operated control females [SHAM females; see Fig. 1, top; $F(1,19) = 9.391, p = 0.006$]. VNX females produced fewer ultrasonic calls in response to odors of other hamsters than they did before surgery (Fig. 1; $t = 4.711, p < 0.0005$) and after surgery they called less than the control females did [$t(20) = 3.47, p < 0.005$]. Before surgery SHAM females and VNX females did not differ in the level of ultrasonic calling.

It can be seen in Fig. 1 that the majority of all ultrasounds were produced on the females' estrous days. Before surgery all females called more frequently on the estrous day than on any other cycle day ($p \ll 0.0005$ for comparisons of estrus and all other cycle days, using $t$-tests and combining all females into one group). After surgery all SHAM females called most frequently when estrous, but some VNX females did not call on the estrous day or on other cycle days. Nonetheless there is still a significant difference within the VNX group in calling frequency between day 1 and all other cycle days (day 1 vs. day 2, $t = 3.19, p < 0.005$; day 1 vs. day 3, $t = 3.24, p < 0.005$; day 1 vs. day 4, $t = 3.31, p < 0.005$). This preponderance of calling during estrus has been reported previously (8,11) and is due to the facilitation of calling by the same hormonal mechanisms that induce behavioral receptivity (8,11).

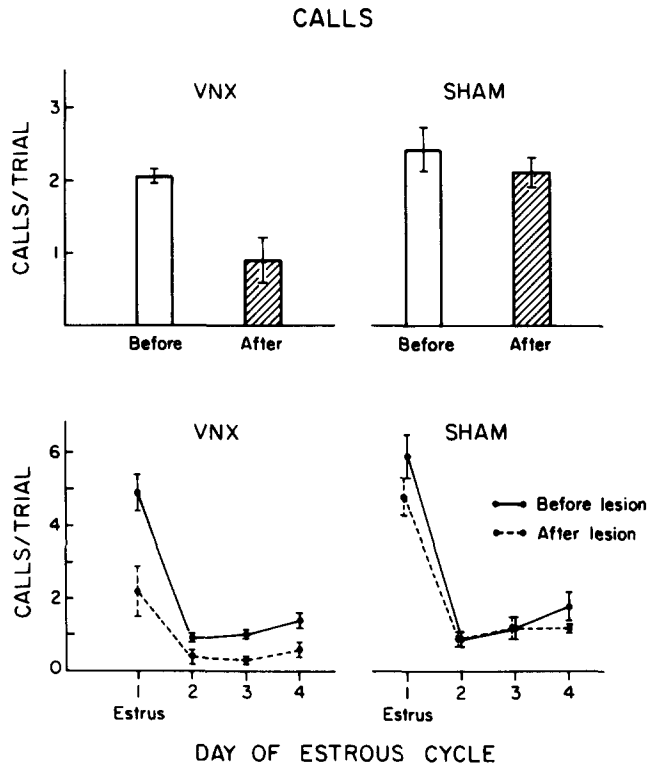


FIG. 1. The mean (±SEM) number of ultrasonic calls produced by females during a 5-min trial in an arena scented by males and females. Females were tested both before and after surgery, which consisted of either vomeronasal organ removal (VNX) or sham removals (SHAM). Top of graph: mean calls per female per trial for all days of the estrous cycle. Bottom of graph: calls per female per trial for each of the 4 days of the estrous cycle. (Experiment 1)

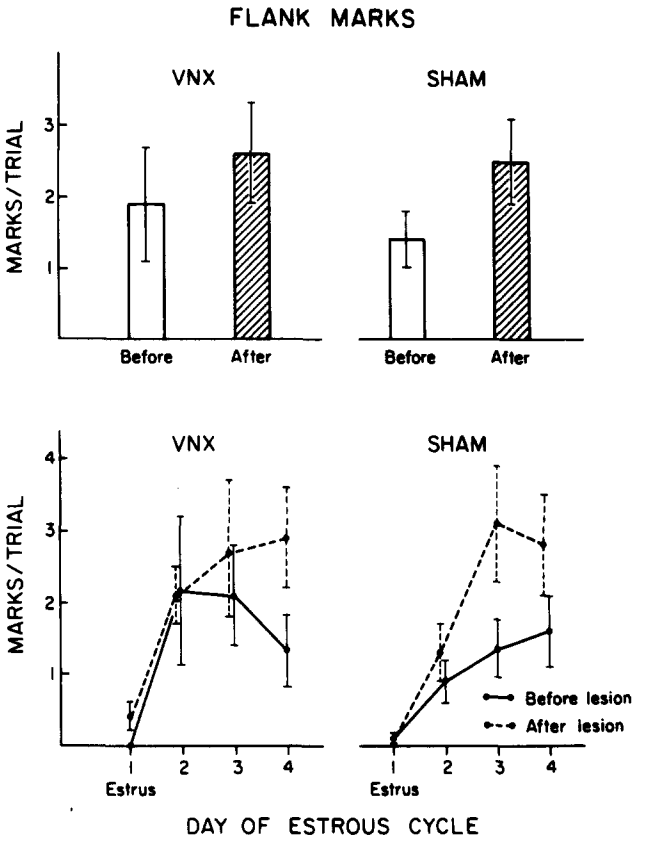


FIG. 2. The mean (±SEM) number of flank marks produced by females during a 5-min trial in an arena scented by males and females. Females were tested both before and after surgery, which consisted of either vomeronasal removal (VNX) or sham removals (SHAM). Top of graph: mean marks per female per trial for days 2–4 of the cycle. Bottom of the graph: mean marks per female per trial for each day of the estrous cycle. (Experiment 1)

Because of the high proportion of calling during estrus a separate analysis was performed for calling on this day. VNX females demonstrated a significantly greater decline after surgery than did the control females [$F(1,19) = 6.742, p = 0.018$]. Before surgery, estrous VNX females called a mean of $4.9 \pm 0.5$ times per 5-minute trial whereas after surgery they called $2.2 \pm 0.7$ times per trial, ($t = 3.74, p < 0.005$); estrous SHAM females called at about the same rate before surgery, $5.9 \pm 0.6$ times per trial, and after surgery, $4.8 \pm 0.5$ times per trial. After surgery the VNX females called significantly less than the SHAM females [$t(20) = 2.885, p < 0.005$]. Thus calling on estrous days alone was significantly decreased by vomeronasal lesions. Although the magnitude of the differences were smaller, females also called significantly less after vomeronasal removal on all other days of the cycle as well (Fig. 1, bottom; day 2, $t = 1.82, p < 0.05$; day 3, $t = 5.03, p < 0.0005$; day 4, $t = 4.73, p < 0.0005$).

Removal of the vomeronasal organ had little influence on flank marking in response to odors of other hamsters [Fig. 2, top; $F(1,20) = 0.008, p = 0.927$]. Lesioned females flank marked at about the same rate that control females did, both before and after surgical treatment. Both groups, however, marked more after treatment than they did before [$F(1,20) = 20.411, p < 0.001$]. This increase is probably due to the increased experience

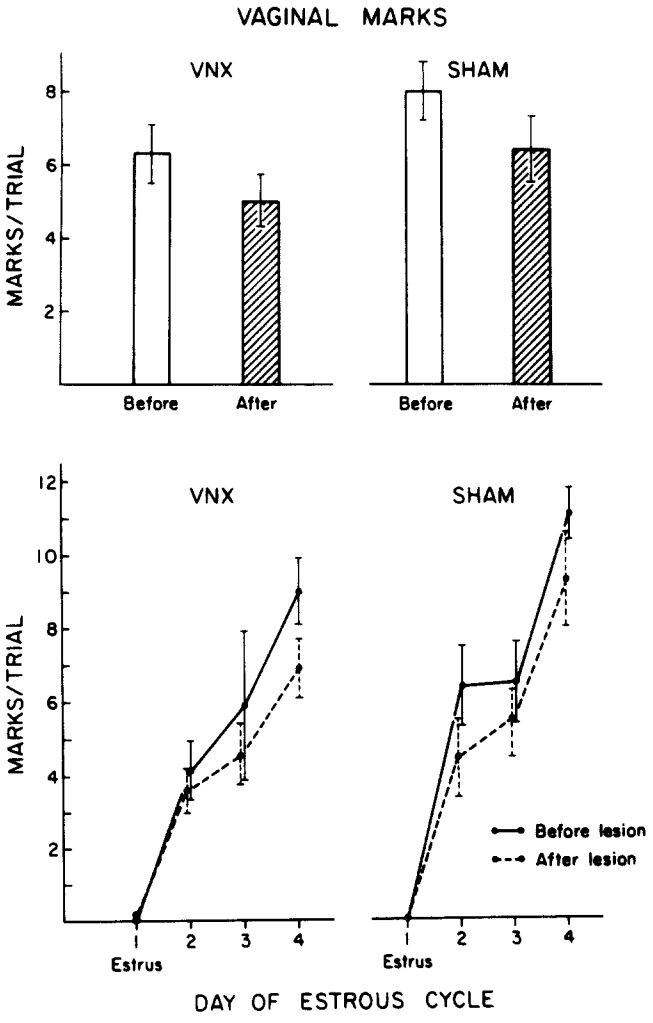


FIG. 3. The mean number of vaginal marks produced by cycling, nonestrous females during a 5-min trial in an arena scented by both males and females. Females were tested both before and after surgery, which consisted of either vomeronasal organ removal (VNX) or sham removals (SHAM). Top of graph: mean marks per female per trial for days 2–4 of the estrous cycle. Bottom of the graph: mean marks per female per trial for each day of the estrous cycle. (Experiment 1)

of the females in the testing situation (15,17). Most flank marking occurred on the three nonestrous days of the cycle (Fig. 2, bottom); when the data for each of these days was analyzed separately, there were no significant effects of the lesions. Marking on each of the nonestrous cycle days increased after surgery, but did so to the same extent in both lesioned and control females.

The differences between flank marking frequency on the estrous day and other days of the cycle are highly significant; combining females from both lesion and control groups the statistics before surgery are: day 1 vs. day 2, $t(21) = 2.52$, $p < 0.01$; day 1 vs. day 3, $t(21) = 3.85$, $p < 0.0005$; day 1 vs. day 4, $t(21) = 7.700$, $p < 0.0005$. After surgery the statistics are: day 1 vs. day 2, $t = 6.09$, $p < 0.0005$; day 1 vs. day 3, $t = 4.56$, $p < 0.0005$; day 1 vs. day 4, $t = 5.08$, $p < 0.0005$. There was no general pattern of differences in flank marking frequencies on other days of the cycle, although marking by SHAM females after surgery was less on day 2 than on either day 3 ($t = 3.51$, $p < 0.01$) or day 4 ($t = 3.28$, $p < 0.02$). These cyclic changes in flank marking by females have been described previously (17,19). In the presence of male scent this cyclic pattern is pronounced, as in the present experiment (17); however in the presence of female scent alone, flank marking does not show such striking cyclic patterns because females mark at relatively high rates on all cycle days (17).

Vomeronasal removal also had no effect on the frequency of vaginal marking in response to conspecific odors [Fig. 3; $F(1,20) = 1.438$, $p = 0.245$]. Females in both SHAM and VNX groups marked less after treatment [$F(1,20) = 15.471$, $p = 0.001$]. The VNX and SHAM groups did not differ, however, in the degree of decrease in vaginal marking or in the absolute level of marking (Fig. 3). The highest frequency of vaginal marking occurs on cycle day 4, approximately one day before the female is behaviorally receptive (17). Analyzing the data for marking on this day alone we found that once again that there was no effect of vomeronasal removal [$F(1,20) = 1.020$; $p = 0.325$].

The cyclic changes in vaginal marking frequency are the most striking of the three behaviors studied in this paper (17,19). Females rarely ever mark when estrous; combining females from VNX and SHAM groups, vaginal marking frequency on cycle days 2, 3, and 4 was significantly greater than on day 1, both before and after surgery (all 22 females marked more on all nonestrous days; using $t$-tests, $p \ll 0.0005$). Vaginal marking frequency was also greater on day 4 than on day 2 or 3: before surgery—day 2 vs. day 4, $t(21) = 7.10$, $p < 0.0005$; day 3 vs. day 4, $t(21) = 5.16$, $p < 0.0005$; after surgery—day 2 vs. day 4, $t(21) = 6.76$, $p < 0.0005$; day 3 vs. day 4, $t(21) = 4.42$, $p < 0.0005$. It should be noted that the frequencies of ultrasonic calling, flank marking, and vaginal marking each have a different relationship to the estrous cycle, suggesting the existence of some subtle and exquisitely calibrated hormonal mechanisms.

Histological examination of the nasal cavities revealed that the vomeronasal pore was present in all animals. In four animals no remnant of the organ remained. In three animals the pore began to enlarge into the organ proper but only a small proportion of the epithelium appeared to be present (it appeared in three sections or less). Since the tissue posterior to this was all removed, it is assumed that axonal connections to the accessory bulb were lesioned, although we do not know if neural connections were reestablished. In one animal the vomeronasal organ on one side remained in good shape for approximately one half of its normal length. For this reason this female was removed from the VNX group for the summary statistics and analysis. It is interesting that she had a higher rate of ultrasonic calling after the lesion than any other female, and was a statistically significant outlier of the lesioned group (as calculated by the SYSTAT program). This female had flank and vaginal scent marking scores that were not unusual, however, thus tending to confirm our conclusion that the vomeronasal organ is important in mediating ultrasonic calling stimulated by conspecific odors, but is not important for scent marking. Except for this one case there was no relationship of any kind between observed behavior and extent of the lesions. There were no deficits observed in the olfactory mucosa of females with vomeronasal organ removal.

## EXPERIMENT 2

Although both males and females produce ultrasonic calls in response to the odors of the opposite sex, the highest rates of calling are observed during actual interactions between a male and an estrous female, most especially during brief separations between the pair (8,10). Does vomeronasal organ removal influence calling at such times, or just in response to odors of another individual? This question was investigated in the following experiment.

## METHOD

Females from Experiment 1 were used again, beginning three days after the end of that experiment. Ten females lacking their vomeronasal organs (VNX) and eight females with sham surgery (SHAM) were used as subjects. Females were observed in the same arena used in Experiment 1, but in the present experiment the arena was initially divided in half by a removable partition. At the beginning of each trial a female subject was placed in one half of the arena and a adult stimulus male was placed in the other half. After 1 minute the partition was removed and the two animals were allowed to interact for 2 minutes. The male was then removed and the female was observed alone in the arena for 5 minutes. Ultrasonic calls, flank marks, and vaginal marks were recorded during the interaction and the postinteraction period, but only data for the postinteraction period are reported here since during the interactions (i) we could not determine if calls were produced by the male or the female and (ii) scent marks were rare. Females were tested for 12 consecutive days (three estrous cycles).

Because of the large amount of vomeronasal tissue remaining in one female (see Experiment 1) the data for this female were dropped from analysis of calling behavior. She did produce more calls than other females in her group, although the significance of the differences between the experimental and control females are the same whether this female is included or excluded.

## RESULTS

The vast majority of ultrasonic calling occurred on the estrous days of the females (Table 1). Estrous VNX females called 4.0 ± 1.2 times per 5-minute trial while estrous sham females called 6.2 ± 2.7 times, a nonsignificant difference ($t = 0.807$). Similarly when calling was examined for all days of the estrous cycle, VNX females called 1.7 ± 0.3 times per trial while SHAM females called 2.1 ± 0.7 times per trial ($t = 0.545$). Removal of the vomeronasal organ did not produce a significant deficit in ultrasonic calling by females immediately after a brief interaction with a male. It is noteworthy that the apparent difference between the mean scores of VNX and SHAM females does not reflect a trend toward less calling by the VNX females. The higher mean calling rate for SHAM females was due primarily to one female that called much more than the others; median calls per female per trial are much closer in value (VNX, 3.3; SHAM, 2.8). Thus vomeronasal lesions do not depress calling in all situations, indicating that such surgery does not influence the ability to produce ultrasonic calls. Presumably the reason that no deficit was seen is that the interaction with the male provided motivation for calling far beyond that provided by the odors in the arena. The results of Experiments 1 and 2 together thus suggest that vomeronasal lesions influence calling specifically in response to chemical cues.

Neither flank marking nor vaginal marking was influenced by vomeronasal removal (Table 1). The mean number of flank marks per trial for VNX females was 2.3 ± 0.7 and for SHAM females it was 1.9 ± 1.1. The mean number of vaginal marks per trial for VNX females was 3.8 ± 0.6 and for SHAM females it was 4.4 ± 0.8. On the day preceding estrous, when vaginal marking was most frequent, the mean number of marks was 6.1 ± 1.4 for VNX females and 6.5 ± 1.4 for SHAM females. Thus the results of this experiment are consistent with those of Experiment 1 in suggesting no role for the vomeronasal organ in the mediation of flank or vaginal scent marking.

For the results of the histological analysis of the lesions of these females see Experiment 1. As in that experiment, there were no significant correlations between observed behavior and differences in the extent of the lesion, except that the one female with one partially intact vomeronasal organ called more than any other female.

TABLE 1

MEAN (±SEM) RATES OF ULTRASONIC CALLING, FLANK MARKING, AND VAGINAL MARKING

| Behavior | VNX | SHAM |
|---|---|---|
| Ultrasonic calls | | |
| Estrous day | 4.0 ± 1.2 | 6.2 ± 2.7 |
| All days | 1.7 ± 0.3 | 2.1 ± 0.7 |
| Flank marks | 2.3 ± 0.7 | 1.9 ± 1.1 |
| Vaginal marks | | |
| All days | 3.8 ± 0.6 | 4.4 ± 0.8 |
| Day 4 | 6.1 ± 1.4 | 6.5 ± 1.4 |

Rates per female per 5-min trial by female hamsters that were intact (SHAM) or had their vomeronasal organs removed (VNX). These behaviors were assessed immediately after separation from an adult male.

# EXPERIMENT 3

The purpose of this experiment was to determine the role of the main olfactory system in the mediation of ultrasonic calling, flank marking, and vaginal marking by female hamsters in response to conspecific odors.

## METHOD

Twenty-one sexually naive female hamsters, 5 months of age, were used as subjects. Ten of these females were in the experimental group and were treated intranasally with zinc sulfate to incapacitate the main olfactory system; 11 females served as controls and were treated in the same manner with isotonic saline. Estrous cycles of females were initially determined by observing them every day during brief interactions with males; throughout the experiment they were checked with males on predicted estrous days to ensure they were maintaining regular cycles.

Experimental and control females were tested in a repeated measures design. First, all females were observed in the testing arena for 5 minutes per day for 8 days to accustom them to the procedures and to determine that all females were marking and calling at reasonable rates. Then they were observed for 5 minutes on 12 consecutive days to establish baseline marking and calling rates; on day 13 they received a treatment (either saline or $ZnSO_4$) and the following day the second period of testing began; this consisted of another series of 5-minute observations on 12 consecutive days. Thus all females were observed for three complete estrous cycles both before and after treatment. The experiment was run in two phases, with approximately half the females in each phase.

One testing arena was used for all females and contained odors of both males and females. On each observation day a male was allowed to investigate, mark, and otherwise deposit scent in this arena for 30 minutes; then trials for 5–6 females were run, followed by another 20–30 minutes of scenting by another male, followed by testing the remainder of the females. The arena was similar to the one used in Experiment 1, namely a 61 × 61 × 30.5 cm painted wooden box with one Plexiglas wall for observation. Tests were conducted 1–3 hours after the

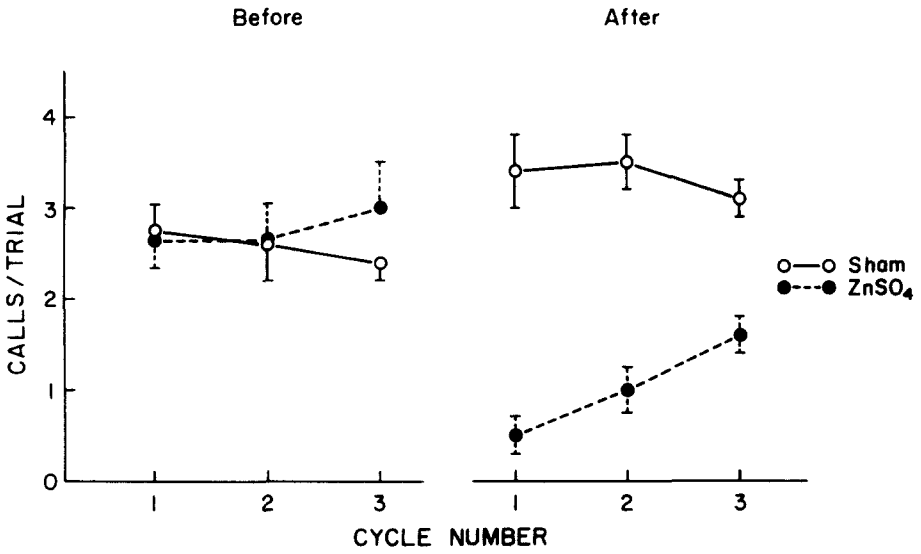


FIG. 4. The mean number (±SEM) of ultrasonic calls per 5-min trial produced by females before and after treatment with saline (SHAM) and with zinc sulfate ($ZnSO_4$). Data are presented separately for the three estrous cycles before and after treatment. (Experiment 3)

beginning of the dark phase of the light cycle in a room containing no other hamsters.

Zinc sulfate treatment was accomplished by methods previously described (23,26,52). A female was lightly anesthetized with ether, secured on her back on an inclined board with her head down, and 0.5 cc of 5% zinc sulfate solution in 0.5% saline was introduced into the nasal cavity via the nasopharyngeal meatus. Excess solution drained out the nares; its removal was aided by gentle aspiration. Control females were treated with saline in the same manner. Zinc sulfate kills the majority of the currently extant receptors in the olfactory mucosa but spares those in the vomeronasal organ (52). After treatment nasal tissues are swollen and the passages are filled with sloughed tissue. Some recovery of function occurs within 4–6 days and is probably initially due to decreased swelling and blockage of the nasal passages; later recovery is due to regeneration of olfactory mucosa (12,23). Thus over a 12-day testing period some recovery of function is expected. Six additional females that were treated with zinc sulfate were not used in the experiment because of adverse reactions to the treatment; three had considerable nasal congestion, had some trouble breathing, and were rather lethargic; three others died, presumably from ingestion of some of the zinc sulfate. The animals used in the experiment all seemed to be normally active and responsive; some exhibited a kind of sniffly breathing. All maintained regular 4-day estrous cycles.

The effects of zinc sulfate treatment on the animals' ability to detect odors were assessed by an independent behavioral test. The time that females took to find a cup containing peanut butter that was buried under approximately 1 cm of bedding was measured 6 and 12 days after treatment. Peanut butter was placed on the bottom of a disposable plastic cup (2 cm diameter, 2 cm high) that was covered with Kimwipe tissue. Ten small holes were punched in this tissue. After a female was removed from her home cage, the cup was buried under the bedding and the female was returned to her cage. The latency to find the cup was measured. The criteria for detection were that the female had to (i) stop and sniff directly over the cup for at least 2 seconds and (ii) uncover at least part of the top of the cup, either by snout movements or digging. These tests were carried out 1–2 hours after the daily behavioral trial.

Effects of the zinc sulfate treatment on the olfactory mucosa were assessed by histology of the nasal cavity. Females were deeply anesthetized with sodium pentobarbital and perfused with saline followed by 10% formalin. The snout was decalcified, embedded in parafin, sectioned at 40 microns and stained with hematoxylin and eosin. Every fifth section was examined for healthy olfactory mucosa. The percentage of potentially functional olfactory mucosa remaining on each side of the nose was estimated (0–5%, 5–10%, etc.) with reference to untreated controls and the average of both sides served as a summary for that section. All percentages were then averaged to reach an overall measure for each animal. Potentially functional mucosa was defined as areas of olfactory mucosa in which a thick layer of cells were observed; whether the receptors were actually functional was not possible to determine.

Behavioral results were analyzed with a repeated measures ANOVA using the SYSTAT statistics package and by *t*-tests for paired comparisons. Since performance changed with time after zinc sulfate treatment the data are presented in blocks of four days, representing the first, second, and third estrous cycles following treatment.

## RESULTS AND DISCUSSION

Treatment of females' nasal cavities with zinc sulfate affected ultrasonic calling, vaginal marking, and flank marking (Figs. 4–6); the results for each behavior are presented separately.

Females treated with zinc sulfate greatly reduced their frequency of ultrasonic calling in response to odors of other hamsters whereas females treated with saline actually called more frequently, yielding a highly significant interaction effect [$F(1,19) = 30.401$, $p < 0.0001$, data collapsed across all three cycles; see Fig. 4]. Before treatment the zinc sulfate group produced a mean of $2.7 \pm 0.4$ calls per 5-minute trial whereas afterward they produced $1.0 \pm 0.2$ calls per trial ($t = 4.333$, $p < 0.005$). In contrast, the sham treatment group called significantly more often after saline treatment ($3.3 \pm 0.2$ calls per trial) than they called before it ($2.6 \pm 0.2$; $t = 3.219$, $p < 0.005$; Fig. 4). The zinc sulfate group called significantly less than the sham group after treatment ($t = 8.852$, $p < 0.0005$) whereas the two groups were not different before treatment.

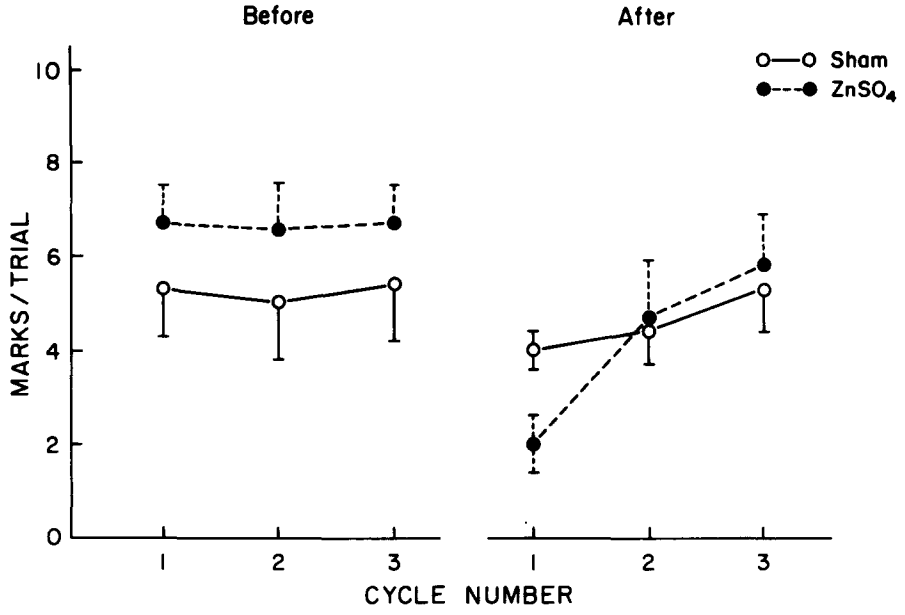


FIG. 5. The mean number (±SEM) of vaginal marks per 5-min trial produced by females before and after treatment with saline (SHAM) and with zinc sulfate ($ZnSO_4$). Data are presented separately for the three estrous cycles before and after treatment. (Experiment 3)

Figure 4 shows the data on the basis of each of the three 4-day estrous cycles before and after treatment. Females maintained a relatively consistent rate of calling during all three cycles before treatment; saline-treated controls called at similar rates after treatment. Those females treated with zinc sulfate dropped to very low levels of calling during the first cycle after treatment but increased calling frequency over the following two cycles [for cycle 1 vs. cycle 3, $t(9) = 3.6$, $p < 0.005$; for cycle 2 vs. cycle 3, $t(9) = 5.81$, $p < 0.0005$]. Nonetheless the experimental group called less than the saline-treated controls in each of these 4-day periods: cycle 1, 0.5 ± 0.2 versus 3.4 ± 0.4 calls per trial, $t = 6.598$, $p < 0.0005$; cycle 2, 1.3 versus 3.5 ± 0.3 calls per trial, $t = 6.998$, $p < 0.005$; cycle 3, 1.6 ± 0.2 versus 3.1 ± 0.2 calls per trial, $t = 4.67$, $p < 0.0005$. Thus despite a tendency for calling rates to recover over time in lesioned females, calling rates remained lower than those observed in control females.

The frequency of vaginal marking was also reduced by treatment of the nasal mucosa with zinc sulfate (Fig. 5). Both groups tended to mark less after treatment, and this is reflected in a significant main effect for treatment [$F(1,19) = 21.6$, $p < 0.0001$]. The zinc sulfate group, however, shows a greater decline after treatment than the sham-lesioned controls [interaction effect, $F(1,19) = 16.43$, $p < 0.02$]. When paired comparisons are made of vaginal marking rates for each group before and after treatment the females in the zinc sulfate group marked more before treatment (6.6 ± 0.7 times per trial) than after treatment (4.2 ± 0.8 times per trial; $t = 5.531$, $p < 0.0005$), whereas the sham treatment group did not show a significant decrease (6.6 ± 0.7 marks/

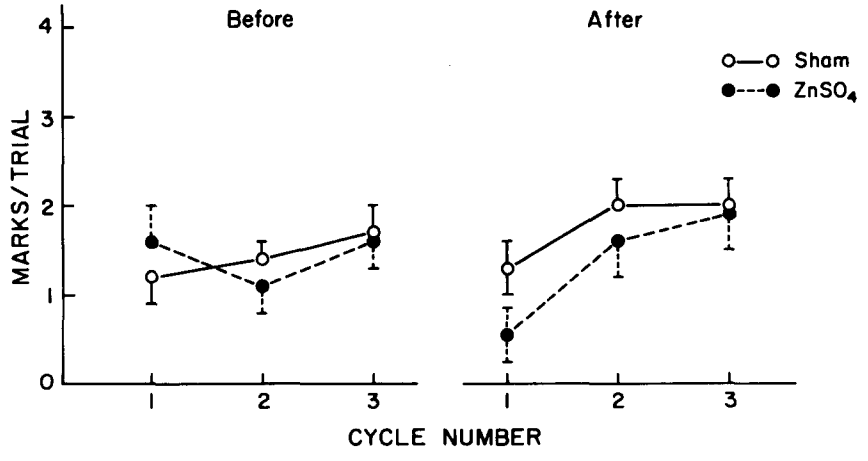


FIG. 6. The mean number (±SEM) of flank marks per 5-min trial produced by females before and after treatment with saline (SHAM) and with zinc sulfate ($ZnSO_4$). Data are presented for the three estrous cycles before and after treatment. (Experiment 3)

trial before treatment vs. 5.3 ± 0.5 marks/trial afterwards; $t = 1.51$, $0.05 < p < 0.1$).

Apparently due to the fact that the experimental groups started off marking slightly more than the control group during the baseline condition there was no overall difference between the treatment and control groups after treatment. However, when the data were analyzed separately for the first, second, and third cycles following treatment, vaginal marking by the zinc sulfate group was significantly less than the sham group during the first estrous cycle after treatment, 2.2 ± 0.6 marks per trial for those with olfactory impairment versus 4.0 ± 0.4 marks per trial for controls ($t = 2.867$, $p < 0.005$; see Fig. 5). The two groups did not differ in vaginal marking rate during the second and third cycles after treatment.

Flank marking was also affected by zinc sulfate treatment, although the effect was somewhat less than that for the other two behaviors (Fig. 6). There was a barely significant interaction effect, indicating that marking rate after treatment depended on whether the females received zinc sulfate or saline infusion into the nose [$F(1,19) = 4.272$, $p = 0.053$]. The effect is slight, however, and paired comparisons of the data summarized over all trials do not indicate significant differences within the zinc sulfate group before and after treatment, nor between the zinc sulfate and control groups. If the data for different cycles after treatment are considered, the zinc sulfate group flank marked less than the control group during cycle 1; the mean number of flank marks per trial during this 4-day period was 0.5 ± 0.3 for zinc sulfate group and 1.3 ± 0.3 for the sham group ($t = 2.167$, $p < 0.025$). Similar comparisons of flank marking rates during cycles 2 and 3 were not significant. The relatively minimal effect of olfactory lesions on flank marking by females is surprising since similar experiments with male hamsters showed striking effects (23). However, the marginal effects of the lesions could be due to a "floor" effect—since relatively few marks were performed it would be difficult to see a depression in these rates. The stimulus conditions in this experiment were not ideal for eliciting flank marking from females, since both female and male odors were present in the test arena. Flank marking by females is quite variable in response to male odors and some females rarely mark in such conditions (17). Females do mark at consistently high rates to odors of other females; the effects of olfactory lesions on flank marking in response to female odors alone is examined in Experiment 4.

The peanut butter location task revealed that $ZnSO_4$-treated females were deficient in detecting the presence of this attractive scent in their cages (Table 2). The saline-treated control females spent 12.6 ± 1.8 seconds and 13.3 ± 1.1 seconds in locating the buried cup containing peanut butter on day 6 and day 12 after treatment, respectively. In contrast the females treated with zinc sulfate spent 176.9 ± 39.4 seconds on day 6 and 85.6 ± 32.5 seconds on day 12 in finding the buried cup. On day 6 four of these females never found it. The differences between saline and zinc-treated females are highly significant: 6 days after treatment, $t = 4.382$, $p < 0.0005$ and 12 days after treatment, $t = 2.336$, $p < 0.025$. Females treated with zinc sulfate showed considerable improvement in the latency to find the cup in the second test (day 6 vs. day 12, $t = 2.321$, $p < 0.025$). This is most probably due to improvement in their olfactory abilities and not to learning about the testing procedure, since the saline-treated females did not show any improvement. Similar tests with males indicated that there was also striking improvement in function between four and six days after zinc sulfate treatment (23).

Histological analysis revealed that a mean of 18% of the olfactory mucosa was potentially functional; the range across animals was 8–30%. These figures are comparable to those obtained previously in hamsters using similar techniques (23,41,52). There were no statistically significant correlations between level of behavioral responses and extent of potentially functional olfactory mucosa. No deficits were observed in vomeronasal organs.

TABLE 2

MEAN LATENCY (±SEM) IN SECONDS FOR FEMALES TO FIND A SCREENED CUP CONTAINING PEANUT BUTTER THAT WAS BURIED IN THE ANIMAL'S HOME CAGE (EXPERIMENT 3)

| | Saline Controls | $ZnSO_4$ Lesions |
|---|---|---|
| Day 6 | 12.6 ± 1.8* | 176.9 ± 39.4† |
| Day 12 | 13.3 ± 1.1 | 85.6 ± 32.5 |

* $p < 0.0005$; † $p < 0.025$; comparisons between controls and lesioned females.

Thus, under these testing conditions, treatment of the nasal mucosa with zinc sulfate clearly reduced ultrasonic calling and vaginal marking by females in response to odors of other hamsters; such lesions also seemed to reduce flank marking, but the effects were considerably smaller and somewhat equivocal.

## EXPERIMENT 4

In this experiment I investigated the effects of olfactory lesions on flank marking by females tested in the presence of the odors of other females, conditions better suited to eliciting such marking than those used in Experiment 3 (17).

### METHOD

Twelve females (15 months of age) were selected for use as experimental subjects; 10 additional females were used as stimulus animals. Estrous cycles were monitored as described in Experiment 3.

Females were observed in a repeated measures design. First they were observed for 12 consecutive days and, after a break of 9 days, for another 6 consecutive days; these two periods together constituted the habituation phase of testing. Since three females exhibited low and quite variable levels of flank marking, they were dropped from the experiment. The day after the end of the habituation period the remaining nine females were treated intranasally with saline solution and observed on the following 6 days. The day after this control condition was completed the females were treated intranasally with 0.5 ml of a 5% zinc sulfate solution, and observed on the following 6 days. One female died, apparently from the zinc sulfate ingestion, leaving eight females in the experimental group.

Observations were carried out in a 61 × 61 cm wooden arena as described in Experiment 3. Prior to the first trial the arena was cleaned thoroughly and then a nonexperimental female was allowed to explore and scent mark the arena for 20–30 minutes. Four experimental females were then observed in the arena for 5 minutes each. Then another nonexperimental female was allowed to mark and explore for 20 minutes, after which the remaining four females were tested. The number of flank and vaginal marks during these 5-minute trials was recorded. The observations began about 2 hours after the onset of the dim phase of the light cycle. On days 4 and 6 of the saline and zinc sulfate conditions, two hours after the end of the marking observations, females were tested for their ability to discover a buried peanut butter cup (see Experiment 3).

Histological verification of the effects of $ZnSO_4$ treatment on the olfactory receptors was carried out as described above in Experiment 3.

The data are presented as mean marks per trial, averaged across all days of each condition; the data could not be grouped according to first and second cycles since there were only 6 days of testing in the saline control and zinc sulfate conditions. Daily means were not appropriate since marking varies with estrous cycle day and the cycles of the females were not synchronized.

## RESULTS

Treatment of the nasal mucosa with zinc sulfate clearly reduced the frequency of flank marking in response to odors of other females (Fig. 7). Females flank marked a mean of 2.4 ± 1.0 times per trial after treatment with zinc sulfate, a rate that was significantly less than that seen either during habituation trials (4.5 ± 1.3; $t = 2.392$, $p < 0.025$) or after treatment with saline (7.2 ± 0.8; $t = 6.094$, $p < 0.0005$). All eight females flank marked less after treatment with zinc sulfate than after treatment with saline.

Flank marking was more frequent following saline treatment than during the habituation period ($t = 4.641$, $p < 0.005$). It seems unlikely that marking in response to odors was actually stimulated by saline treatment, since we have not seen any similar results in other experiments and there are no reports of any effects of saline treatment in the literature. It is more likely that this reflects the general tendency for flank marking to increase in frequency with increasing familiarity of the animals with the testing situation (15).

Vaginal marking was also clearly influenced by zinc sulfate treatment (Fig. 7). The vaginal marking rates during habituation and saline treatment conditions were very similar but were greatly reduced in the zinc sulfate condition (habituation vs. zinc, $t = 6.937$, $p < 0.0005$; saline vs. zinc, $t = 9.799$, $p < 0.0005$). All eight females marked less following zinc sulfate treatment than following saline treatment or in the habituation condition.

The independent measure of olfactory function indicated that during the saline condition females took a median of 27 and 21 seconds to find the peanut butter cup on days 4 and 6 of the testing and a median of 58 and 38.5 seconds to find the peanut butter on the two tests during the zinc sulfate condition. The difference between the saline day 4 test and the zinc sulfate day 4 test was significant, indicating a loss of ability to detect a novel and moderately attractive odor in the home cage (Wilcoxon matched pairs signed ranks test, saline day 4 vs. zinc day 4, $p < 0.05$). The second, post-zinc sulfate peanut butter test was not statistically different than the tests run during the saline condition, whereas the difference between the day 4 zinc test and the day 6 saline test just missed significance ($p = 0.074$) due to one female in the saline condition that took an exceptionally long time to locate the peanut butter. These tests thus show that zinc sulfate treatment caused at least a temporary decrement in olfactory function, although the deficits did not appear to be as severe as those in Experiment 3.

Histological analysis revealed that in the lesioned animals a mean of 17.2% of the olfactory mucosa was potentially functional; the range was 2.6–47.5%. The unusual animal at the high end of the distribution was not, however, unusual on any of the behavioral measures, suggesting that much of the olfactory deficits caused by this treatment may be due to nasal congestion (23). No deficits were observed in the vomeronasal organs.

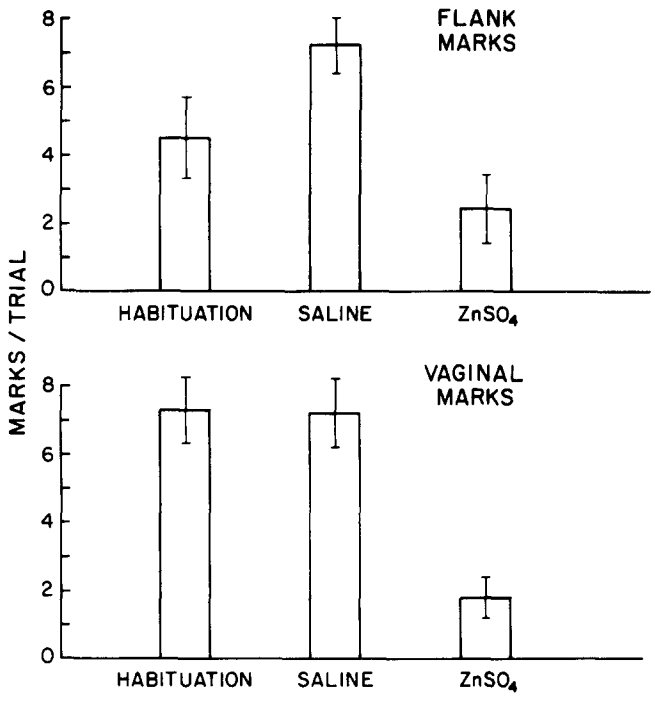


FIG. 7. The mean number (±SEM) of flank marks and vaginal marks by female hamsters during three phases of daily 5-min exposures to odors of other females–a habituation or baseline condition, after treatment with saline, and after treatment with zinc sulfate ($ZnSO_4$). (Experiment 4)

## GENERAL DISCUSSION

These experiments indicate that in female hamsters, the stimulation of both flank and vaginal scent marking by odors of conspecifics is mediated by the olfactory system, but not by the vomeronasal system, whereas ultrasonic calling in response to such odors is mediated by both the olfactory and vomeronasal systems. Previously it had been shown that removal of the olfactory bulb, a procedure that eliminates both olfactory and vomeronasal function, reduced the frequency of ultrasonic calling and both marking behaviors (27,29). Thus the current findings refine our knowledge about the neural mechanisms underlying these three behaviors. The mediation of flank marking in male hamsters is similar to that in females; flank marking in males is reduced by olfactory lesions but is not influenced by vomeronasal lesions (23).

It is noteworthy that in all of these lesion studies with hamsters, both those in which only one system was selectively lesioned and those in which the entire olfactory bulb was removed, neither scent marking behaviors nor ultrasonic calling were eliminated completely (23,27,29). This indicates that there is some level of all of these behaviors that is endogenously generated and that does not depend on conspecific scent. Endogenous causation makes sense for all of these behaviors, since they all function to some extent as advertisements of an individual's presence in the area. It would not make sense for an individual to cease such advertisement completely because of a temporary loss of olfactory function or the temporary absence of conspecific scent in the environment (10,15,17,19).

The degree to which we understand the effects of the two lesion techniques differs considerably. Vomeronasal organ removal is much easier to interpret than treatment of the nasal cavity with zinc sulfate. Surgical removal of the vomeronasal capsule is relatively straightforward, the removal is usually complete and any remaining vomeronasal tissue is easy to notice in histology of the nasal cavity. The main uncertainty with this

technique is whether any function remains in the few cases in which a small fraction of normal-looking vomeronasal tissue is observed in the most anterior portion of the organ. In this and other studies we have not observed any behavioral differences between animals with complete lesions and those with small pieces of remaining tissue (23–26). A new staining technique that indicates the presence of functional connections to the accessory olfactory bulb from the vomeronasal organ should help clarify this issue in future studies (54). Zinc sulfate lesions of the olfactory mucosa are more problematic. One difficulty is that this substance is toxic and can kill or debilitate animals if it gets into the stomach. We observed females after treatment and eliminated any from the experiment that were sick or lethargic; all the animals we used seemed to be normally active and responsive. Although we did not measure activity quantitatively it seems unlikely that the deficits could be due to generalized lethargy or depression. If this were true one would expect more animals to exhibit no marking or calling, whereas all animals in our experiments did continue to mark and call to some extent. In Experiment 3, for example, there was only a slight reduction in flank marking by females, whereas ultrasonic calling and vaginal marking were reduced substantially. Furthermore, in other studies similar lesions have had no effect on male mating behavior or female maternal behavior (32,38), suggesting the lack of general deficits.

Another problem with the zinc sulfate method is that such treatment does not kill all of the cells in the olfactory mucosa and it is not always clear whether the remaining tissue is functional. In our histological analysis we erred in a conservative direction, calling any tissue that had some cells remaining in the olfactory mucosa potentially functional. We may thus have greatly overestimated the percentage of functional tissue, especially since access of odors to the receptors may be blocked by nasal congestion. When no deficits are seen after zinc sulfate treatment it is not clear if the lack of effects are due to no involvement of the olfactory system or to lack of effect of the technique. In the present studies we observed effects on the behaviors in question as well as on the independent measures of olfactory function, indicating clear deficits due to the lesions.

The sensory mediation of ultrasonic calling and of scent marking behaviors seems to differ in different species of rodents. Ultrasonic calling by male house mice in response to other mice is not affected by lesions of the olfactory mucosa with zinc sulfate but is reduced after vomeronasal lesions or nerve cuts (1,55). Urine marking by mice is also influenced by the vomeronasal system; for male mice that have had their vomeronasal organs removed, urine marking in a clean arena was less than for intact males and was also less than when either a male or a female was present across a screen barrier (35). Reductions in urine marking by male mice have also been observed after treatment of the nasal cavity with zinc sulfate, suggesting that the main olfactory system may be involved as well (33). Unfortunately in this latter study no histology of the vomeronasal organ was performed so one cannot rule out the possibility that the zinc sulfate damaged the vomeronasal receptors.

Correlated with these differences in sensory mediation of marking and calling in mice and hamsters are differences in the stimulus conditions that stimulate these behaviors. Hamsters scent mark at low to moderate rates in clean areas and are stimulated to mark by odors of conspecifics (15–17). In contrast male and female house mice urine mark at high baseline rates all the time and, although urine marking can be stimulated by odors or the presence of conspecifics, any novel stimulus, such as a clean test chamber or an individual of another species, can stimulate high levels of marking. It is believed that novelty, rather than a specific chemical signal, is the primary feature that elicits increases in urine marking in house mice (2,33,34). In the case of ultrasonic calling, male and female hamsters call in response to odors or other stimuli from members of the opposite sex; females call most when estrous (8,11). The sources of scent from male hamsters that stimulate female calling are not known, but vaginal secretion scent is the most important female odor that stimulates male calling (22). Hamsters use such calls to locate potential mates and maintain proximity to them (10). In house mice, socially experienced males call in response to urine odors of females but not those of males; the relevant cues appear to be heat-resistant, relatively large molecules that are modulated by hormones (43,51). These calls are apparently involved in male–female interactions but their precise communicative functions are not understood. Thus, for ultrasonic calling and scent marking behaviors there are differences across species in the stimuli that cause them and probably in their functions, so it is not surprising that the sensory mediation of these behaviors also differs across species. On the other hand it is not clear why the role of the two chemosensory systems differ—why the types of behaviors mediated primarily by the vomeronasal or olfactory system are not the same.

In hamsters we are beginning to develop a catalogue of odor-mediated responses that can be classified as being mediated by the vomeronasal system, the olfactory system, or both. The vomeronasal system is necessary for the increases in circulating testosterone levels seen in males after exposure to female odors (24,25,45), a result that is consistent with results in other species, all indicating that the vomeronasal system is especially important for hormonal responses to scent (39,53,54). In contrast the olfactory system in hamsters is necessary for odor-mediated flank marking by males (23), flank and vaginal marking in females (this study), and male attraction to female odors from a distance (46). Both systems seem to be involved in odor-mediated ultrasonic calling and in male sexual behavior (38).

What hypotheses can explain the roles of the olfactory and vomeronasal systems? Can differences in function be explained by the size of the molecules that serve as the relevant signal? This hypothesis could possibly explain the role of the vomeronasal system in mediating male copulatory behavior, since the vomeronasal system has been implicated in mediating responses to a high molecular weight compound from vaginal secretions that stimulates copulatory behavior (4,49); it is also known that volatile components of the secretion contribute to sexual arousal, which could be explained by a parallel role for the olfactory system (18,44). However, the vomeronasal system could be sensitive to volatile components of the vaginal secretion as well (44). Furthermore, the molecule-size hypothesis cannot entirely explain the vomeronasal mediation of hormonal responses by male hamsters to vaginal secretions, since such responses can be obtained when males do not contact these secretions and thus do not have access to large molecules (31). What about the mediation of scent marking by the olfactory system and ultrasonic calling by both systems? The chemical nature of the cues stimulating calling and scent marking are not presently known, although in the case of flank marking by males it is known that the odors of the flank gland itself are the most important source of such cues (16). It is also known that in ultrasonic calling by males in response to female odors, the vaginal secretion is the scent of most importance (22).

Another hypothesis that has been advanced to explain some of the differences between the two systems is that the main olfactory system may be more likely to mediate behaviors that depend on pattern recognition mechanisms, such as individual or species recognition (20,23). Both vaginal marking and flank

marking are stimulated by conspecific odors but not by odors of a closely related species (21); it could be that discrimination of conspecific from heterospecific scent is mediated through pattern recognition mechanisms (e.g., discrimination of differences in odor qualities of complex mixtures of compounds). This hypothesis is supported by results implicating the main olfactory system in individual discrimination by hamsters and spiny mice (26,36). However, in hamsters the vomeronasal organ may also be involved in recognition of individual odors. Steel and Keverne (50) have shown that removal of the vomeronasal organ eliminates a male hamster's ability to distinguish the scent of a female whose vaginal secretions were familiar from scent of female whose vaginal secretions were unfamiliar. They also claimed that the main olfactory system was not involved because the discrimination abilities of males with zinc sulfate lesions were not affected; unfortunately in this latter experiment they did not actually test males' abilities to discriminate familiar versus unfamiliar individuals so the experiment cannot be interpreted as a lack of effect of zinc sulfate treatment on individual discrimination.

It is clear that at present we do not yet understand the degree to which the functions of the vomeronasal and olfactory systems are distinct and the degree to which they overlap, even in one species. The current experiments contribute to this endeavor by suggesting that in female hamsters odor-mediated flank marking and vaginal marking primarily depend upon the olfactory system, whereas odor-mediated ultrasonic calling depends on both the vomeronasal and olfactory systems. Once we understand the causation of these and other behaviors more thoroughly we may be able to develop a more general theory of the functions of the two systems.

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