Female odors evoke ultrasounds from male mice*

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Four experiments investigated the stimulus properties of female mice that influence ultrasound production by adult males. Female urine alone was less effective in evoking ultrasounds than was the female in male-female pairs. Visual cues were not necessary, since there was no difference in the incidence or latency of ultrasounds from male-female pairs when tested in light and dark conditions. A presently unspecified chemical cue produced by females, whose effectiveness is mediated by olfaction, was sufficient to evoke ultrasounds from male mice. The role of this chemical cue in a multimodal sequential communicative chain is discussed.

Substantial evidence has accumulated in recent years to suggest that ultrasonic emissions are associated with mating behavior in a variety of rodents. For instance, Sales (1972) reported the detection of ultrasounds during mating behavior from all of 11 species of myomorph rodent that she investigated. Among adult laboratory mice (*Mus musculus*), ultrasounds are often detectable almost immediately when opposite-sex individuals are placed together (Whitney, Stockton, & Tilson, 1971). These very short latency ultrasounds are probably produced by the male member of a pair, since ultrasonic emissions were frequently detected from unlike-sex pairs when the female was anesthetized but never when the male member of a pair was anesthetized. The suggestion that these short latency ultrasounds detectable from unlike-sex pairs are related to male mating behavior is supported by the finding that they are eliminated by castration and reinstated following testosterone propionate treatment in some males (Coble, Whitney, & Alpern, 1973).

A communicative function in connection with early stages of male mating behavior, akin to a courtship display, has been hypothesized for these ultrasonic emissions. Briefly, the suggestion was that, since postparturition female mice are quite aggressive against strange males and since infant mice produce ultrasounds that may direct maternal approach and inhibit too severe grooming or "aggression" from lactating females, the male ultrasonic emissions might represent an infant-like courtship display that functions to inhibit female aggression and, thus, facilitate mating during the postparturition estrous period (Whitney, Coble, Stockton, & Tilson, 1973). However, active female aggression is not a necessary prerequisite for male ultrasound production, since males produce ultrasounds upon initial encounter with a nonaggressive female and even when paired with an anesthetized female.

Since female movement does not appear to be

necessary to evoke ultrasounds from at least some males, the experiments reported here were conducted to gain some initial information concerning the characteristics of female stimuli that are involved in the production of short latency ultrasounds by male mice.

EXPERIMENT I

Since chemical constituents of both male and female mouse urine have been implicated in the control of aggressive behavior (Connor, 1972) and male urine is known to affect female reproductive performance (Gleason & Reynierse, 1969), the efficacy of female urine to evoke ultrasonic emissions from males was investigated.

Method

Subjects

A total of 24 male mice served as Ss. Ten were of the DBA/2J inbred strain and were 84-95 (range) days of age when tested, 10 were C57BL/6J males 63-74 days of age, and four were genetically heterogeneous offspring derived from a selection experiment (Coble, 1972) that were over 100 days of age when tested. All males were individually housed for at least 14 days before testing.

Ten adult female mice over 100 days of age served as stimulus animals and were donors of the stimulus urine. They were eight C57BL/6J $\times$ A/J F_1 hybrids and one each from the I^S/Bi and 101 Bag/R1 inbred strains.

Apparatus

Ultrasounds were detected with a Holgate ultrasonic receiver mk IV set to a center frequency of 70 kHz. The setting was selected on the basis of prior results indicating that adult male ultrasounds were most intense at about 70 kHz. The receiver had been calibrated against Bruel and Kjaer equipment consisting of a 1/4-in. condenser microphone (No. 4136), microphone amplifier (No. 2604), and bandpass filter (No. 1612). The Holgate was sensitive to signals within about 10 kHz of indicated frequency and was adjusted so that all scored signals were of an intensity greater than 60 dB (re .0002 dynes/cm²).

The test chamber was constructed of clear Plexiglas and measured 24.5 x 12.3 x 25.4 cm high. Opposite each other across the long dimension of the test chamber were startboxes 6.5 x 6.7 x 15.3 cm high. The startboxes were separated from

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the test chamber by metal guillotine doors. The floor of the apparatus was a table surface covered by brown paper toweling. Maryland Plastics Model 110 metabolic cages were used to collect urine. For testing, a urine-saturated cotton ball was placed in a 6.4-cm-deep x 1.9-cm-diam glass vial. A hand-operated stopwatch was used to measure latencies.

Procedure

Stimulus females who also served as urine donors were placed singly in metabolic cages with ad lib food and water 48 h before a test session. The F_1 hybrid females were used with the C57BL/6J and DBA/2J males who were tested in alternate order on each of 2 test days.

On each day each male was tested twice, with at least 20 min between test occasions. For each male one of the tests was conducted in the presence of female urine and the other test was in the presence of the individual female who was the urine donor for that male on that day. Order of presentation was counterbalanced so that half the males were first tested with a female and then tested with that female's urine; the other half of the males were tested with urine before the female. The second test day, which was 48 h later, was an exact replication of the first test day, except that each male encountered a different female and her urine.

For testing with a female, the male was placed in one startbox and the female in the other startbox. After 60 sec had elapsed, the guillotine doors on both startboxes were raised simultaneously. Immediately after detection of ultrasounds, or 3 min after the doors were raised, the stopwatch was stopped and the test Ss were removed from the chamber. If no ultrasounds were detected during the 1-min pretest period or during the 3-min test, the latency for that occasion was recorded as 180 sec.

For testing with female urine, a cotton ball was saturated with urine from the collection receptacle of the metabolic cage and then inserted in the vial. The vial was then placed in the center of the test chamber at the same time that the test male entered the startbox. The rest of the procedure was identical to that during a test with a female. No animal other than the test S(s) was in the experimental room during testing, the test chamber was swabbed with a 70% ethanol solution, and paper floor was changed between each test. All testing was conducted with fluorescent room lights providing about 43 fc incident illumination, and all animals were tested at about the same time each day.

The procedure for testing the four heterogeneous males was similar, except that each male was tested on 4 different days, 2 days with each of the two inbred strains of female.

Results

Urine from a female was less potent as a stimulus, to evoke detectable ultrasounds than was the female herself for all genotypes of male that were tested.

On the first test day, all 10 DBA/2J males produced detectable ultrasounds during the 3-min test, with a mean latency of 23.95 ± 2.52 sec when tested with a female. However, only five males produced ultrasounds (mean latency 30.68 ± 15.09 sec) when tested with urine from the same female. The difference between test conditions across individual males approached significance ($p < .172$) by the sign test. On the second test day, only two DBA/2J males produced ultrasounds in the presence of the urine, although ultrasounds were detected from all 10 male-female pairs (mean latency 22.01 ± 6.60 sec, $p < .002$ by the sign test).

The results for the C57BL/6J males were in the same direction but were not statistically significant due to the low incidence of ultrasonic emission under both test conditions. C57BL/6J males produced ultrasounds on none of the 20 test occasions with urine, although ultrasounds were detected on 7 of the 20 test occasions with females. A generally low incidence of short latency ultrasound production by C57BL/6J males has been reported from previous investigations (Coble, 1972).

The results were similar for the four genetically heterogeneous males who were tested with inbred strain females and their urine. Ultrasonic emissions were detected on all 16 tests in the presence of a female, with a mean latency of 9.08 ± 1.39 sec. However, ultrasounds occurred during only 3 of the 16 tests with the urine of the same females.

A number of interpretations are possible from these data. One is that, whatever it is about a female that evokes ultrasonic emissions from males early in male-female encounters, it is not primarily some chemical substance contained in female urine. During the tests virtually all males orientated toward and actively investigated the vial containing female urine, but usually without detectable ultrasonic emissions. However, ultrasounds were frequently detected on the same test days when the males were in the presence of the urine-donor female. It should be noted that estrous state of the females was not controlled, and it may be that the efficacy of female urine varies as a function of estrous cycle. However, urine alone vs the urine-donor female was tested, and ultrasounds were detected from the tests conducted on the same day with these females much more frequently than from tests with their urine.

A second possible interpretation from this experiment is that the female is frequently the animal producing the ultrasounds detected from unlike-sex pairs, since short latency ultrasounds were frequently detected from male-female pairs but not from male-female urine pairs. By this interpretation, previous experiments in which ultrasounds were not detected when normal females were tested in the presence of anesthetized males (Whitney et al, 1973) could be taken to indicate that it is the behavior of the male that evokes ultrasounds from females. However, the presence of an awake female is not a necessary condition for a high incidence of ultrasounds to be detectable from pairs of adult mice since, in the previous experiments, ultrasounds were frequently detected when normal males were tested with anesthetized females.

EXPERIMENT II

From previous results, it is unlikely that some active component of female behavior or auditory stimuli from the female member of a pair is important in the production of short latency ultrasounds by males in male-female pairs, since ultrasonic emissions were frequently detected from pairs in which the female but

not the male was anesthetized (Whitney et al, 1973). Experiment I above suggests that chemical cues associated with female urine are at best very weak stimuli for male ultrasound production. Perhaps some combination of stimuli involving more than one sensory channel is most effective. However, the primary remaining single sensory modality that has not been tested is the visual. It is possible that an important component of female recognition preceding ultrasound production by male mice is mediated visually. This experiment was conducted to investigate the contribution of visual cues to ultrasound production from male-female pairs of mice.

Method

Subjects

Eight DBA/2J males 103 ± 2 days of age at the initiation of testing served as Ss. The stimulus females were eight C57BL/6J $\times$ A/J F₁ hybrids who were 107 days of age. All animals had been individually housed for at least 14 days before initiation of testing.

Apparatus

The apparatus was identical to that of Experiment I, except that the males' home cage was used as the test chamber. The 29 x 18 x 13 cm cage was constructed of transparent plastic, and the floor was covered with about 1 cm of wood chip bedding.

Procedure

Each male was tested once at about the same time each day for 8 consecutive days. The test condition (light or dark) was alternated daily so that each male was tested four times in each condition. The order of conditions was counterbalanced across Ss so that half were first tested under each condition. Each female was used once on each day, and the order of male-female pairing was rotated so that each unique pair occurred only once. The order of testing within each day was also varied so that each male was tested only once in each of the ordinal positions within a day.

The test condition (light or dark) was manipulated by having the fluorescent ceiling lights of the experimental room either on or off during the test procedures. This resulted in an incident light level in the test chamber of 43.0 fc during the light condition and not more than 0.001 fc during the dark condition. Light levels were determined with a Tektronix J16 digital photometer. At the time of testing, light levels outside the experimental room were similar to the light-on test condition.

For each test, the male to be tested was carried into the prearranged (light or dark) experimental room in his home cage from which the top, containing food and water, had been removed. The cage was positioned under the microphone for a 1-min adaptation period. In the dark condition, the E monitored the ultrasonic receiver output from outside the experimental room. If any ultrasounds were detected during the 1-min adaptation time, a score of zero latency was given for that trial and the trial was terminated. Upon termination, the cage bedding was changed and the cage returned to the colony room until the next day.

If no ultrasounds were detected during the adaptation period, the appropriate female was taken into the test room in her home cage. The test trial was then initiated with introduction of the female into the male's cage. The trial was terminated upon detection of ultrasounds or after 180 sec had elapsed without ultrasounds. Upon termination of a trial, the female was

returned to her home cage and scores were recorded. The bedding of the male's cage was changed before being returned to the colony room. No animals other than those being tested were in the experimental room during testing and the testing area was swabbed with a 70% ethanol solution between trials.

Results

Visual cues are not necessary for the occurrence of short latency ultrasounds from male-female pairs. The score used in primary analysis was the mean latency of the four trials under each light condition for each male. The group mean latency to ultrasound production was 8.60 ± 2.27 sec in the light condition and 8.68 ± 2.09 sec in the dark condition; there was no significant difference between conditions by a repeated-measures *t* test ($t = -.31$, $df = 7$, $p > .7$). Zero scores were obtained on only 1 of the 16 trials during the first 2 days of testing and on 5 of the 16 trials during the last 2 days. Thus, zero latency scores did not occur frequently enough to invalidate the present comparison between light and dark test conditions. However, the increase in incidence of zero latency scores over the 8 days of testing was great enough to suggest that some learning may have occurred. A comparison of the number of zero latency scores on the first 2 days vs the last 2 days for each male by a Wilcoxon matched-pairs signed-ranks test was significant at $p < .05$ ($T = 2.5$, $N = 8$).

EXPERIMENT III

Visual cues, female-produced auditory cues, and cues provided by specific topographies of active female behavior are not necessary for the occurrence of a high incidence of short latency ultrasounds from male-female pairs of mice. At the same time, female urine is rarely sufficient to evoke any detectable ultrasounds from males. The next experiment was conducted to see if female chemical cues not specifically urine based might be involved in the occurrence of male ultrasonic emissions.

Method

Subjects

The test Ss were 15 male DBA/2J mice. Fourteen were 146 days of age on the day of testing and one was 130 days. They had been individually housed for at least 14 days prior to testing. Seven of these animals had been Ss in Experiment II above.

Apparatus

The apparatus was identical to that of Experiment I, except that the test chambers were 29 x 18 x 13 cm transparent plastic cages containing about 2 cm of wood chip bedding. Three types of test chamber were used. One type was a clean cage containing fresh bedding. The other two were soiled cages containing soiled bedding, in which either females or males other than the test Ss had been living for 5 days prior to the test. Eight female and three male soiled stimulus cages were used; these cages housed

three to five adult females and one or two adult males, respectively.

Procedure

The 15 Ss were randomly assigned to two groups such that 8 were tested in a male-soiled cage before being tested in a female-soiled cage; the other 7 Ss were first tested in a female-soiled cage. The stimulus cages were randomized within sex for order of utilization and used repeatedly in that order until all Ss completed testing. Thus, each of the three male cages was used five times, while seven of the eight female cages were used twice, and one female cage was used only once. The animals who had been living in a stimulus cage were removed to a holding cage immediately prior to use of that cage in an individual test and were returned to their "home" stimulus cage between trials involving that cage.

Each S was first tested for 1 min in a clean cage with fresh bedding. If any ultrasounds were detected during the 1-min period, further testing of that S was precluded. If no ultrasounds were detected, the clean cage containing the S was removed from the experimental room. The first soiled stimulus cage (either male or female, depending on the order assigned to that S) was immediately positioned under the Holgate microphone, and the S was then introduced into the stimulus cage. Upon detection of ultrasounds, or after 180 sec in the first soiled stimulus cage, the cage with the S was removed from the experimental room and the latency was recorded. The second soiled stimulus cage was then positioned and the test procedure repeated for that cage. If a S produced no detectable ultrasounds when in either the male- or female-soiled cage, then that S was immediately tested with an adult female in a clean cage until ultrasounds were detected or 180 sec had elapsed. All animals were tested at about the same time of day and the experimental room lights were on (light-on condition of Experiment II) during testing.

Results

Chemical cues from adult female mice are sufficient to evoke ultrasonic emissions from adult males. None of the 15 Ss emitted detectable ultrasounds during the 1-min pretest in the clean cage or during the 180-sec test in the male-soiled stimulus cage. However, 13 of the 15 Ss produced detectable ultrasounds with a mean latency of 45.80 ± 12.63 sec when tested in the female-soiled cage. The difference in ultrasound production when tested in male- or female-soiled cages was significant at $p < .001$ by the sign test ($N = 13$, $d = 0$).

Two of the 15 Ss produced no detectable ultrasounds during the 180-sec tests in either the male or female stimulus cage. Both of these Ss were immediately tested in the presence of an adult female to see if they would produce ultrasounds to a female. Ultrasounds were detected after 110.7 sec from one of the resultant male-female pairs and the pair containing the other male produced no ultrasounds during the 180-sec test.

EXPERIMENT IV

The above experiment demonstrated that chemical cues from a cage with soiled bedding in which adult females had been living are sufficient to evoke ultrasonic emissions from adult males. However, since the males had been simply placed in the soiled cage, there was no

way to further specify the sensory modality involved. The sufficient chemical stimulation might involve a volatile substance with detection mediated by olfaction, or some nonvolatile female product that is primarily detectable by taste could be involved. The present experiment was conducted to see if olfactory mediation is sufficient or if contact (taste) is necessary to stimulate male ultrasonic emissions in the presence of chemical products from females.

Method

Subjects

The Ss were eight of the adult male DBA/2J mice that had been Ss in Experiment III. They were individually housed for 18 days between experiments.

Apparatus

The ultrasonic detection apparatus was identical to that described in Experiment I. The testing chamber consisted of a 10 x 24 x 13 cm high stainless steel cage with a 1-cm mesh hardware cloth bottom that was placed inside a 29 x 18 x 13 cm transparent plastic cage. Fresh bedding, along with a clean stainless steel cage was used for the adaptation period. Four soiled cages with soiled bedding, in which adult female mice had been group housed for 5 days, were used in the test conditions, with a separate stainless steel cage for each test condition. The group-housed females whose cages were used on Day 1 ranged in age from 114 to 206 days, with three to four females per cage. For Day 2 the females were 131 to 178 days of age, with three to five females per cage.

Procedure

Each male was tested twice at about the same time each day on 2 consecutive days. On one test occasion each male was tested with the stainless steel chamber supported on small blocks, so that the hardware cloth floor was about 2 cm above 1 cm of bedding material. This distance was sufficient to preclude contact with the bedding. On the other test occasion, the chamber was positioned in the bedding material so that some of the wood chip bedding protruded through the hardware cloth floor of the chamber. Order of testing was counterbalanced within each day, so that four of the Ss were tested above the bedding on the first occasion and in contact with the bedding on the second occasion; the other four Ss were tested in reverse order. At least 30 min elapsed between each test within a day, and the order of testing was reversed between days for each S.

For each test, the S was first tested in the appropriate condition for 60 sec with fresh bedding material and then immediately transferred to the soiled bedding condition. A test was terminated upon detection of ultrasounds or after 180 sec in the presence of the soiled bedding. Following each test, the S was returned to his home cage, and the soiled cage and bedding was returned to a separate room between tests. Each female-soiled cage and bedding was used four times. No animal other than the test S was in the experimental room at the time of testing, and the test surface was swabbed with a 70% ethanol solution between each test.

Results

Olfactory detection of female-produced chemical cues is sufficient to evoke ultrasonic emissions from adult males. It is not necessary that males be in contact with

the bedding material from female cages for males to produce detectable ultrasounds.

In the present experiment, ultrasounds were never detected during the pretest with fresh bedding, although they were detected with a mean latency of 42.63 ± 19.82 sec from six of the tests when the S was in contact with soiled bedding, and ultrasounds were detected with a mean latency of 99.78 ± 27.68 sec from four of the tests when the S was not in physical contact with the soiled bedding. Thus, under the conditions of the present experiment, olfactory stimulation alone leads to about as much ultrasound production as does direct contact with the stimulus material. We cannot account for the generally low incidence of detectable ultrasounds in the present experiment compared to the results of Experiment III. However, two procedural differences may be implicated. First, in Experiment IV the group-housed females from each stimulus cage were not returned to that cage after each test; therefore, it is possible that the effectiveness of the stimuli produced by the females diminished over the test sessions. Some support is found by comparing the results of the tests conducted during the first half of the daily sessions with the results of the second half of the sessions. Ultrasounds were produced in 9 of the 16 tests comprising the first half of the sessions, while only 1 of the 16 tests among the second half of the sessions resulted in detectable ultrasounds. A sign test between the first half and second half was significant ($N = 9$, $d = 0$, $p < .002$). Second, the unfamiliar conditions associated with the stainless steel test chamber and hardware cloth floor may have tended to inhibit ultrasound production.

GENERAL DISCUSSION

The results of the present experiments combined with previous work demonstrate that chemical cues from females, detectable as odors by males, are sufficient to evoke ultrasonic emissions. The source of the active substance is unknown at present, and any or all of the secretory and excretory products of female mice may be implicated. Whatever the substance is, it is either available at greatest concentration from females or else its efficacy in evoking ultrasounds from males is inhibited by other male chemical products since soiled bedding from female cages but not from male cages did evoke short latency ultrasounds. It is tempting to suggest that the active chemical substance is not primarily carried by the urine, since urine from a female was much less effective than was the female herself in evoking ultrasounds in the first experiment. Additionally, urine was of only occasional weak effectiveness in Experiment I, while in Experiment III the female soiled bedding was very effective. This may suggest that urine collected from the metabolic cage in Experiment I owes its degree of effect to occasional contamination with some sufficient secretion that is not a usual constituent

of female urine. However, a cautionary note is required, since under the altered conditions of Experiment IV the overall incidence of ultrasounds evoked by soiled bedding was also quite low.

Although female odor is sufficient to evoke ultrasounds from male mice, there is some indication that a responsive female provides a more effective stimulus configuration than does odor alone. Whitney et al (1973) reported no difference in the incidence of ultrasounds in male-female pairs compared to male-anesthetized female pairs. However, the latency to produce ultrasounds was consistently shorter in the former than in the latter condition. In Experiment I of the present study, the male-female urine pairs resulted in less frequent production of ultrasounds and, when produced, the latencies from these pairs were longer than in the male-female pairs. In Experiments III and IV, a female-produced chemical cue was sufficient; however, the mean latencies in the presence of this isolated cue were longer than those produced by male-female pairs both in this study and in previous work. It may be that an active female merely provides a more efficient presentation of the sufficient chemical cue than do any of the other methods of presentation employed in the above studies, in which case this single cue may be said to mediate the male's response. Alternately, the situation may be more complex in that other specific stimuli mediated by other sensory modalities may in combination provide a more potent stimulus configuration than does the female odor alone.

Whatever the sufficient chemical substance may be, two links in a sequential communicative process that involve different communicative channels are indicated in the control of early phases of mating behavior among mice. Chemical cues from females are detected olfactorily by males; the males then emit ultrasounds that presumably effect female responsiveness, and so on. Further analysis of the communicative processes that proximally mediate the coordination of conspecific interactions in biologically important situations such as mating interactions will be necessary for a satisfactory understanding of the behavior of these animals.

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