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Mice Respond Differently to Urine and Its Major Volatile Constituents from Male and Female Ferrets

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Abstract Our previous chemical investigation showed that the concentrations of urinary volatiles from males were much higher than those from females in the ferret (*Mustela furo*). The current study was designed to examine the behavioral significance and ecological relevance of this difference for one of the main prey of the ferret, the house mouse (*Mus musculus*). Our data showed that male mice displayed no difference in their response to raw male and female ferret urine. However, they showed significantly less response to female mouse urine mixed with ferret urine than to pure female mouse urine, and to female mouse urine mixed with male ferret urine than to female mouse urine mixed with female ferret urine. Furthermore, high levels of the three major volatiles (quinoline, 2,5-dimethylpyrazine, and 4-hepatanone) in male ferret urine were as effective as raw male ferret urine was in inhibiting the response of male mice. We discuss the ecological and behavioral significance of these findings in terms of chemical mimicry and cognitive feature extraction of predator odors in mice.

Keywords Mouse · Ferret · Urine · Odor · Pheromone · Quinoline · 2,5-Dimethylpyrazine · 4-Heptanone · Mimicry · Predator · Prey

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Introduction

Numerous studies have shown that predator odor can modify rodents' behavior and physiology. Further investigation has revealed that some key volatiles from carnivore feces, specialized skin gland secretions, and urine play crucial roles in the process, although they are usually not as effective as the natural scent sources in their entirety (Mason et al., 1994; Müller-Schwarze, 1995; Apfelbach et al., 2005). Few attempts have been made to investigate whether male and female predator' odors can elicit differential responses from their prey (Apfelbach et al., 2005). One exception is the study by Stoddart (1980), although he failed to find any difference in the response of rodents to male and female cat odors. Our recent chemical analysis of ferret (*Mustela furo*) urine revealed that the total amount of volatiles in male urine was 10 times that in female urine. This dramatic difference may provide a source for rodent prey to respond differentially so as to exploit the behavioral difference between male and female ferrets (Zhang et al., 2005).

Although sympatric predators possibly exert a stronger effect than allopatric predators do on their rodent prey (Mappes et al., 1998), the prey's response to the odors of predators is generally considered to be innate due largely to sulfur compounds common in carnivores (Mason et al., 1994). For this reason, search for carnivore scent-based rodent repellents has mainly focused on sulfur-containing compounds (Vernet-Maury et al., 1984; Sullivan et al., 1988a,b; Epple et al., 1995; Burwash et al., 1998; Bramley and Joseph, 2001). However, chemical investigation has revealed that sulfur compounds in secretions or excretions from some carnivores are often minor components or even absent (Preti et al., 1976; Brinck et al., 1983; Schildknecht and Birkner, 1983; Raymer, 1984; Raymer et al., 1985; Service et al., 2001; Buesching et al., 2002a,b). This suggests that nonsulfur compounds may be important in the response of rodents to their predators. Indeed, Nolte et al (1993) reported that several nonsulfur-containing compounds repel mice. One such compound is o-aminoacetophenone, a volatile chemical commonly found in the anal gland secretions of several *Mustela* species (Brinck et al., 1983; Zhang et al., 2003, 2005).

Ferret urinary volatiles are nonsulfur compounds, with quinoline, 2,5-dimethylpyrazine (2,5-DMP), and 4-heptanone as the major constituents (Zhang et al., 2005). The proportions of these three compounds in total urinary volatiles are 39.0%, 25.0%, and 22.4% in males, and 41.9%, 9.2%, and 6.8% in females, respectively (Zhang et al., 2005). Previous work showed that sulfur compounds from the anal glands of ferrets can elicit aversive response from rodents (e.g., Sullivan et al 1988a,b; Mason et al., 1994; Epple et al., 1995). However, some findings suggest that urine (without sulfur compounds) from *Mustela* species, such as American minks (*M. vison*) and ferrets, can also alter the behavior of rodents (Epple et al., 1993; Roberts et al., 2001; Cloe et al., 2004).

Interestingly, both 2,5-DMP and 4-heptanone are also found in urine from female house mice. The former is a female-specific pheromonal compound, whereas the latter is associated with pregnancy and lactation (Novotny et al., 1986; Jemiolo et al., 1987). This coincidence of chemical compounds between predator and prey raises the question of how male mice respond to potentially conflicting information from female conspecifics and their main predator. Our present work, therefore, is aimed at answering the questions of whether ferret male and female urine can elicit differential responses and whether the synthetic analogues of the three major volatile constituents can inhibit the response of house mice. Regarding the first question, because the major urinary volatiles are different between male and female ferrets, we hypothesize that mice can differentiate between male and female ferret urine. For the second question, since the presence of a major predator poses a high risk to mice, the potential attraction of male to female mice could be overcome by the presence of a life-

threatening predator. For this reason, we hypothesize that the responses of mice will be more strongly affected by chemicals unique to predator urine than by chemicals common to the urine of both mice and predator.

Methods and Materials

Experimental Animals

Mice We used 23 naïve male and 12 female ICR/Albino house mice, age 9–10 mo. They were purchased from Harlan Sprague–Dawley, Inc., Indianapolis, IN, USA, and were kept individually in polypropylene cages (27×12×17 cm) in Indiana University Animal Facility. The mice were housed at 21±0.2°C and 50–70% RH. A 12L:12D reverse light cycle regime was used with light on at 2200 hours. Purina Mouse Chow and water were supplied *ad libitum*. The bedding material (sawdust) was changed weekly. Test males used as odor recipients were randomly assigned to two groups for experiment series 1 (12 males) and experiment series 2 (11 males), respectively. The subjects were used in random order. Four estrous females were selected as urine donors.

Ferrets Four adult male and five adult female ferrets in breeding condition (about 1 yr of age) were purchased from Marshall Farms (North Rose, NY, USA). Upon arrival at the Boston University Animal Facility, they were individually housed in modified rabbit cages under a long-day (16L:8D) photoperiod. Ferrets were fed with moistened Purina Ferret Chow once a day, whereas water was available *ad libitum*. The handling procedure for mice and ferrets complied with the institutional guidelines for animal use and care for both Indiana University and Boston University.

Sample Preparation and Experimental Procedure

Quinoline, 2,5-DMP, and 4-heptanone were purchased from Aldrich Chemical Company. They were each diluted to 0.5% in deionized water for the behavioral test. We roughly estimated the concentrations of quinoline, 2,5-DMP, and 4-heptanone to be 0.96, 0.64, and 0.54 ppm, respectively, in male ferret urine by comparing their gas chromatographic peak areas with those of concentration-known internal standards (Zhang et al., 2005). Various odor samples of the synthetic chemicals were created by adding the synthetic compounds to either estrous female mouse urine or female ferret urine, and tested using male mice.

Experiment series 1 To characterize the responses of mice to male and female ferret odor, responses of male mice to a binary choice of raw male or female ferret urine were measured over 4 consecutive days. Next, we tested the responses of male mice to a binary choice of female mouse urine (FMU) with added male or female ferret urine at concentrations of 50%, 20%, and 10%. After finding that all concentrations were effective, we tested the responses of male mice to a binary choice of 10% raw ferret urine (either male or female) in FMU against FMU alone.

Experiment series 2 To investigate the roles of the three major volatile constituents in ferret urine on the responses of male mice to male and female ferret urine odor, we first tested FMU with only one of the synthetic chemicals, at about 40 times its natural level in male

ferret urine, against FMU. Next, we tested the permutations of FMU spiked with one of the three compounds (at about 40 times its level in male ferret urine) against each other in binary choices. Then, we tested all three chemicals (at 40 times their levels in male ferret urine) added to FMU against either FMU or FMU with quinoline (at 40 times the level in male ferret urine). Finally, we tested combinations of FMU contaminated by 10% (v/v) one of the following: (A) spiked female ferret urine (FFU) by the three synthetic chemosignals at 40 times their natural levels in male ferrets; (B) spiked FFU by them at the natural levels in males; (C) 10% raw FFU; and (D) 10% raw male ferret urine.

Urine Collection

Female mouse urine collection We determined female estrus by examining the vaginal smear. Four estrous females, out of 12, were randomly selected and each kept in a metabolic cage for urine collection for 6 hr. Urine was fed to a vial immersed in dry ice. Urine samples were stored at −20°C until used in a behavioral test.

Ferret urine collection As described by Zhang et al. (2005), ferrets were placed individually in a clean cage with a grid floor and a clean collecting pan underneath to collect urine. Fresh urine from each of four males and three females was collected and immediately stored on ice. Urine that was deposited with or next to feces was not collected. All urine samples were stored at −20°C until use. Pooled urine samples of either sex were used.

Behavioral Test

The preference of male mice to two aqueous scent samples was tested in the dark. Immediately before each trial, a test mouse in its home cage was transferred to the test room under dim light. Scent samples were presented to the mouse by a disposable glass capillary (i.d. 1.1–1.2 mm; o.d. 1.3–1.4; 15 cm length; Drummond Scientific Co., Broomall, PA, USA), similar to the methods used by Lai et al. (1996) and Zhang et al. (2001). Each capillary contained a 10-μl sample aliquot. The opening of the capillary was sealed by Tube Sealing Compound N:O 510 (Chase Instruments Corp., Rockwood, TN, USA) so as to suspend the aqueous sample inside the capillary 1 cm from the sample-containing end. The sample-containing end of the capillary was presented to a mouse, whereas the other end was held by an observer wearing disposable plastic gloves. Two aqueous samples were presented simultaneously to the test subject for 3 min after it first showed a sniffing response. The two capillaries were lowered through the wire lid and kept approximately 2 cm apart. The time that the mouse spent sniffing within 1 cm from the tip and licking the end of the capillary was recorded for both treatments. A mouse was used only once a day. Subjects whose investigating time was less than 1 s on a day were excluded for that day.

Statistical Analysis

Wilcoxon matched-pairs signed-rank test was used for behavioral response using the program SPSS (Version 10.0). The level of significance was set at $\alpha=0.05$ for all tests.

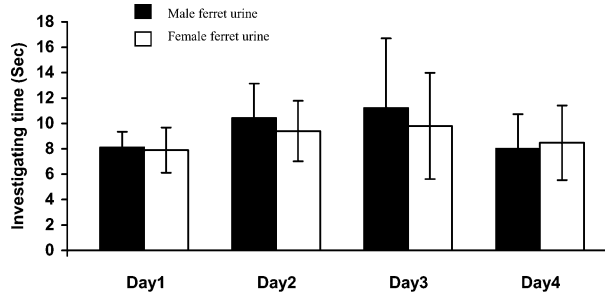

Fig. 1 Responses (mean ± SE) of male mice to male (black) and female (white) ferret urine over four consecutive days

Results

Experiment Series 1. Effects of Male and Female Ferret Urine on Responses of Male Mice

Male mice did not show a differential response to male and female ferret urine in the 3-min daily test on each of 4 successive days (Day 1: N=12, Z=0.079, P=0.938; day 2: N=12, Z=1.177, P=0.239; day 3: N=12, Z=1.098, P=0.272; day 4: N=12, Z=0.314, P=0.754) (Fig. 1). However, when 50%, 20%, or 10% of ferret urine was added to FMU, male mice spent less time investigating FMU mixed with male ferret urine than that mixed with female ferret urine (N=12, Z=2.824, P=0.005; N=11, Z=2.667, P=0.008; N=11, Z=2.756, P=0.005) (Fig. 2). Compared with FMU, adding either 10% male or female ferret urine to FMU reduced the responses of male mice (N=12, Z=3.060, P=0.002 and N=11, Z=2.667, P=0.008, respectively, Fig. 2).

Experiment Series 2: Roles of Three Major Constituents in Eliciting Responses of Mice

Effects of Single Major Components

Male mice reduced investigating time to FMU with quinoline (N=12, Z=2.667, P=0.008), 2,5-DMP (N=10, Z=2.497, P=0.013), or 4-heptanone (N=11, Z=2.134, P=0.033) compared with pure FMU (Fig. 2). Furthermore, male mice spent less time investigating FMU with quinoline compared with FMU with either 2,5-DMP (N=12, Z=2.667, P=0.008) or 4-heptanone (N=10, Z=2.293, P=0.022). They did not respond differently between FMU containing 2,5-DMP and that containing 4-heptanone (N=10, Z=0.968, P=0.330) (Fig. 3).

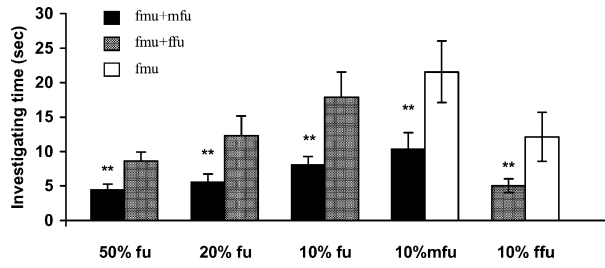

Fig. 2 Responses (mean ± SE) of male mice comparing binary choices between female mouse urine (fmu) mixed with various concentrations of ferret urine (fu), either male (mfu) or female (ffu). Additionally, binary choices between fmu and fmu plus 10% mfu or 10% ffu were compared. **P<0.01; *P<0.05

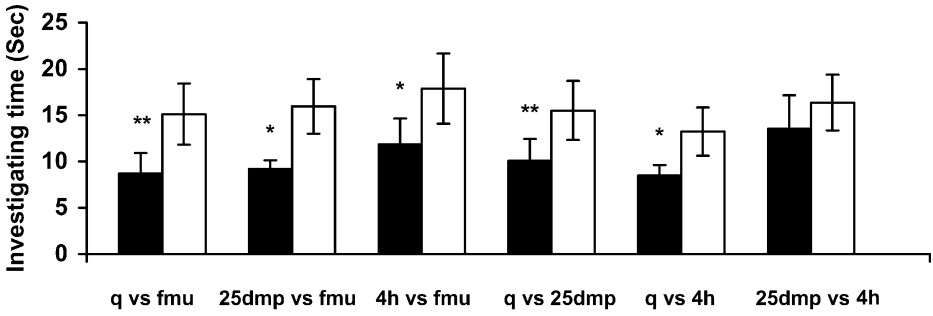

Fig. 3 Responses (mean ± SE) of male mice to female mouse urine (fmu) spiked with quinoline (q), 2,5-dimethylpyrazine (25dmp), or 4-heptanone (4h) in various binary choices. $^{**}P<0.01$; $^{*}P<0.05$

Combined Effect of the Three Major Components

FMU with quinoline, 2,5-DMP, and 4-heptanone together, reduced the investigative response of male mice compared to pure FMU ($N=10$, $Z=2.803$, $P=0.005$) or FMU with quinoline ($N=12$, $Z=2.432$, $P=0.015$) (Fig. 4).

Effect of Mimicking Male Ferret Urine by Spiking Female Ferret Urine with the Three Compounds

Male mice showed less response to FMU scented by 10% female ferret urine containing the three synthetic compounds at about 40 times their natural levels in male ferret urine, than to the same treatment with the three synthetic compounds at roughly the natural levels of male ferrets ($N=10$, $Z=2.089$, $P=0.037$). The response of mice to FMU mixed with 10% female ferret urine, with the three compounds at roughly their natural levels in male ferret urine, was also reduced compared to their response to FMU mixed with 10% raw female ferret urine ($N=10$, $Z=2.497$, $P=0.013$). There was no difference in response to FMU plus the

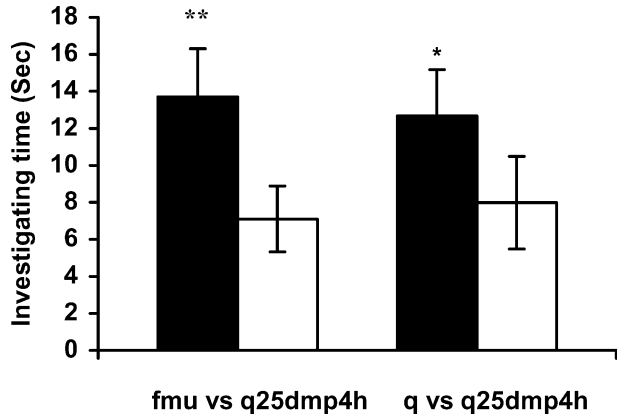

Fig. 4 Responses (mean ± SE) of male mice to female mouse urine (fmu) spiked with quinoline (q), 2,5-dimethylpyrazine (25dmp), and 4-heptanone (4h) together, versus either fmu alone or fmu with *q* only. $^{**}P<0.01$; $^{*}P<0.05$

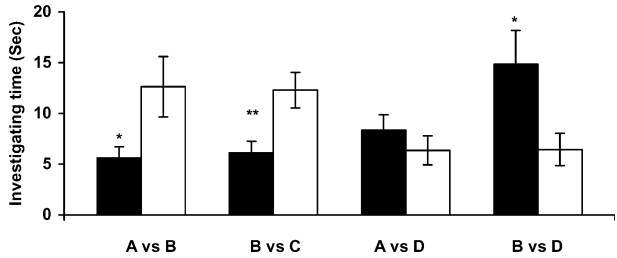

Fig. 5 Binary choices testing the responses (mean ± SE) of male mice to female mouse urine (fmu), mixed with 10% of the following: A= female ferret urine (ffu) plus quinoline (q), 2,5-dimethylpyrazine (25dmp), and 4-hepatanone (4h), all at concentrations 40 times that found in natural male ferret urine (mfu); B= ffu plus *q*, 25 dmp, and 4h, all at natural concentrations found in male ferret urine (mfu); C= ffu; D= mfu. $^{**}P<0.01$; $^{*}P<0.05$

treated female ferret urine with three synthetic compounds, at about 40 times their natural levels in male ferret urine, compared to FMU plus 10% male ferret urine ($N=10$, $Z=1.274$, $P=0.226$). In contrast, mice showed a greater response to FMU plus the three synthetic compounds, at about their natural levels in male ferret urine, compared to FMU plus 10% male ferret urine ($N=10$, $Z=2.089$, $P=0.037$) (Fig. 5).

Discussion

In experiment series 1, we found that mice did not show a difference in response to pure male and female predator odor, consistent with previous work (Stoddart, 1980). However, when female mouse urine was used as a background odor, male mice showed a differential response between sexually dimorphic male and female ferret urine (Zhang et al., 2005). Hence, our hypothesis that mice are able to discriminate between male and female ferret urinary odors is supported. A reason for the apparent discrepancy between the results may be that the response of mice to ferret odor is context-dependent. Urine of male and female ferrets by itself might represent an equal level of threat to mice. However, when mixed with female mice urine, a situation was created for male mice to leverage between a potential benefit (female mouse as a mate) and a cost (the presence of a ferret), and male mice showed behavioral differentiation between the male and female ferret urine.

In experiment series 2, our results suggest that adding any of the synthetic versions of the three compounds to the urine of estrous female mice reduced investigation by male mice. In particular, quinoline, present in ferrets but absent in mice, exhibited a stronger inhibitory effect than the other two chemicals (which are present in both ferrets and female mice) (Andreolini et al., 1987; Jemiolo et al., 1987; Zhang et al., 2005). Thus, our second hypothesis is also supported. Researchers have shown that titers of 2,5-DMP and 4-heptanone in female mice are related to the nonreceptive condition (Novotny et al., 1986; Andreolini et al., 1987; Jemiolo et al., 1987). The compound 2,5-DMP is a mouse pheromone component, which is excreted at a high level by crowded female mice, with the vital behavioral function of the Lee-Boot effect. Its level goes up during diestrous days and down on the estrous day (Novotny et al., 1986; Andreolini et al., 1987; Jemiolo and Novotny, 1993). 4-Heptanone is found in the urine of female mice, and attains a high level in pregnant and lactating females (Jemiolo et al., 1987). That is, these compounds exist in ferrets [both males and females, albeit in different quantities, see Zhang et al. (2005)] and also characterize nonreceptivity of

female mice. This may be the reason why their increases in the urine of estrous females reduced the investigative response of males. Furthermore, the coincidence in the timing of the increase of 2,5-DMP and 4-Heptanone in the urine of female mice and when male mice are unwanted supports the contention that females might chemically deceive males by mimicking the presence of ferrets. This deception could cause an opposing selection pressure for males to evolve an anti-deceptive response. Indeed, our study shows that males exhibited a stronger response to quinoline, which is present in ferrets but not female mice, than to either 2,5-DMP or 4-heptanone. In other words, quinoline may convey unambiguous information about the presence of ferrets. This presumed chemical mimicry, in terms of the function served for the mimicker, would be fundamentally different from other known mimicry systems (e.g., Pasteur, 1982; Dettner and Liepert, 1994). Further studies are needed to substantiate this as well as to investigate how such a system might evolve.

Of the three major volatile compounds in ferret urine, quinoline had the strongest deterring effect. This result demonstrates an evolutionary convergence in the response of a variety of prey animals because quinoline is also an effective repellent for ants, spiders, cockroaches, and frogs (Eisner et al., 1997). Interestingly, apart from ferret urine, quinoline is found mainly in the thoracic glands of phasmid insects (*Oreophoetes peruana*) as a defensive substance (Schildnecht et al., 1986; Zhang et al., 2005).

Our study supports the general rule that for prey recognition, individual components of an odor are less effective than the whole (Müller-Schwarze, 1995; Mason et al., 1994; Schulte et al., 1994; Apfelbach et al., 2005). This is shown by the combination of the three components being more effective than the most effective single compound (quinoline), and by the three compounds together, at their natural concentrations, being less effective than male ferret urine. With regard to the latter point, the three component blend was only as effective as male ferret urine when presented at levels much higher than their natural levels. This suggests that other minor constituents in ferret urine may also play a role in eliciting the aversive response. One possible compound is *o*-aminoacetophenone, which is a repellent for birds and mice (Nolte et al., 1993).

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