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An ephemeral sex pheromone in the urine of female house mice (Mus domesticus)

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by

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Acknowledgments

I dedicate this thesis to my parents, Laszlo and Denise Sipos, and family with all my love. I also dedicate this work to my brother, Eric, for being the perfect role model for a younger brother to follow.

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Abstract

From previous research, the ultrasonic vocalizations of male house mice (*Mus domesticus*) to female mouse urine were hypothesized to be learned as a result of classical conditioning during adult heterosexual encounters. According to this interpretation, a previously neutral conditioned stimulus (CS) in female urine comes to elicit vocalizations as a result of its association with some other unknown unconditioned stimulus (UCS) associated with adult females. However, the research from which this hypothesis was derived utilized urine collected in metabolic cages. The research presented in this thesis reevaluated the classical conditioning interpretation utilizing freshly voided urine.

In Experiments 1-3, evidence is presented that male vocalizations to freshly voided urine do not parallel the previous findings which gave rise to the classical conditioning interpretation and that freshly voided urine may actually contain a second, more potent chemosignal not present in metabolic-cage-collected urine. This second chemosignal would appear to be a UCS for the elicitation of ultrasonic vocalizations.

Experiments 4-6 assessed whether the rapid loss of pheromonal activity of the UCS in freshly voided urine was due to volatilization or degradation. The findings from these studies were consistent with the UCS being nonvolatile and its loss from urine being caused by degradation.

Thus, the results of this thesis suggest that two chemosignals exist in female mouse urine that elicit vocalizations from males: (1) a potent, nonvolatile but easily degraded (by heat and possibly other factors) UCS to which males vocalize without sexual experience and (2) a nonvolatile, chemically stable CS.

Chapter 1: Introduction & Literature Review

This thesis consists of six experiments that compare the physical characteristics of two urinary sex pheromones of female house mice. Both pheromones cause sexual arousal and elicit 70-kHz ultrasonic vocalizations from males. One pheromone has a fade-out time measured in weeks or months, is learned as a result of experience, and has been extensively studied (Nyby & Whitney, 1978). The other pheromone has a fade-out time measured in hours, is innately recognized, and is described for the first time in this thesis. In order to better understand and interpret my results, a brief review of literature is presented first.

Pheromones: A Source of Communication

The term pheromone is commonly defined as "an external chemical secretion having conspecific communication functions (Gleason & Reynierse, 1969)." Although the concept of pheromones grew out of early experimentation with sex attractants in insects (Kullenberg, 1956), the concept is now widely applied to mammals as well. Doty (1986) enumerated the types of information that most likely would be conveyed by mammalian pheromones (see Table 1).

Doty (1986) points out that although each type of message in Table 1 has visual and auditory analogs in most species, potential benefits exist for using chemosignals in many situations. First, odors can be used in situations in which either auditory or visual signals are ineffective (e.g., at night or near a source of loud sounds). Second, odors can be deposited quickly and easily, making them ideal for providing information about

territory occupation. Territorial marking often occurs prior to physical encounters and may prevent physical conflicts by minimizing intermale contact. Such non-violent displays can also serve in maintaining dominance. Mice often advertise their territory ownership by scent marking. Third, odors can remain in the environment for longer periods of time than visual or acoustic signals without endangering the signalling individual. If an animal emitted a continuous noise or visual signal, it might fall victim to predators. Finally, odors allow for communication even if the sender and receiver are not in close proximity. For example, odors could provide important information about the resident from a distance such as physical condition, group size, group constituency, and reproductive state. Many female mammals use olfactory signals to advertise their readiness to mate (Johnston, 1983). For example, sexually receptive female golden hamsters deposit vaginal secretion scent marks when males enter their territories (Johnston, 1975).

Table 1

Types of messages most likely conveyed by means of olfaction (Doty, 1986)

Age appraisal	Individual appraisal
Alarm	Pain indication
Attention seeking	Predator
Defense	Prey
Distress-signalling	Reproductive stage indication
Encouraging approach	Social status appraisal
Frustration	Species membership
Gender appraisal	Submission
Greeting	Territory marking
Gregariousness	Trail marking
Group membership appraisal	Warning
Identification with home range	

A dichotomy in mammalian pheromonal function, originally based on entomological constructs (Wilson & Bossert, 1963), can be defined in terms of the type of effect produced in the recipient: (1) Priming pheromones act on endocrine systems "priming" the body thereby causing long-term changes in metabolism and (2) Signalling pheromones produce immediate and specific behavioral responses.

This classification system is now widely used for mammals as well (Bronson, 1971). The effects of urinary odors on the mouse estrous cycle are examples of primer effects, and the chemosensory sex attractants found in many species are examples of signaller effects (Bronson & Desjardins, 1974). While it is theoretically possible for the same chemical substance to have both primer and signalling effects, evidence exists that some of the different functions of chemosignals may be subserved by different chemosignals (Nyby, 1983b). For example at least two separate chemosignals have been postulated to exist in female mouse urine (Dixon & Mackintosh, 1975) and in hamster vaginal secretions (Johnston, 1977; Macrides, Johnson, & Schneider, 1977).

Many behavioral and physiological responses in mammals can be elicited by exposure to conspecific urine (Bronson, 1971). In fact, urinary pheromones of male mice and the responses they elicit are among the most studied pheromonal communication systems in mammals (Maruniak, Owen, Bronson, & Desjardins, 1974).

Several primer and signalling effects on various mammals have been studied and are presented below. Since house mice (*Mus domesticus*) are the focus of this thesis, the effects of both primer and signalling pheromones on house mice are given greater consideration.

Primer Pheromones

Four extensively studied effects of primer pheromones include the Whitten Effect, the Bruce Effect, the Vandenbergh Effect, and the Lee-Boot Effect.

(1) *The Whitten Effect:* Estrous cycles of females become synchronized within three days following exposure to the odor of a male mouse (Whitten, 1956; Whitten, 1958).

(2) *The Bruce Effect:* The odor of a strange male causes a recently mated female to fail to implant and thereby abort the fetus (Bruce, 1959; Bruce, 1960).

(3) *The Lee-Boot Effect:* The odor of group-housed female mice disrupts the estrous cycles of such females and leads to increased pseudo-pregnancy (Lee & van der Boot, 1955). The phenomenon may be due to the mutual depression of ovarian activity in order to facilitate subsequent synchronization of breeding (Whitten, 1966).

(4) *The Vandenbergh Effect:* The onset of puberty in either males or females is accelerated by the presence of odors from adults of the other gender (Vandenbergh, 1967; Vandenbergh, 1969; Vandenbergh, 1971).

Signalling Pheromones

Responses to signalling pheromones are behavioral and occur shortly after detection (Bronson, 1971). The response may involve either increases

or decreases in behavior. The ultrasound-eliciting pheromone, the focus of this thesis, is an example of a signalling pheromone.

The following are important effects of signalling pheromones in mammals (Doty, 1986):

(1) *Individual Recognition:* Mammals use chemical cues in individual recognition and preferences. Individual discrimination has been demonstrated in rodents (e.g., house mice, rats, gerbils, hamsters, wood mice, and guinea pigs), carnivores (e.g., dogs and wolves), lagomorphs (e.g., the European rabbit), ungulates (e.g., pigs and blacktailed deer), and many non-human primates (e.g., ring-tailed lemurs and marmosets) (Doty, 1986).

(2) *Sexual Preference:* Evidence exists that individuals often prefer the odors of potential mates that are in reproductive readiness. For example, male mice prefer estrous females over non-estrous females (Hayashi & Kimura, 1974) and estrous female mice prefer dominant males over subordinate males (Jones & Nowell, 1974).

(3) *Gender Identification:* Rodents often respond quite differently to olfactory stimuli of males and females. For example, male house mice emit ultrasonic vocalizations when exposed to female urine, but not to male urine (Nyby, Dizinno, & Whitney, 1977).

(4) *Aggressive Behavior:* Male mice attack other males based upon male odor (Denenberg, Gaulin-Kremer, Gandelman, & Zarrow, 1973). In contrast, female mouse urine is known to inhibit the aggression of males (Mugford & Nowell, 1970).

(5) *Territorial Avoidance:* The concept of territoriality includes marking as well as defense (Hediger, 1950). Urine seems to be a particularly well suited marking agent, and is used for such purposes by a wide variety of animals (Hediger, 1950). Jones & Nowell (1973) found an aversive factor in male mouse urine which discourages investigation of an area marked with such urine. Similarly, male rats prefer to investigate the odors of submissive males more than those of dominant males (Krames, Carr, & Bergman, 1969).

(6) *Communication of Stress and Alarm:* Animals behave differently in the presence of odors from conspecifics that have recently experienced stress-producing events (Doty, 1986). For example, mice that received electric foot shocks or injections of NaCl produced an odor that was avoided by other mice (Carr, Martorano, & Krames, 1970). This finding suggests the presence of a relatively specific signalling pheromone in the urine of stressed animals that functions to communicate danger to other members of the same species.

Scent Marking: Pheromonal Advertising

Although odors often emanate from various sites on an animal's body, in many mammalian species, the release and distribution of pheromonal material is further accomplished through scent marking. Vertebrate pheromones are found in a variety of secretions and excretions including urine, feces, vaginal secretions, and cutaneous scent gland secretions many of which are deposited in the environment.

Specialized behavior and morphology have evolved to aid in the distribution of odors. For example, urinary marking is common in many rodents and may be the major mechanism used in the broadcast of pheromones in house mice. In mice, dominant male mice typically deposit their urine by dragging or touching the prepuce to the substrate (Maruniak, Desjardins, & Bronson, 1975). Maruniak and his colleagues studied morphological adaptations for urinary marking behavior in rodents (in house mice, deermice, gerbils, and hamsters) and postulated an adaptational advantage for a long penis sheath and for a particular configuration of the prepuce. The tip of the house mouse prepuce appears bifurcated with many long hairs which may act as brushes during urinary marking behavior (Maruniak, Owen, Bronson, & Desjardins, 1975).

The possible function of scent marks (Johnson, 1973) include: (1) a deterrent or substitute for aggression (marks warn conspecifics away from an occupied territory); (2) a navigational system used by an individual within its own territory; (3) a sex attractant or stimulant; (4) an indicator of gender, sexual status, age, dominance, and individual identity, and (5) an alarm signal to conspecifics.

Pheromonal Volatility

The time frame over which scent marks are broadcast to other individuals is regulated in part by pheromone volatility (Regnier & Goodwin, 1977). A highly volatile pheromone can evaporate in a few minutes while a pheromone of low volatility may last for days or months. A pheromone's chemical and physical properties govern its volatility, which in turn determines the type of message it can convey (Regnier & Goodwin, 1977). For example, an alarm signal must be communicated quickly in

order to warn conspecifics of impending danger and fade quickly in order to minimize the period of heightened arousal. Consequently, one might expect an alarm signal either to be highly volatile or quickly degraded. However, a territorial mark must persist in order to be an effective signal of territory ownership. Thus, territorial marks of low volatility would last longer and would not need to be replaced as frequently as territorial marks of high volatility.

Pheromonal Persistence

Chemical signals can persist for days or even months. Wilson & Bossert (1963) demonstrated this feature of persistence, which was measured in terms of fade-out time, to be specialized for different functions. Although persistence of mammalian pheromones found in scent marks has not been thoroughly investigated, some information is available.

Johnston & Schmidt (1979) suggested that male hamsters may use the changing odor quality of female scent marks to gauge how recently a female was in the area. For example, freshly deposited flank marks or vaginal marks of female hamsters were better at eliciting male licking and sniffing than were marks that were one day old (Johnston & Schmidt, 1979). Nonetheless, the male hamsters continued to show some level of responsiveness for up to 45 days to flank marks and for up to 100 days to vaginal marks. Whether the change in male hamster responsiveness was mediated by a rapidly disappearing but potent chemosignal and a more persistent but less compelling chemosignal was not entertained by those workers.

Other examples of mammalian chemosignals that persist for only short periods of time include male attraction to the urine of estrous females which disappears by 24 hours after being voided in rats (Lydell & Doty, 1972) and by 48 hours in guinea pigs (Beauchamp & Berüter, 1973). Assuming some adaptive value for some chemosignals to degrade rapidly (*i.e.*, Wilson & Bossert, 1963) a variety of mammalian pheromones may exist whose message degrades quickly after deposition.

Johnston & Schmidt (1979) suggest that the odor quality of most mammalian scent marks should change in a predictable manner with time. However, few experiments other than those described here examined the abilities of animals to discriminate the freshness of odors, and even fewer have examined differences in responses to pheromones of different ages.

The Role of Context and Experience

Beauchamp, Doty, Moulton, & Mugford (1976) point out that the pheromone concept seems to imply, to many workers, both a single chemical entity and a single mode of communication relatively uninfluenced by other chemical and nonchemical signals. Likewise, a tendency exists to classify pheromones according to function (i.e., as sex attractants, estrus inducers, aggression inhibitors, etc.). The danger in classifying pheromones in such a fashion is that consideration may not be given to the possibility that the same substance has other effects (Bronson, 1971). The chemical message in different contexts may be identical, but the meaning associated with it may vary depending on the recipient or on the context (Beauchamp et al., 1976). Smith (1968) stated that "since displays, received in different circumstances, can lead to different meanings for a

recipient, a variety of messages would exist to correspond to the variety of meanings. But all of these messages would still be encoded by the same single display, and the recipient could recognize them only by knowing the contexts [p. 49]."

Male copulatory experience either facilitates or is necessary for the preference of one female conspecific odor over another (e.g., (Hayashi & Kimura, 1974; Lydell & Doty, 1972). Experience may be important for an animal to learn the various contexts in which chemical messages have different meanings. The role that social/sexual experience plays in the regulation of ultrasonic courtship vocalizations to female urine is considered below.

Male Ultrasonic Courtship Vocalizations to Female Urine in Mice

Male mice emit 70 kHz ultrasonic vocalizations to females during courtship and copulation (Nyby, 1983a; Sales, 1972). However, male mice will emit ultrasounds not only to females, but also to female odors (Nyby & Whitney, 1979). Many aspects of this "pheromonal" communication system have been examined (*e.g.*, (Nyby, 1983b; Nyby & Whitney, 1978; Nyby & Whitney, 1979; Whitney & Nyby, 1983).

The female-produced chemosensory cues that elicit male ultrasounds appear to be located all over the female's body. For example, cotton swabs containing female urinary, vaginal, or facial secretions were all effective stimuli in eliciting male ultrasounds whereas male and control substances were ineffective (Nyby, Wysocki, Whitney, & Dizinno, 1977). Whether the same chemosignal is present in these different odor sources or whether

different chemosignals have the same ultrasound-eliciting quality has yet to be determined.

Most of the research examining the physical characteristics of ultrasound-eliciting chemosignals has focussed on urine for at least three reasons: (1) clear comparisons can be made between male- and female-produced cues (*i.e.*,vaginal odors do not have an immediately obvious male collection site); (2) urine amount is much easier to quantify and control than the amount of facial or vaginal cues; and (3) much of the mouse pheromone literature has concentrated on urine, thus allowing direct comparisons to be made. Previous work (Nyby, Wysocki, Whitney, Dizinno, & Schneider, 1979) indicated that the ultrasound-eliciting substance found in female urine is a water-soluble, heat-resistant, relatively nonvolatile substance whose production appears to be ovarian-independent and primarily regulated by pituitary factors.

One surprising feature of the urinary elicitation of ultrasounds was that in previous research (Dizinno, Whitney, & Nyby, 1978; Nyby, Bigelow, Kerchner, & Barbehenn, 1983), sexually naive adult males typically did not vocalize to the urine of adult females. Only after social/sexual experience did males begin to vocalize to female urine alone. In contrast, such males did vocalize when first encountering a female in adulthood. Moreover, if a sexually experienced male repeatedly encountered female urine, in the absence of a female, his vocalizations to urine gradually declined. These findings led to the conclusion that male vocalization to female urine was an instance of classical conditioning in which some factor in female urine served as a conditioned stimulus (CS) for eliciting vocalizations with some other unknown aspect of the female serving as an unconditioned stimulus

(UCS). This CS was nonvolatile (Nyby & Whitney, 1979) and chemically stable (Nyby & Zakeski, 1979).

Previously published research examining the acquisition and extinction of vocalizations to female urine utilized urine collected in metabolic cages (*e.g.*, (Dizinno et al., 1978; Kerchner, Vatza, & Nyby, 1986; Nyby, 1983b; Nyby & Whitney, 1978; Nyby, Whitney, Schmitz, & Dizinno, 1978; Whitney & Nyby, 1983). Since this urine was typically collected over a 12 hour period, the possibility existed that some urinary components with chemo-communicatory value may have been lost due to either volatilization or degradation.

The experiments presented in this thesis suggest that male vocalizations to freshly voided urine do not parallel the previous findings which gave rise to the classical conditioning interpretation and that freshly voided urine may actually contain a second, more potent chemosignal not present in metabolic-cage-collected urine. This second chemosignal would appear to be an unconditioned stimulus for the elicitation of ultrasonic vocalizations.

This thesis asks two questions. First, how do freshly voided and aged (metabolic) urine differ in their elicitation of ultrasonic vocalizations from male mice? Experiments 1-3 examine these differences. Second, do differences in volatility cause these differences in behavioral response to emerge? Experiments 4-6 address this issue.

Chapter 2: Methods and Results

Methods common to all experiments are presented first. Additions or deviations to the general methods are described within the respective experiments.

General Method

Animals: House mice *(Mus domesticus)* of the following strains were used: (1) CF-1 males (purchased from Charles River Labs), and (2) C57BL/6J X AKR/J ("CK") hybrid males and females (bred in the Central Animal Facilities, Lehigh University).

Three different categories of animals were used: (1) "Subjects" consisted of adult CK hybrid male mice whose ultrasonic vocalizations were the dependent variable. All male subjects were group housed with other males following weaning at 21 days of age. Thus these males did not encounter a female after weaning. After reaching sexual maturity the males were individually housed in transparent plastic cages (29 x 18 x 13 cm) with hard wood chips for beddings and a wire top containing a water bottle and food; (2) "Stimulus donors" were CK hybrid male and female mice that provided the urine stimuli used in the vocalization tests; (3) "Social-experience animals" consisted of adult CF-1 males and CK hybrid females that were systematically placed in the adult subjects' home cages prior to testing. The stimulus donors and social-experience animals were housed in groups of two to four in opaque cages similar to those of the subjects.

The CF-1 male social-experience animals were used because they were white and easily differentiated from the black subjects. CF-1 males received bilateral olfactory bulbectomies (OBX) under sodium pentobarbital (Nembutal) anesthesia. OBX males were used because, while neither initiating nor responding to attack, they reliably elicit aggression comparable to intact males (Denenberg et al., 1973).

Apparatus: Vocalizations were monitored with a QMC S-100 ultrasonic receiver tuned to 70 kHz with the microphone centered 25 cm above the floor of the subject's home cage (29 x 18 x 13 cm), which served as the test chamber. Stimuli were presented on cotton swabs.

Maryland Plastics Metabolism Units (Model E110) were used to collect metabolic-cage collected urine.

Procedure: Food (Purina Mouse Chow) and water were provided *ad libitum*. The colony room was maintained at a constant temperature (23° C) and on a 12:12 light:dark cycle with lights on at 7 am.

Urine Collection: Urine was collected in two ways:

(1) Metabolic-cage collection (METABOLIC): Twelve female urine donors were group housed overnight (approximately 12 hrs) in metabolic cages. The pooled urine from the participating females was then placed into a glass syringe and stored until used. Thus the urine was voided sometime between 1/4 and 13 hours prior to its use.

(2) Manual collection (FRESH): Urine was collected by grasping a female urine donor by the loose skin of the dorsal neck. The act of handling the animal in this fashion was often sufficient to induce urination.

However, if urination did not occur, it could usually be induced by gently palpating the bladder. The donor was positioned so that its urine fell into a glass collection vial. While the amount of urine from individual animals varied, approximately 0.05 to 0.1 ml of urine per donor was typical. After several donors urinated, the pooled urine was quickly placed into a glass syringe and stored until used. Thus the time intervening between urination and use ranged from 1/4 hour to 1 1/2 hours.

Social Experience Regimen: Unless otherwise stated, 2 weeks after being individually housed, the subjects were systematically exposed, over an eight-day period, to adult social-experience animals. Briefly, male and female social-experience animals were sequentially placed for 3-min each per day into the subject's home cage. The order of presentation of social-experience animals was counterbalanced across subjects and reversed each day. This regimen allowed the subject to be exposed to at least one complete 4-day estrus cycle. The social-experience regimen has been described in detail elsewhere (Nyby & Whitney, 1978; Nyby et al., 1977).

Vocalization Measurement: The subject's home cage was taken from the main colony room to an adjacent testing room and placed under the QMC microphone for 1 min to ensure that the subject was not emitting ultrasonic vocalizations to random aspects of the test situation. If a vocalization was detected during this 1-min habituation period, the animal was returned to the main colony room, and was retested 10-15 minutes later.

The stimulus was prepared during the 1-min habituation period by placing 0.1 ml of stimulus (urine or water) on a cotton-tipped swab. The part of the stimulus-impregnated swab touched by the experimenter was

broken off and the remainder briefly stored in a test tube (15-20 secs) until use.

A 3-min test began by placing a stimulus into the subject's home cage. The amount of vocalization was quantified by dividing the 3-min trial into 36 5-secs intervals. The number of intervals containing vocalizations was recorded yielding possible scores ranging from 0-36. Tests always occurred during the light portion of the 12/12 light/dark cycle and were separated by 24 hours to minimize carryover effects. The experimenter recording the amount of vocalizations was blind to the treatment of the subject and the type of stimulus presented.

Experimental Design: Subjects were randomly assigned to treatment groups. The data of Experiments 1, 3, and 4 were analyzed by a 1-between, 1-within analysis of variance, while selected contrasts employed simple means comparisons. The data of Experiments 2, 5, and 6 were assessed with the Kruskal-Wallis H-test, while selected contrasts employed Mann-Whitney U-tests.

Experiments 1 - 3

Female urine collected in metabolic cages is believed to contain a nonvolatile, long-lasting pheromone that is a CS for ultrasonic vocalizations (Nyby & Zakeski, 1979). The first three experiments presented in this chapter demonstrated that freshly voided urine of females may, in addition, contain a second, more potent, chemosignal not present in metabolic-cage-collected urine. This second chemosignal would appear to be a UCS for the elicitation of ultrasonic vocalizations.

Experiment 1

Sexual experience is necessary for male mice to emit vocalizations to female urine collected in a metabolic cage (METABOLIC) (Dizinno et al., 1978; Nyby et al., 1983). In this experiment, the extent to which sexual experience was also necessary for male vocalizations to freshly voided (FRESH) urine was examined.

Method

Animals: Subjects were 40 sexually naive males, 65 $\pm$ 5 days of age on the first urine test. Fifteen females of the same age served as stimulus donors.

Apparatus: The ultrasound receiver and metabolic cages described earlier were used.

Procedure: Subjects were randomly assigned to one of two stimulus conditions defined by whether they repeatedly encountered METABOLIC (N=20) or FRESH (N=20) urine. This experiment was run as two equal replicates, offset by two weeks.

Results

The mean levels of vocalizations to either FRESH or METABOLIC urine in the two replications did not statistically differ ($F(1,37) = .001, p$ = n.s.)

and were combined for graphical presentation and statistical analysis. The ultrasonic courtship vocalizations of the two groups are seen in Figure 1.

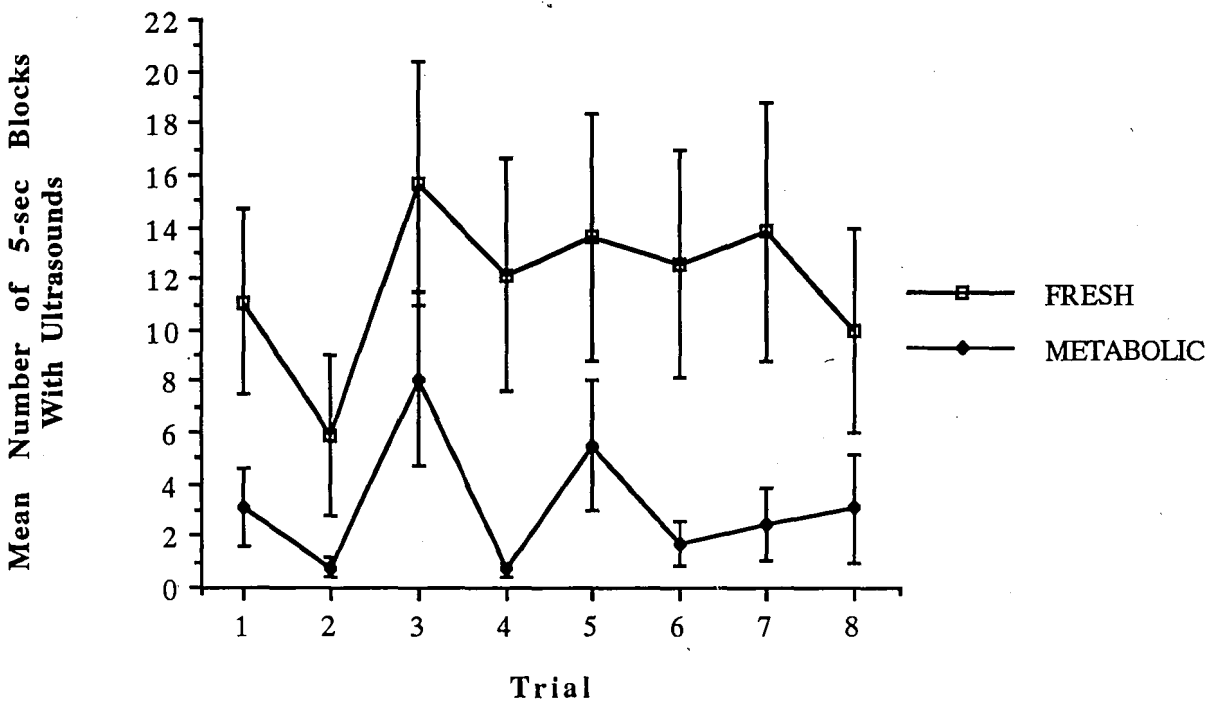

Figure 1. Amount of ultrasound (mean ± SEM) emitted by sexually naive male mice to either freshly voided (FRESH) or metabolic-cage-collected (METABOLIC) urine of females.

Socially inexperienced males repeatedly encountering FRESH urine emitted more vocalizations than those encountering METABOLIC urine ($F(1,37) = 5.411, p < .04$). The variability in vocalizations across trials resulted in a significant trial effect ($F(7, 126) = 4.897, p < .0001$). The group by trial interaction was not significant ($F(7, 126) = 1.135$, p = n.s.).

Experiment 2

Repeated presentations of female METABOLIC urine to a male resulted in a gradual decline in their vocalizations over tests (Dizinno et al., 1978; Nyby et al., 1983). In this experiment, the extent to which males extinguish their vocalizations to FRESH urine with repeated testing was examined.

Method

Animals: Subjects were 20 males, 75 ± 3 days of age on the first test. Social-experience animals and stimulus donors were adult males and females.

Apparatus: The ultrasound receiver and metabolic cages described earlier were used.

Procedure: Subjects were randomly assigned to two groups. Approximately two weeks later the males received the social-experience regimen described earlier. Two days after completing the social-experience regimen, all subjects were given a pretest with FRESH urine to ensure that both groups had comparable baseline vocalization levels.

The two groups were defined by the stimuli subsequently presented. Thereafter, the males of the FRESH group (N=10) were repeatedly presented with freshly voided urine and males of the WATER group (N=10) were repeatedly presented with water. Trials 1, 2, and 3 were separated by 2 days;

Trials 3, 4, 5, and 6 were separated by 3 days; and Trials 6, 7, and 8 were separated by 5 days.

Results

As seen in Figure 2, the two groups initially had similar levels of vocalizations during the pre-test when presented with FRESH urine ($F(1,18) = 0.013, p$ = n.s.). However, over the eight days of stimulus presentation, the FRESH group emitted significantly more vocalizations than the WATER group (Mann-Whitney U, z = 3.83, p<.0001). Moreover, visual inspection of Figure 2 suggests that males of the FRESH group did not decline in vocalization amount over the eight trials. Thus, no extinction to FRESH urine was seen over the time frame of this experiment. In contrast, in previous research (Dizinno et al., 1978) vocalizations had fallen to very low levels in males repeatedly tested with metabolic-cage-collected urine in a similar experiment.

Across the 8 days of testing, the FRESH animals began to anticipate the presentation of the urine stimulus by vocalizing during the habituation period preceding stimulus presentation. Such behavior was never seen in the WATER animals. To examine whether the propensity to vocalize had diverged in the two groups as a result of this experience, all subjects were given 2 post-tests. The first post-test employed FRESH urine and the second employed WATER.

The groups did not significantly differ on the first post-test where both groups received FRESH urine ($F(1,18) = .105, p$ = n.s.), thus indicating that both groups remained equally likely to vocalize to an appropriate stimulus.

Surprisingly, the two groups were significantly different on the second post-test in response to WATER (Mann-Whitney U, $z = 3.173$, $p < .002$). I interpret the difference as arising because for the FRESH males, the cotton swabs used to present the stimuli had been repeatedly encountered previously when containing FRESH urine. Thus repeated pairings with the FRESH urine (UCS) caused the cotton swabs (a CS) to become effective stimuli. On the other hand, the WATER males encountered swabs impregnated with either water or urine and thus such conditioning was much less likely. These findings indicated that FRESH urine can serve as a reinforcer for ultrasonic vocalizations to a previously neutral stimulus (cotton swab).

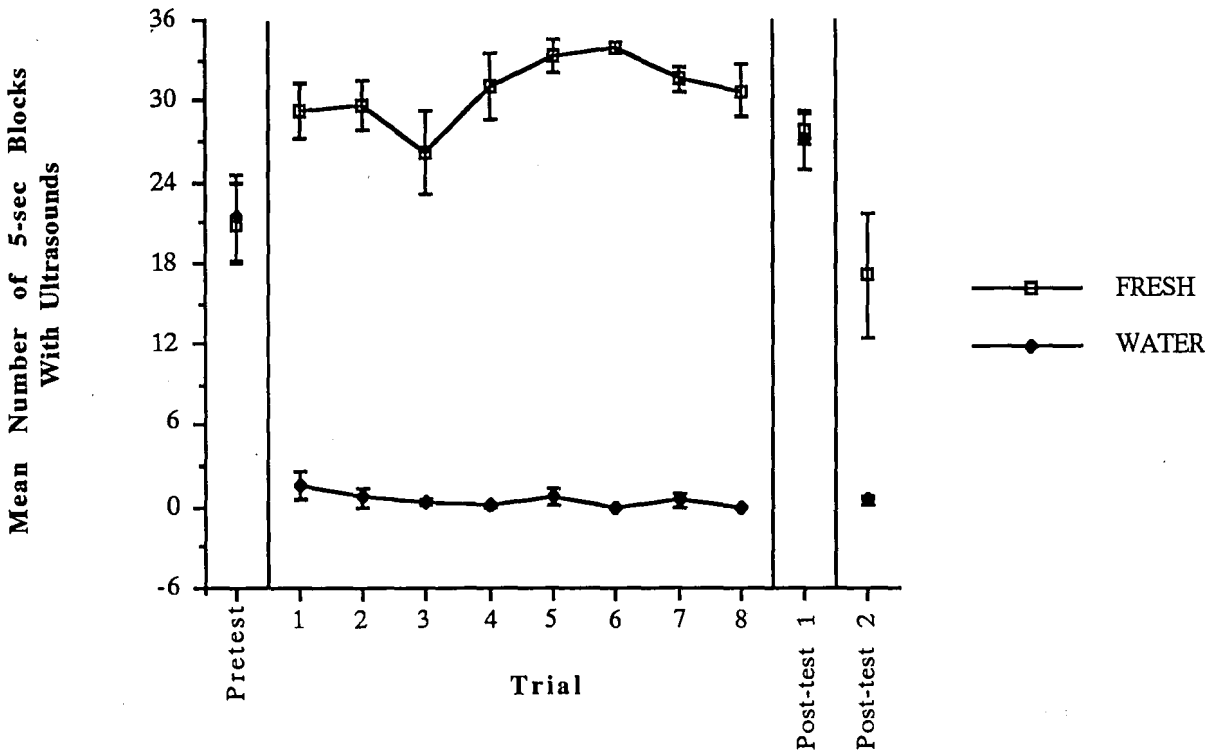

Figure 2. Amount of ultrasound (mean ± SEM) emitted by socially/sexually experienced male mice in response to either freshly voided (FRESH) urine or WATER. The pretest and post-test 1 employed FRESH urine as the stimulus. Post-test 2 employed WATER as the stimulus.

Experiment 3

In previous work (Dizinno et al., 1978; Nyby et al., 1983), repeated presentations of METABOLIC urine from females to a male in the absence of the female herself resulted in a gradual decline in vocalizations whereas in Experiment 2 repeated presentation of FRESH urine from females did not. In this Experiment, the ultrasonic responsiveness of males to repeated presentations of either METABOLIC or FRESH urine was directly contrasted.

Method

Animals: Subjects were 14 males, 60 $\pm$ 5 days of age on the first test. Social-experience animals and stimulus donors were 9 adult males and females.

Apparatus: The ultrasound receiver and metabolic cages described earlier were used.

Procedures: Subjects were randomly assigned to two groups defined by whether the male repeatedly received FRESH (N=7) or METABOLIC (N=7) urine. The subjects were given a pretest with FRESH urine to ensure comparable baseline vocalization levels. Trials 1 and 2 were separated by 2 days; trials 2 and 3 were separated by 7 days; trials 3, 4, 5, 6, 7, and 8 were separated by 1 day.

Results

As seen in Figure 3, the groups did not differ on the pretest ($F(1,12)$ = 0.02, p = n.s.), indicating that they had comparable propensities to vocalize at the beginning of the experiment. Although the amount of vocalizations to FRESH and METABOLIC urine were virtually identical during the first two stimulus presentations, the two groups diverged thereafter. A significant group by trial interaction ($F(5,60) = 4.529$, $p < .002$) reflected the gradual decline in responsiveness to METABOLIC urine, and the unaltered high responsiveness to FRESH urine over the six trials.

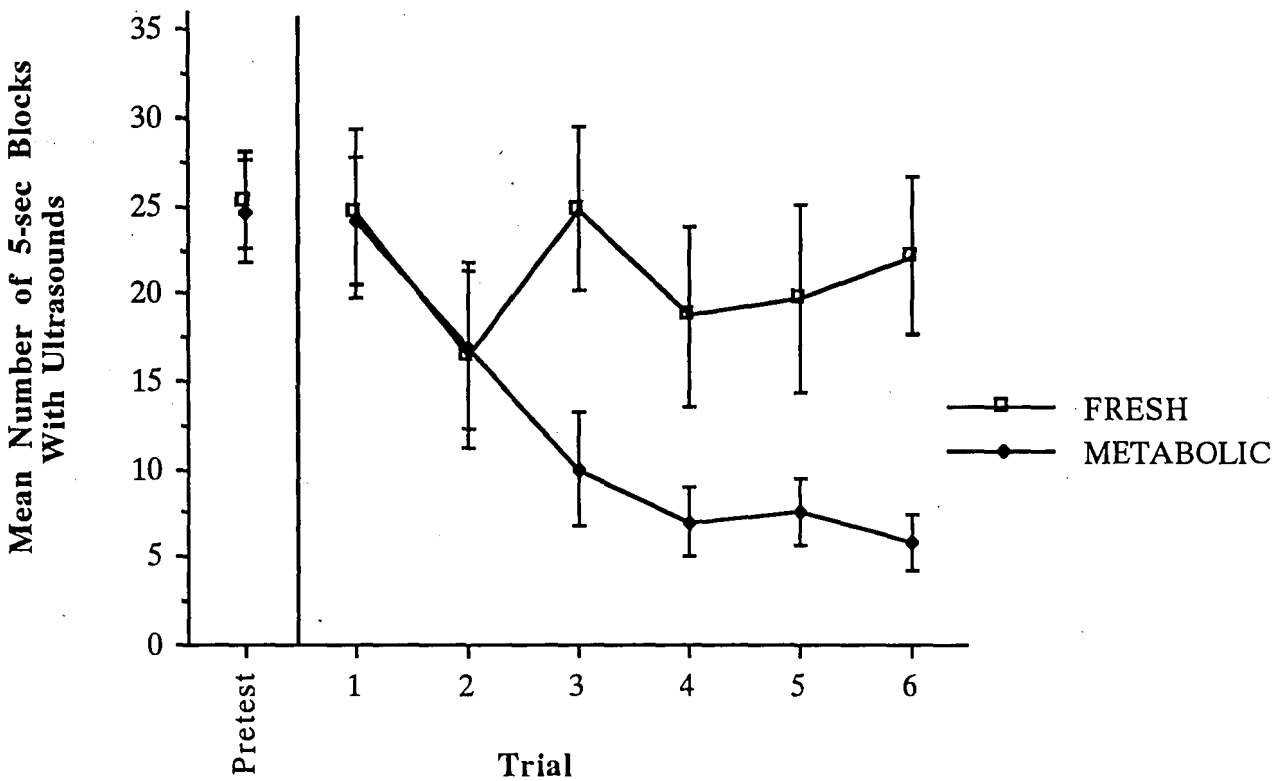

Figure 3. Amount of ultrasound (mean ± SEM) emitted by socially/sexually experienced male mice in response to either freshly voided (FRESH) or metabolic-cage-collected (METABOLIC) urine. The pretest employed FRESH urine as the stimulus.

Experiments 4 - 6

Previous research (Kerchner, 1988; Nyby et al., 1979) indicated that the ultrasound-eliciting substance in METABOLIC female urine was molecularly stable and relatively nonvolatile. Experiments 1-3 suggested that FRESH female urine also contained another more potent pheromone that serves as a UCS for vocalization elicitation. Thus female urine would appear to possess at least two chemosignals that have the property of eliciting ultrasounds.

Although the UCS for vocalization elicitation is potent, it also disappears rapidly. Several factors that may contribute to the decreasing potency of the UCS following urination include volatilization and degradation.

The following two experiments examined the volatility of the UCS found in FRESH female urine.

Experiment 4

This study investigated the capacity of FRESH and METABOLIC urine to elicit vocalizations when they could be smelled but not contacted. Thus if the males vocalized, one could infer that the chemosignal must be volatile.

Method

Animals: Subjects were 45 sexually experienced CK hybrid males, 105 $\pm$ 10 days of age on the first urine test. The social-experience animals and stimulus donors were as described in the General Method.

Apparatus: The ultrasound receiver and metabolic cages previously described were used. The urinary stimuli were presented in a glass vial (5.3 cm in length, 1.5 cm outer diameter). The center of the plastic screw cap had a circular hole covered with a wire mesh barrier which prevented contact with the urinary stimuli inside while at the same time permitting the passage of odors from the vial.

Procedure: A stimulus was prepared by placing 0.1 ml of FRESH or METABOLIC urine on a cotton-tipped swab. The part of the swab touched by the experimenter was broken off and the remainder placed into the glass vial, cotton-tipped end first. Care was taken to ensure that the swab did not come in contact with the vial until well inside. After the experimenter washed his hands, the cap was screwed onto the vial, and inspected to ensure that the wire-mesh completely covered the hole in the cap. The vial was then stored upright until used. Each type of vial was handled using different tongs to minimize possible odor contamination. Following behavioral testing, the glass vials were washed with soap and water, with acetone, and finally were allowed to air dry.

Male subjects were individually housed at approximately 95 days of age and randomly assigned to one of three groups defined by the type of stimulus they repeatedly encountered. Social experience began as described in the General Method. Forty-eight hours following the last day of social experience, subjects were given a pretest with FRESH female urine on a cotton swab to ensure that all groups had comparable baseline vocalization levels. Twenty-four hours later, repeated daily testing for ultrasonic responsiveness to either FRESH VIAL urine (N=15),

METABOLIC VIAL urine (N=15), or FRESH urine (N=15) began. This experiment was run as two replicates, offset by approximately six weeks.

Results

The levels of vocalizations in the two replications did not statistically differ ($F(1, 43) = .653, p$ = n.s.) and the group by replication interaction was not significant ($F(2, 39) = .810, p$ = n.s.), so the replicates were combined for graphical presentation and statistical analysis. The ultrasonic courtship vocalizations of the three groups are seen in Figure 4. The three groups initially had similar levels of vocalizations during the pre-test when presented with FRESH urine ($F(2, 42) = .263, p$ = n.s.), thus indicating that all three groups were equally likely to vocalize to an appropriate stimulus. However, over the three days of stimulus presentation, males emitted significantly more vocalizations to FRESH urine than to the FRESH VIAL and METABOLIC VIAL stimuli ($F(2, 42) = 23.446, p$ =0.0001). Further analysis revealed no significant difference in male responsiveness to FRESH VIAL or METABOLIC VIAL stimuli ($F(1, 42) = .408, p$ = n.s.).

A significant group by trial interaction ($F(4,84) = 4.323, p = 0.0031$) reflected a gradual decline in responsiveness to FRESH urine and a gradual rise in responsiveness to both FRESH VIAL and METABOLIC VIAL stimuli. Further analysis, however, showed no effect of trials on vocalization levels in males exposed to FRESH urine, reflecting that these males did not decline significantly in vocalization amount over the three trials ($F(2,28) = 2.068, p$ = n.s.).

These findings suggest that in order to vocalize at high levels to FRESH urine, socially experienced males must physically contact the

urine. These findings also suggest that the UCS in FRESH urine is of low volatility.

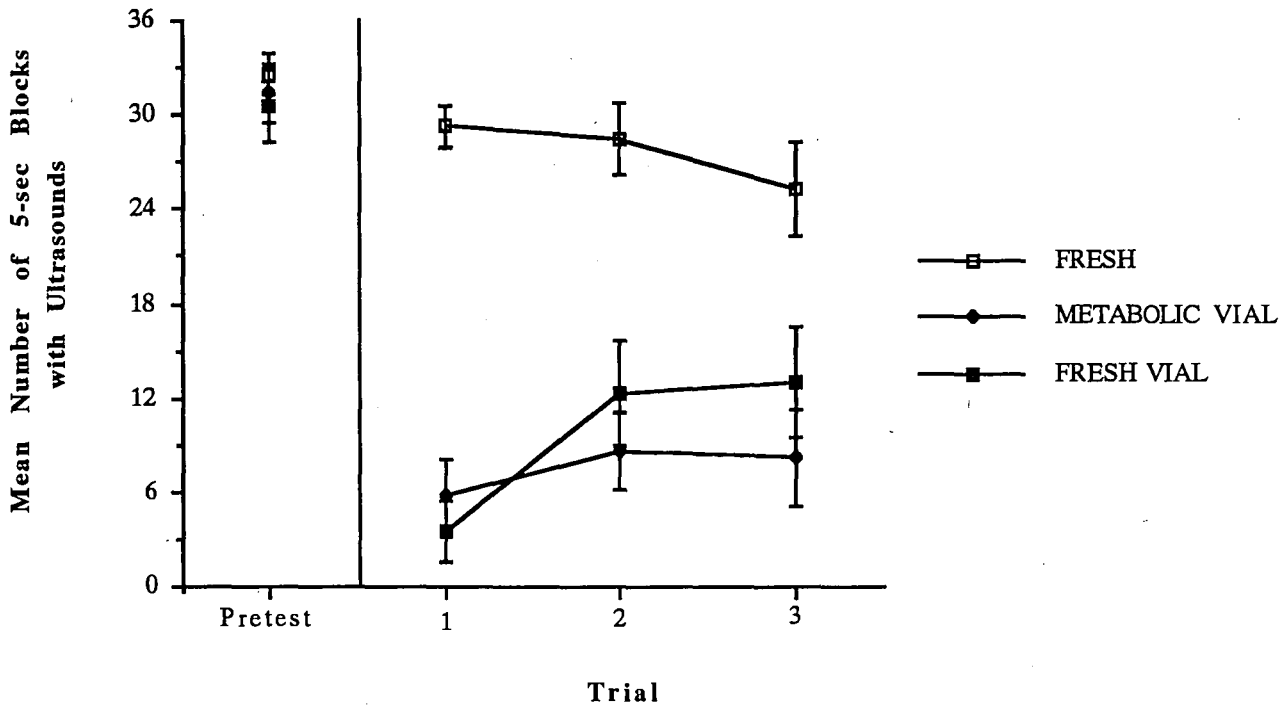

Figure 4. Amount of ultrasound (mean ± SEM) emitted by socially/sexually experienced male mice in response to either freshly voided (FRESH) urine presented on a cotton swab, freshly voided (FRESH VIAL) urine presented in a glass vial, or metabolic-cage-collected (METABOLIC VIAL) urine presented in a glass vial. The pretest employed FRESH urine as the stimulus.

Experiment 5

This study further examined the volatility of the chemosignal in FRESH urine by examining the ultrasonic responsiveness of sexually naive males to the three types of stimuli used in Experiments 4.

Method

Animals: Subjects were 30 sexually naive CK hybrid males, 120 $\pm$ 15 days of age on the day of the test. The stimulus donors were as described in the General Method. These subjects did not receive the social experience regimen.

Apparatus: The ultrasonic receiver, metabolic cages, and the glass vials described earlier were used.

Procedure: Sexually naive male subjects were individually housed at approximately 100 days of age and randomly assigned to one of three groups defined by the type of stimulus they received. Subjects assigned to FRESH VIAL and METABOLIC VIAL groups were habituated to the glass vial. The glass vial was placed into the males' home cage for 3-min over a four day period. Forty-eight hours later, a single test for ultrasonic responsiveness to either FRESH VIAL (N=10) urine, METABOLIC VIAL (N=10) urine, or FRESH (N=10) urine began. Stimulus preparation was the same as in Experiment 4. Repeated trials were not used, because Experiment 1 demonstrated sexually naive males vocalize at fairly constant levels to urinary stimuli.

Results

The ultrasonic courtship vocalizations of the three groups are seen in Figure 5. Socially inexperienced males receiving FRESH urine emitted significantly more vocalizations than those encountering either FRESH VIAL or METABOLIC VIAL stimuli (Kruskal-Wallis H test, $H(2) = 14.528$, $p < .0008$). These findings suggest that sexually naive males must physically contact FRESH urine from females before emitting vocalizations. It should be noted, however, that the levels of vocalizations emitted by sexually naive males are substantially lower than levels emitted by sexually experienced males (See Figure 2).

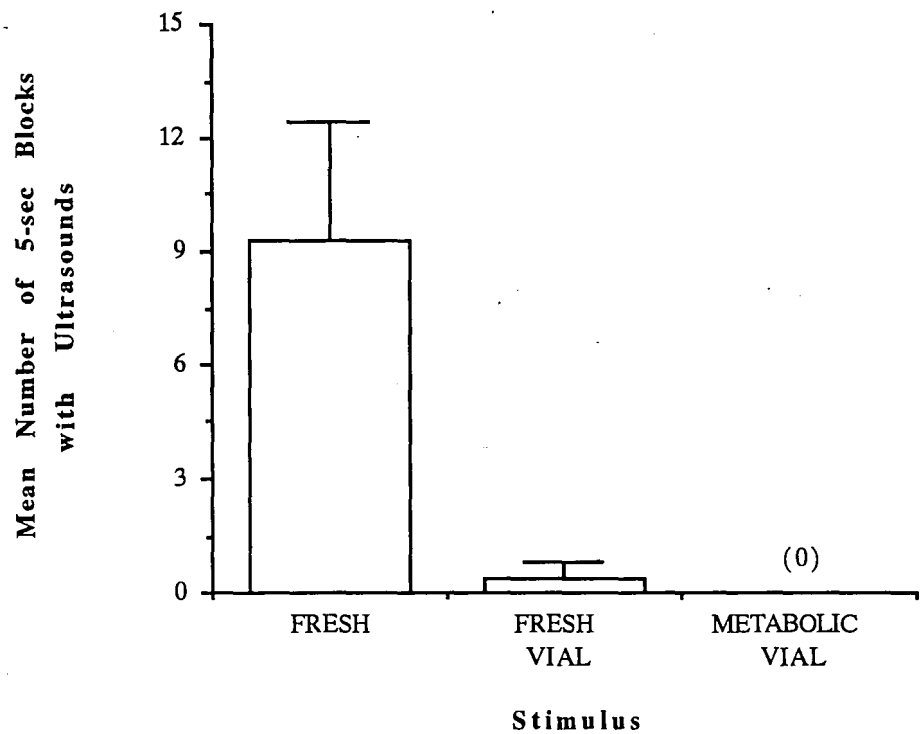

Figure 5. Amount of ultrasound (mean ± SEM) emitted by socially/sexually naive male mice in response to either freshly voided (FRESH) urine presented on a cotton swab, freshly voided (FRESH VIAL) urine presented in a glass vial, or metabolic-cage-collected (METABOLIC VIAL) urine presented in a glass vial.

Experiment 6

Previous research (Nyby et al., 1979) indicated that the ultrasound eliciting factor in METABOLIC urine was heat resistant (although extreme heat destroyed it). For example, placing the urine in a boiling water bath for 2 hours had no observable effect upon the potency of the urine to elicit ultrasounds. Placing the urine in an autoclave and subjecting it to extreme heat did reduce its ability to elicit ultrasounds. This experiment examined the effect of heat on the chemosignal found in FRESH urine.

Method

Animals: Subjects were 40 sexually experienced CK hybrid males, 120 $\pm$ 5 days of age on the first urine test. The social experience and stimulus donors were as described in the General Method.

Apparatus: The ultrasound receiver and metabolic cages previously described were used. A boiling water bath was used to heat the urine stimuli.

Procedure: Male subjects were randomly assigned to four groups defined by the type of stimulus they repeatedly encountered. Subjects were individually housed at approximately 100 days of age. Social experience then began as described in the General Method. Forty-eight hours after the last day of social experience, subjects were given a pretest with FRESH urine to ensure that all groups had comparable baseline vocalization levels. Twenty-four hours later, testing for ultrasonic responsiveness to either

FRESH female urine, FRESH female urine that had been heated in a boiling water bath (BOILED FRESH), METABOLIC female urine, or METABOLIC female urine that had been heated in a boiling water bath (BOILED METABOLIC) began.

The BOILED urine stimuli were prepared by placing approximately 1.5 ml of female urine in a test tube and suspending the test tube, so that the urine level was covered, in a boiling water bath for 2 hours. Stimuli were presented as described in the General Method.

Results

The ultrasonic courtship vocalizations of the four groups are seen in Figure 6. Socially experienced males receiving FRESH and METABOLIC urine emitted significantly more vocalizations than those encountering either BOILED FRESH or BOILED METABOLIC stimuli ($H(3) = 26.784, p = 0.0001$). FRESH urine elicited significantly more vocalizations than METABOLIC urine (Mann-Whitney U, $z = 3.107, p < .002$) and BOILED FRESH and BROILED METABOLIC urine did not significantly differ (Mann-Whitney U, $z = 1.42$, p= n.s.). These findings suggest that both the UCS found in FRESH urine and the CS found in METABOLIC urine are lost when exposed to heat.

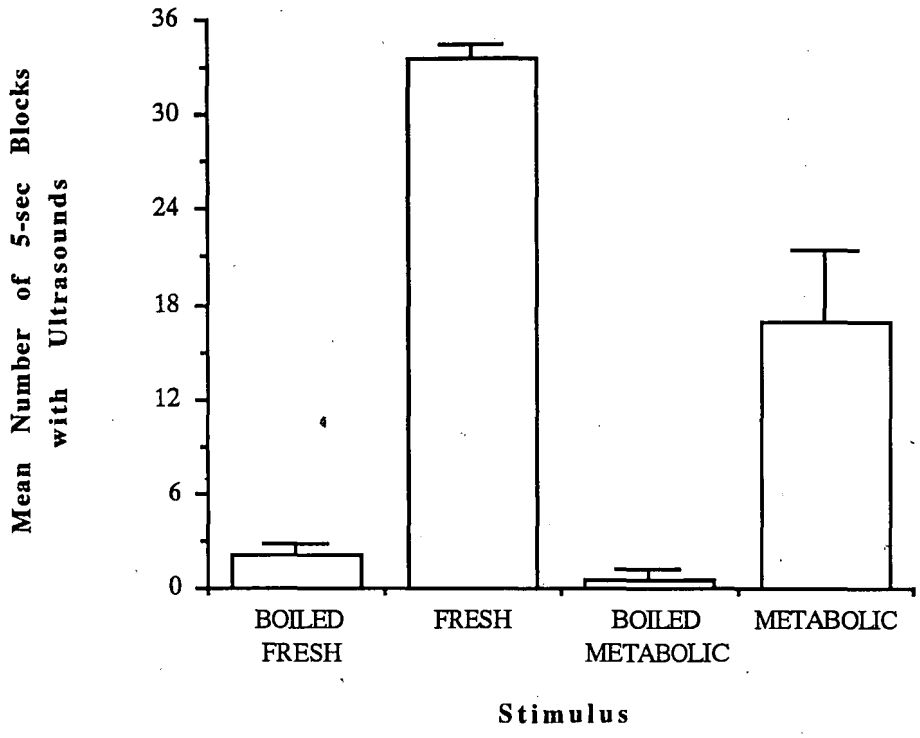

Figure 6. Amount of ultrasound (mean ± SEM) emitted by socially/sexually experienced male mice in response to either freshly voided (FRESH) urine , boiled freshly voided (BOILED FRESH) urine, metabolic-cage-collected (METABOLIC) urine, or boiled metabolic-cage-collected (BOILED METABOLIC) urine.

Chapter 3: General Discussion

Experiments 1-3 replicate previous findings (Nyby et al., 1983; Nyby & Whitney, 1978; Nyby & Whitney, 1979; Whitney & Nyby, 1983) that METABOLIC urine of female mice is a CS for the elicitation of ultrasonic vocalizations from male mice. Sexually naive mice emitted few ultrasounds to such urine (Experiment 1) and only after social/sexual experience were high levels of vocalizations emitted to urine alone (Experiment 3). Furthermore, during repeated exposure to such urine, the ultrasonic vocalizations of males gradually declined (Experiment 3).

In contrast, males vocalized to FRESH urine quite differently. First of all, sexually naive males vocalized significantly more to FRESH urine than to METABOLIC urine (Experiment 1). Thus males appeared to recognize the sex signalling value of freshly voided urine prior to their first adulthood encounter with an adult female. Furthermore, unlike METABOLIC urine (Kerchner et al., 1986; Nyby & Whitney, 1979), FRESH urine remained a potent stimulus for eliciting vocalizations with repeated testing (Experiments 2 and 3). Finally, FRESH urine appeared to act as a reinforcer to cause a previously neutral stimulus (cotton swabs) to acquire ultrasound-eliciting properties (Experiment 2).

These findings suggest that FRESH urine contains a potent but ephemeral pheromone that serves as a UCS for vocalization elicitation while METABOLIC urine contains a molecularly stable, nonvolatile, but less compelling, chemosignal that can, under certain circumstances, serve as a CS for eliciting vocalizations for at least 30 days following urination (Nyby & Zakeski, 1979).

These results bear a remarkable similarity to findings from hamsters. For example, male hamsters showed greater responsiveness to flank marks or vaginal marks of females that were freshly deposited than those that were one day old. Nonetheless, male hamsters continued to show some lowered level of responsiveness for up to 45 days to flank marks and for up to 100 days to vaginal marks (Johnston & Schmidt, 1979). Johnston & Schmidt (1979) suggested that male hamsters may use the changing odor quality of the female scent-marks to gauge how recently a female was in the area. Perhaps male mice use odor qualities of female urine to gauge the age of the urine in a fashion similar to hamsters.

Whether male mouse responsiveness to FRESH urine is innate (Kirchoff-Glazier, 1979; Schumacher & Molz, 1982) or is determined by prenatal experience (Pederson & Blass, 1982; Stickrod, Kimble, & Smotherman, 1982) was not examined in this thesis. However, the vocalizations by sexually naive males to freshly voided urine in Experiment 1 were not quite at the levels seen following sexual experience in Experiments 2 and 3, suggesting that yet other unknown factors operating in adulthood may also influence male ultrasonic responsiveness. One such factor is social/sexual experience. A sexually naive male may not fully "realize" the value of the UCS and may require heterosexual experience before showing high levels of responsiveness.

Similar to my findings, Maruniak & Bronson (1976) reported that previous adult contact with a female was not necessary for male LH secretion in response to female urinary odors. Closer examination of their data, however, suggests that the LH response of naive males was not as prolonged as that of sexually experienced males. Thus even "innate"

responsiveness to the urinary pheromones of female mice may be further augmented by sexual experience.

The results of Experiments 1-3 raise questions about why the UCS in FRESH urine disappears. Several ways exist that FRESH urine could lose its ultrasound-eliciting potency. For example, bacteria present in urine may degrade the UCS, resulting in decreased potency. Another possibility is that the UCS may be an unstable chemical compound that upon exposure to air oxidizes into less potent products. Finally, the UCS may simply be volatile. Experiments 4-6 examined this last possibility.

In Experiment 4, subjects vocalized significantly more to FRESH urine presented on a cotton swab than to either FRESH or METABOLIC urine presented in a vial. During Trial 1, the subjects approached and investigated the vial with caution. The novelty of the test situation may have affected their ultrasound levels. Trials 2 and 3 examined whether the subjects' vocalization levels would change as they became habituated to the glass vials. As seen in Figure 4, subjects receiving FRESH VIAL and METABOLIC VIAL urine continued to emit significantly lower levels of vocalizations than males receiving the FRESH urine. Furthermore, the FRESH VIAL urine did not significantly differ from the METABOLIC VIAL urine in it's ability to elicit vocalizations. This finding suggested that the males receiving FRESH VIAL and METABOLIC VIAL urine may have emitted vocalizations to another property of the urine (e.g., the odor of the urine itself) to which they had become conditioned.

Experiment 5 addressed the question of odor conditioning by using sexually naive subjects. Experiment 1 demonstrated that sexually naive subjects vocalize to FRESH urine, but not to METABOLIC urine. It was logical to assume that if the UCS in FRESH urine were volatile, the volatile urinary odors should elicit vocalizations from sexually naive subjects when

presented in a glass vial. As seen in Figure 5, sexually naive subjects vocalized to FRESH urine, but did not respond at appreciable levels to either FRESH VIAL or METABOLIC VIAL urine. Taken together, Experiments 4 and 5 suggest that the UCS found in FRESH urine is of relatively low volatility at room temperature.

Placing the urine stimuli into vials in Experiments 4 and 5 prevented the subjects from physically contacting the stimuli. If the UCS was volatile, the subjects would have detected it and emitted high levels of vocalizations. The fact that they did not indicates that the UCS is relatively nonvolatile. One could argue, however, that Experiments 4 and 5 were not properly controlled. The FRESH swab should also have been presented in a vial, but in such a way that the subject could contact it. Consequently, the presence of the glass vial may simply have reduced ultrasonic vocalizations. Although this interpretation is unlikely, future research will examine this possibility.

Experiment 6 showed that extreme heat destroyed the ultrasound-eliciting factors found in both FRESH and METABOLIC urine. Three likely reasons are that boiling the urine may have accentuated normally occurring degradative processes, heat denatured the molecules comprising the pheromone, or volatilized off the pheromone.

In contrast to my results, Nyby et al. (1979) found that heating METABOLIC urine in a boiling water bath did not destroy its ability to elicit ultrasounds. The difference could have arisen because of the different methodologies employed. For example, in this thesis, each stimulus was presented to an independent group of subjects. In the study by Nyby et al. (1979), all subjects experienced all stimuli in a repeated-measures, counter-balanced fashion. Pilot studies not reported in this thesis demonstrated

that a male previously tested with FRESH or METABOLIC urine would subsequently vocalize to boiled female urine. Thus, the higher levels of vocalization seen to boiled female urine by Nyby et al. (1979) may have been due to carry-over effects from previous exposure to female urine.

At least one other investigator has also found that heat adversely affects the activity of a mouse urinary pheromone (Ingersoll, 1986). Heating male urine to 37° C in a temperature controlled water bath for 1 hr, decreased its aggression-eliciting properties, perhaps by facilitating enzyme and/ or bacterial action as well as by evaporating the more volatile chemosignal(s).

Recent work in our lab suggests that the time by which the pheromonal activity of the UCS is almost completely lost following urination is approximately 15-18 hours (Unpublished observations). The speed by which the pheromone would be lost under natural conditions, however, might be even faster. For example, in naturalistic settings female urine is deposited over a larger surface area and may evaporate or oxidize at a much faster rate. In contrast, in our laboratory setting, much larger volumes of FRESH female urine are pooled, which greatly reduce the surface area/volume ratio.Thus the speed with which volatilization and oxidative degradation can occur is substantially reduced.

It is interesting, in this regard, that METABOLIC urine, usually collected over 12 hours, appears devoid of the UCS. This is somewhat surprising in that some of the urine collected in this fashion may have been voided within minutes or hours of collection. Perhaps the method of collection, whereby the urine flows down a funnel, which greatly increases the urine's area/volume ratio, serves to deactivate the UCS very rapidly.

Thus under "normal" circumstances, the UCS may indeed be ephemeral and serve as a "biological marker" for the very recent presence of a female.

In conclusion, the results of this thesis suggest that two chemosignals exist in female mouse urine that elicit vocalizations from males: (1) a potent, nonvolatile but easily degraded (by heat and possibly other factors) unconditioned stimulus to which males vocalize without sexual experience and (2) a nonvolatile, chemically stable conditioned stimulus.

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August, 1991 to present: Recitation Coordinator, Teaching Assistant & Lab Coordinator: Currently responsible for coordinating Introduction to Psychology recitations and assist teaching laboratory and surgical techniques for Behavioral Neuroscience Laboratory, and supervise daily progress of undergraduate research progress.

May, 1991 to December, 1991: Recitation Instructor & Graduate Assistant: Coordinated the daily operation and maintenance of the Central Animal Facility, Lehigh University. Taught three Introduction to Psychology recitations.

August, 1990 to May, 1991: Recitation Instructor & Teaching Assistant: Taught three Introduction to Psychology recitations and assisted teaching Neuroanatomy of Behavior.

June, 1990 to August, 1990: Graduate Assistant: Coordinated the daily operation and maintenance of the Central Animal Facility, Lehigh University.

January, 1990 to May, 1990: Apprentice Teacher: Assisted teaching Experimental Design and Statistics.

Education

1992- Second year PhD student (Dr. John G. Nyby-Advisor).

1990-1992 Lehigh University, Bethlehem, PA 18015, M.S., Psychology

1986-1990 Lehigh University, Bethlehem, PA. 18015, B.A., Psychology

Military Experience

1986-1990 Reserve Officer's Training Corps Cadet.

June 1990 Commissioned as a Second Lieutenant in the United States Army.

Awards

1990 Elizabeth Major Nevius Award

1990 John Steckbeck Memorial Award

Membership

American Psychological Society

Senior Honors Thesis

Hormonal Regulation of Urinary Marking Behavior in Male House Mice (*Mus domesticus,* **Linn.**) Senior Honors Thesis (1990). Lehigh University.

Posters

Sipos, Jubilan, Poppito, & Nyby (1990). Hormonal Regulation of Male-typical Behaviors in Male House Mice (Mus Domesticus). Poster presented at the 22nd Annual Conference on Reproductive Behavior.

Sipos & Nyby (1991). Evidence for a Volatile Pheromone in Female House Mouse Urine. Poster presented at the 23rd Annual Conference on Reproductive Behavior.

Sipos & Nyby (1991). Hormonal Regulation of Four Male-typical Reproductive Behaviors in House Mice (Mus Domesticus). Poster presented at the 23rd Annual Conference on Reproductive Behavior.

Presentations

Nyby, Matochik, Sipos, & Barfield (1991). Intracranial Hormonal Stimulation of Androgen-dependent Behaviors in House Mice. Presentation at the 23rd Annual Conference on Reproductive Behavior.

Publications

Sipos, Kerchner, & Nyby (1992). Two Urinary Chemosignals in the Urine of Female House Mice (Mus domesticus) that Elicit Ultrasonic Vocalizations from Males? *Behavioral and Neural Biology,* In review.

END

OF

TITLE