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Profiles of Volatiles in Male Rat Urine: The Effect of Puberty on the Female Attraction

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Abstract

Rat urine contains many volatile constituents that may be used for chemical communication. The levels of certain urinary volatiles are strongly dependent on the sex and endocrine status (e.g., puberty). We performed chemical and behavioral studies to identify the volatiles in adult male rat urine that attract mature females. Our results demonstrated that adult male rats have higher levels of 2-heptanone (2-HP), 4-methylphenol (4-MP), and 4-ethylphenol (4-EP) than prepubescent male rats; furthermore, female rats are more attracted to the odor of adult male rat urine than that of prepubescent males. When prepubescent rat urine was supplemented with 2-HP, 4-MP, and 4-EP to the levels found in adult male urine, the attractiveness of the urine to females was markedly enhanced. Our results suggested that this attraction is due to an increased level of chemosignaling.

Key words: 4-methylphenol, pheromones, puberty, rat, urinary volatiles, urine odor

Introduction

The odors in the urine of rodents are a rich source of chemical sensory information for other animals. For example, an animal can determine the species of another animal, as well as its stress levels, age, individual identity, and whether it has an infection, solely on the basis of the odors in its urine (Yamaguchi et al. 1981; Yamazaki et al. 1994, 2002; Osada et al. 2003, Osada, Tashiro, et al. 2008; Gutierrez-Garcia et al. 2007). There is also evidence that the gender, reproductive state, and sexual experience of an animal can be detected on the basis of its urine scent (Jemiolo et al. 1985; Novotny et al. 1986; Yamazaki et al. 1989; Beauchamp et al. 1994, 1995). Moreover, previous behavioral studies have shown that the urinary pheromones of male mice (*Mus musculus*) can induce the estrous cycle in conspecific animals (Whitten et al. 1968). These urinary pheromones can also induce the failure of implantation and the return to the estrous cycle (Bruce 1960; Bruce and Parrott 1960). Conversely, female urinary pheromones tend to suppress the estrous cycle and delay the onset of puberty (Van Der Lee and Boot 1955, 1956). In addition, some male urinary pheromones appear to act in a highly specific manner to alter the secretory patterns of luteinizing hormone and prolactin, as well as the production of sex steroids whose secretion is regulated by the latter tropic hormones and that serve to accelerate the onset of puberty (Vandenbergh 1967).

The chemical basis for these effects can, at least in part, be attributed to urinary volatiles. The pheromonally active compounds in house mouse urine are largely volatile substances that tend to bind to proteins excreted into the urine (Novotny 2003). These include 2-sec-butyl-4,5-dihydrothiazole (BT) and 3,4-dehydro-exo-brevicomin (DB), which together induce estrous and accelerate the onset of puberty in female mice (Jemiolo et al. 1986) and potentiate aggression in male mice (Novotny et al. 1985). In addition, the urinary sesquiterpenic volatiles α-farnesene and β-farnesene accelerate the onset of puberty in females (Novotny et al. 1999) and signal male dominance (Harvey et al. 1989). These volatile chemicals in male mouse urine appear to be androgen-dependent mouse pheromones that impart endocrinological messages: These messages not only activate female mouse sexual attraction but they also accelerate the onset of female puberty and induce the synchronization of the estrous cycle, among other effects. In addition to these pheromones in male mouse urine, the 2-heptanone (2-HP) and 2,5-dimethylpyrazine levels in

female urine increase during the proestrus and estrus phases of the estrous cycle in the female mouse (Jemiolo et al. 1989). These volatiles are known to be female mouse pheromones that control the timing of puberty and induce menstrual cycle synchronization in sniffer female mice (Novotny et al. 1986).

Like mice, although rats (*Rattus norvegicus*) are a worldwide pest, they are also very useful as animal models in biological research. In particular, the inbred and closed colony strains of rats have been used extensively to determine the molecular underpinnings of chemical communication (Brown 1985). It has been shown that rats use urine odors to discriminate males from females and sexually active from sexually inactive adults. For example, female rats prefer the urine odor of adult and pup males to those of females; they also prefer the urine odor of intact males to those of castrated males (Brown 1977; Lucas et al. 1982). In addition, rats can determine the stress levels and the individual identity of another rat solely on the basis of its odor (Brown et al. 1989; Kiyokawa et al. 2005; Inagaki et al. 2008). However, compared with the studies examining murine urine, fewer studies have sought to identify the volatile semiochemicals in rat urine. Nevertheless, earlier definitive reports have shown that the urine of conventional rats has a higher number of volatiles, including 2-HP and phenols, than the urine of germ-free rats (Holland et al. 1983). In addition, when diabetes is induced by alloxan treatment, the types of rat urinary volatiles that are present change (Rhodes et al. 1982). These studies were the first to systematically investigate rat urinary volatiles. However, the semiochemicals in male rat urine that attract female rats have not yet been identified.

One recent report showed that some chemicals, including 2-HP and 4-ethylphenol (4-EP), are present at higher levels in normal male Sprague–Dawley (SD) rat urine than in female and castrated SD male rat urine and showed that these chemicals activate female attraction (Zhang et al. 2008). However, adding these chemicals to castrated male urine did not fully restore the female preference for urine from intact males, which suggests that other compounds may also play a crucial role in female attraction. To our knowledge, additional studies have not yet been performed that identify these female attractant chemicals in male rat urine and clarify the mechanism underlying these chemicals action.

To better understand the attraction to adult male rat urine by female rats, we first used gas chromatography (GC) to analyze the volatiles in prepubescent (5-week-old [5W]) and adult male (8-week-old [8W]) rat urine. We also analyzed the chemicals in the urine of young adult (11-week-old [11W] and 14-week-old [14W]) male rats that had or had not had sexual experience at 9 weeks of age. We then performed a behavioral study using a 2-choice preference test to determine the response of mature female rats to the odor of prepubescent and adult male rat urine. We then examined the response of female rats to the odor of intact prepubescent male rat urine relative to the same intact urine spiked with chemicals that increase with puberty.

Materials and methods

Animals

The rats belonged to the Donryu (DN) strain and were bred at the Health Sciences University of Hokkaido. They were maintained in a room at 22 °C with a photoperiod of 12:12 light:dark and were provided with continuous food (Lab Chow, MF, Oriental Yeast) and water. The animals were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals. The experimental protocols were approved prior to experimentation by the Animal Ethics and Research Committee of the Health Sciences University of Hokkaido.

Collection of urine samples

For the first GC experiment, urine samples were collected from healthy 5W (n = 10) and 8W (n = 10) male rats kept in metabolic cages. For the second GC experiment, urine samples were collected in a similar manner from 5W (n = 12), 8W (n = 12), sexually experienced 11W (n = 6), sexually inexperienced 11W (n = 6), sexually experienced 14W (n = 6), and sexually inexperienced 14W (n = 6) male rats. To obtain sexually experienced rats, the 11W and 14W male rats had been mated at the age of 9 weeks with female DN rats for 5 days. For the behavioral studies, the test samples consisted of large pools of urine samples collected from the same age groups. To avoid diurnal fluctuation, urine collection was performed between 18:00 to 8:00 (overnight). The urine samples were stored in sterile tubes at –80 °C.

Design of GC experiments

In the first GC experiment, the differences between 5W and 8W rats in terms of their volatile urine components were determined. For this, the peak areas of 32 representative compounds present in both 5W and 8W male rat urines were analyzed. This experiment revealed that 5W and 8W rats differ in their urinary levels of 2-HP, 4-methylphenol (4-MP), and 4-EP (see Table 1). In the second GC experiment, the levels of 2-HP, 4-MP, and 4-EP in urine samples from 5W, 8W, sexually experienced and inexperienced 11W, and sexually experienced and inexperienced 14W rats were measured. In all, 200 ng of 7-tridecanone (dissolved in hexane) served as the internal standard (IS).

Preparation of the samples for GC analysis by headspace solid phase micro extraction

The samples were prepared for GC analysis by headspace solid phase micro extraction (HS-SPME). To concentrate

Table 1 Results of Mann–Whitney *U*-test of the average peak area (×10) of identified urinary chemicals

Peak number		RT	5W urine interquartile		8W urine interquartile		Mann–Whitney *P* value
	Peak identity		Median	Range	Median	Range	5W versus 8W
1	Acetone	1.8	878	525–1870	354	72–748	$P < 0.001$
2	Butanone	2.5	608	286–1755	174	97–794	$P < 0.01$
3	6H6M3H (lactole)	3.0	23	11–180	107	10–285	
4	Pentanone	3.7	614	368–1976	710	421–1911	
5	2-Hexanone	6.4	29	10–89	32	27–82	
6	4-Heptanone	7.5	12	10–21	21	10–66	
7	2-Ethyl-2-pentenal[a]	7.7	55	10–93	62	37–87	
8	2-Heptanone	8.8	450	303–938	1819	1404–4946	$P < 0.0001$
9	5-Hepten-2-one[a]	10.3	41	18–142	55	24–133	
10	Octanal	11.1	81	10–146	36	13–80	
11	3-Hepten-2-one	11.8	24	16–69	80	49–261	$P < 0.001$
12	Nonanal	12.6	51	21–176	30	17–127	
13	5-Methyl-2-hexenal[a]	13.6	34	11–57	33	10–50	
14	Acetic acid	13.7	73	26–173	116	77–264	
15	2-Ethyl-hexanol	14.3	87	46–151	23	10–165	
16	Decanal	14.4	53	38–253	41	23–56	
17	Benzaldehyde	14.8	48	20–82	74	39–144	
18	Butanol	16.1	97	46–203	69	20–90	
19	Acetophenone	16.5	29	15–69	36	18–60	
20	Methyl-butyric acid	16.6	84	51–230	82	72–150	
21	Benzyl methyl ketone	17.5	32	10–128	33	11–85	
22	Hexanol	17.6	197	140–380	81	10–149	$P < 0.01$
23	Capronic acid	18.7	74	12–297	51	35–161	
24	2-Methoxy-phenol	19.0	77	59–241	86	53–162	
25	Dimethyl sulfone	19.6	37	27–72	24	11–83	
26	2-Methyl-hexanoic	19.9	82	15–263	71	21–363	
27	Heptanoic acid	20.1	31	10–66	36	22–147	
28	Phenol	20.5	194	83–475	327	158–1154	
29	4-Methylphenol	21.4	16 049	4204–38 078	96 983	48 210–145 260	$P < 0.0001$
30	4-Ethylphenol	22.4	2117	780–4108	9732	4730–20 336	$P < 0.0001$
31	2,6-Di-tert-butyl-phenol[a]	24.3	36	19–68	17	10–27	
32	Indole	25.0	61	29–628	122	69–277	

Median height of 10 urine samples. The underlined and not underlined *P* values signify significant age-related decreases and increases Peaks were identified by mass spectrum (quadrapole) and by retention time matching their retention times against authentic chemicals.
[a]Tentatively identified by mass spectrum (quadrapole).

the volatiles from the urine, an SPME fiber (2 cm–50/30 μm DVD/Carboxen/PDMS StableFlex, Supelco) was inserted for 60 min into the headspace of a 4 ml vial with a Teflon septum (Supelco) containing 150 μl of rat urine that was then saturated with NaCl, mildly heated to 37–40 °C, and constantly stirred.

Chemical analysis performed with flame ionization detector–gas chromatography and gas chromatography–mass spectrometry

The chemical analysis was carried out on a Hewlett Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) (Agilent technologies). The gas chromatograph was fitted with a Restek Stabilwax column (30 m × 0.32 × 0.5 μm; Restek). The carrier gas was helium, and the column flow was 2.4 ml/min. The oven temperature was maintained at 40 °C for 5 min, increased by 10 °C/min to 200 °C, and then elevated by 5 °C/min to 240 °C. The injector temperature was held constant at 230 °C.

The identification of odor compounds was performed by gas chromatography–quadrupole mass spectrometry (GC-MS) QP5000 ver.2 (Shimadzu). The column and oven conditions were identical to those described for the chemical analyses. The identification of the structures of representative peaks was performed by using both the NIST92 library and a manual interpretation of mass spectra based on comparisons with those reported in the literature. In addition, a comparison of relative retention times and mass spectra of authentic chemicals was performed by employing a 6-hydroxy-6-methyl-3-heptanone sample (Tashiro et al. 2008) and commercial chemicals. These authentic chemicals served as a hexane solution (1000 parts per million [ppm] v/v) and were preserved in a deep freezer.

Behavioral study

Fifty-eight sexually experienced 5- to 6-month-old female DN rats served as the test animals. These females were housed 2 per cage in stainless steel wire cages in our sterile animal facility. The preference tests were conducted in a rectangular open-bottomed arena (45 × 30 cm). For odor presentations, 2 stainless steel cylinders covered on top by stainless steel wire mesh (4 cm in height and 5 cm in diameter) were placed in the centers of the 2 short sides of the arena. A Petri dish (3.5 cm diameter) containing a 0.5 ml urine sample was fitted into the bottom of each cylinder so that the respondent animal could sniff the opening but could not access its contents. The animal was considered to be sniffing at the top of a cylinder when its snout was oriented toward the stainless wire mesh and held within 1 cm of it. To avoid data confounding due to learning, the female rats were only tested once. Prior to each test, a 3-min period was provided for habituation. Following habituation, the Petri dishes containing male rat urine were fitted into the bottom of each cylinder. The test period was 3 min long and an observer used separate timers to record the amount of time the animal spent sniffing each of the cylinders.

Each test was conducted between 9:00 AM and 12:00 PM. The floor of the test area was replaced with a clean bench coat between each trial to eliminate residual cues that could influence the next rat. All the behavioral studies were performed as blind tests.

In this study, we conducted 4 types of odor preference tests. Experiment 1 was designed to determine whether the age of the male rat affects the attractiveness of its urine to mature females. Thus, the urines from 5W and 8W male rats were placed in the 2 cylinders (n = 16). To demonstrate the attractability of adult male rat urine for female rats, we processed the behavioral data as described below. First, the relative sniffing time of 8W urine was taken to be that of the sniffing time of 8W urine over that of sniffing time of 5W urine. Next, we confirmed that there were no significant differences between the sniffing times of control 5W urine and those of 5W urine (n = 6). In fact, the relative sniffing time of 5W urine was nearly zero. Hereafter, we compared the differences between the average relative sniffing time of 8W urine and that of 5W urine (Figure 3A).

Experiments 2 asked whether supplementing 5W urine with sufficient 2-HP, 4-EP, and/or 4-MP (which are present at higher levels in 8W urine than in 5W urine, see Table 1) to generate postpuberty levels induces the same level of female interest as 8W urine. Thus, 5W urine supplemented with 2-HP, 4-MP, and 4-EP was placed at one end of the arena and 5W urine spiked with equal amounts of hexane was placed at the other end (n = 10). Next, we confirmed that there are no significant differences of the sniffing times between the control 5W urine plus hexane and that of the 5W urine plus hexane (n = 6). These data were processed in the same manner as in experiment 1.

To generate the postpuberty levels of 2-HP, 4-MP, and 4-EP in 5W urine, 10, 10, and 40 ppm v/v of these chemicals were added to 5W urine, respectively. The differences in the levels of 2-HP, 4-MP, and 4-EP between 5W urine and 8W urine were estimated by referring to the standard curve. The chemical concentrations used for each standard curve were 6, 18, 60 ppm. Experiments 3 and 4 (n = 10, each other) were the same as Experiment 2 except that the 5W urine was only supplemented with 2-HP and 4-EP in Experiment 3 and only with 4-MP in Experiment 4.

The statistical significance of the differences in the responses between the urine samples was assessed by the chi-square test. The statistical significance of differences between the relative sniffing times was assessed by the Mann–Whitney U-test (Figure 3A) or the Kruskal–Wallis test with a post hoc test (Steel–Dwass; Figure 3B).

Results

Chemical studies

Typical examples of the FID-GC chromatograms of prepubescent (5W) and young adult (8W) male DN rat urine extracts are shown in Figure 1. The FID-GC analysis did not detect any absolute differences in the composition of the extracts, but the amounts of the urine components did differ depending on the age of the rat. Of the 47 representative peaks that were chosen for comparison because we could

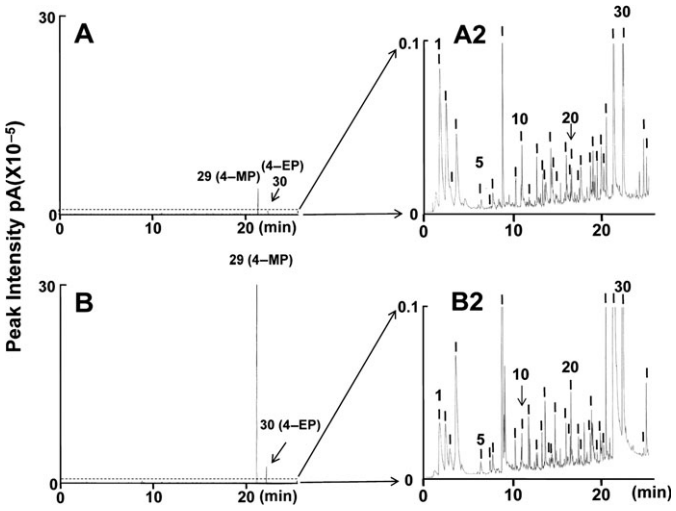

Figure 1 Typical gas chromatograms of HS-SPME of urine samples from 5-week-old **(A)** and 8-week-old **(B)** male DN rats. The 32 peaks that were chosen for comparison are indicated with tick marks and sequential numbers. To show the many small peaks that were detected in these chromatograms, the rectangular strip areas indicated by dotted lines on **(A** and **B)** were magnified to A2 and B2.

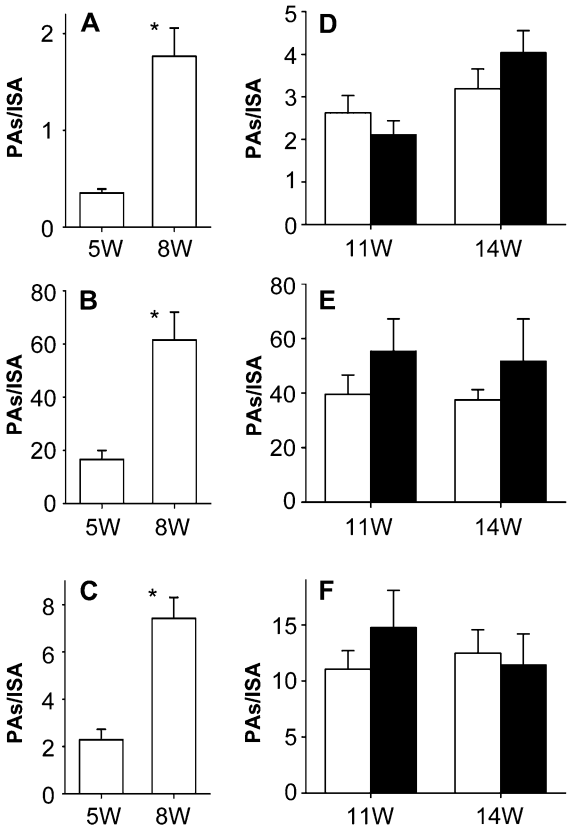

Figure 2 Effect of maturation on the levels of 2-HP **(A**, **D)**, 4-MP **(B**, **E)**, and 4-EP **(C**, **F)** in male rat urine. Urine samples were individually collected from male rats at 5, 8, 11, and 14 weeks of age (n = 12 per age group). The ordinate (PAs/ISA) indicated the ratio of peak areas of 2-HP, 4-MP, and 4-EP divided by IS peaks. Among the 11- and 14-week-old rats, half of the rats in these groups were allowed to mate at 9 weeks of age, and thus, at 11 and 14 weeks of age, these rats were divided into a sexually experienced group (n = 6; filled bar) and a sexually inexperienced group (n = 6; open bar). The statistical significance of the differences between the groups was assessed by either the Wilcoxon test (*$P < 0.01$) or the Mann–Whitney U-test.

reliably detect them, 32 were identified by GC-MS (Table 1). Of these, the 5W and 8W urine samples differed significantly in terms of 6 peak areas (Table 1). 2-HP, 4-MP, and 4-EP were associated with the most prominent differences, with the 8W urine having significantly higher levels of these 3 chemicals than the 5W urine. 4-MP was the most abundant urinary chemical for both prepubescent and young adult male DN rats. On the other hand, acetone, butanone, and hexanol are present at higher levels in prepubescent urine than in adult urine (Table 1).

The second GC experiment examined the changes in the urinary levels of 2-HP, 4-MP, and 4-EP as the male rat progresses from prepubescence (5W) to young adulthood (8W). In Figure 2, the ordinate (PAs/ISA) indicated the ratio of peak areas of 2-HP (A), 4-MP (B), and 4-EP (C) divided by IS peaks. The urinary levels of 2-HP, 4-MP, and 4-EP significantly increased as the rats aged from 5W to 8W (Figure 2A–C). In the same experiment, we assessed the effect of sexual experience at 9 weeks of age on the urinary levels of 2-HP, 4-MP, and 4-EP at the 11 and 14 weeks old rats but found that the sexually experienced group did not differ significantly from the sexual inexperienced group in terms of the levels of these chemicals (Figure 2D–F).

Behavioral studies

Experiment 1

The average total time mature female rats spent sniffing the whole urine from 5W and 8W male rats during the 3-min test was 29.6 ± 2.2 (SE) s. The average times spent sniffing the 8W urine and the 5W urine were 20.9 ± 1.4 versus 9.7 ± 1.3 s ($P < 0.01$), respectively. On the other hand, there were no differences between the sniffing times of the test sample (5W urine) and those of the control sample (5W urine). The differences between the average relative sniffing times of the 8W urine and 5W urine indicates that the females preferred the urine from the adult males over the urine from the prepubescent males (11.2 ± 1.6 vs. 0.7 ± 2.1, $P < 0.01$; Figure 3A). In the experiment 1, the estrous status of these females was assessed by performing standard vaginal cytology just before the behavioral experiment, which resulted in the assignment of 4 females to each of the proestrus, estrus, metestrus, and diestrus groups. There were no significant differences in the relative sniffing times for 8W urine among the 4 estrous status groups (proestrus; 12.4 ±3.7 s, estrus; 12.1 ± 2.1 s, metestrus; 8.5 ± 3.4 s, and diestrus; 11.9 ± 4.1 s). Therefore, the other behavioral experiments were performed without assessment of the estrous status.

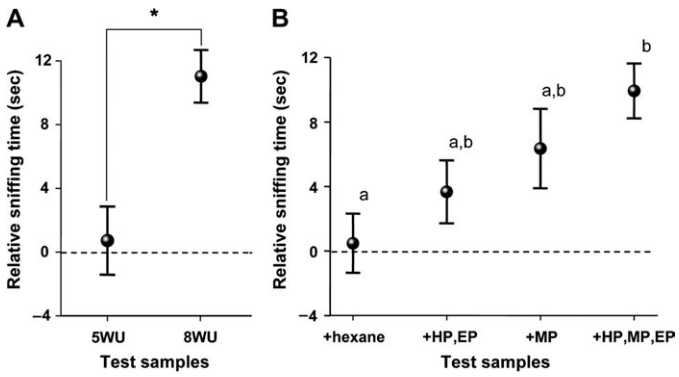

Figure 3 Olfactory preference of mature female rats exposed simultaneously to 2 types of male urine samples. The mean (±standard error of the mean) relative sniffing times of female rats are shown in regard to **(A)** the difference between 5W and 8W urine and **(B)** the differences between (1; whole 5W urine spiked with hexane) and each of the test samples (2: 5W urine supplemented with 2-HP, 4-MP, and 4-EP; 3: 5W urine supplemented with 2-HP and 4-EP; and 4: whole 5W urine supplemented with 4-MP). The statistical significance of the differences between the relative sniffing times was assessed by the Mann–Whitney *U*-test (**A**: $*P < 0.01$) or the Kruskal–Wallis test with a post hoc test (Steel–Dwass; **B**). A different letter between the groups means a significant difference ($P < 0.05$).

Experiment 2

The average total time during the 3-min test that the mature females spent sniffing 5W plus hexane urine to which 2-HP, 4-MP, and 4-EP had or had not been added was 22.5 ± 2.3 (SE) s. The average time spent sniffing the 5W urine plus 2-HP, 4-MP, and 4-EP or the 5W urine plus hexane was significantly different between the test and control samples (16.2 ± 1.5 vs. 6.4 ± 1.5, $P < 0.02$). On the other hand, there were no differences between the sniffing times of the test sample (5W urine plus hexane) and those of the control sample (5W urine plus hexane). The differences between the average relative sniffing time of 5W plus 2-HP, 4-MP, and 4-EP urine and that of 5W plus hexane urine indicate that females preferred the odor of the experimental sample rather than the odor of the control sample (9.8 ± 1.7 vs. 0.5 ± 1.8, $P < 0.05$; Figure 3B).

Experiment 3

The average total time during the 3-min test that the females spent sniffing 5W urine to which 4-EP and 2-HP had or had not been added was 17.5 ± 2.7 (SE) s. The females tended to spend more time sniffing the 2-HP + 4-MP–supplemented 5W urine than the hexane-supplemented control 5W urine (10.6 ± 2.0 vs. 6.9 ± 1.4), but this did not achieve statistical significance. There were no significant differences in the relative sniffing times of the 5W plus 4-EP and 2-HP urine and the 5W plus hexane urine (3.7 ± 2.0 vs. 0.5 ± 1.8, not significant [NS]; Figure 3B).

Experiment 4

The average total time during the 3-min test that the females spent sniffing the 5W urine to which 4-MP had or had not been added was 18.0 ± 3.5 (SE) s. The females spent significantly more time sniffing the 4-MP–supplemented 5W urine than the control 5W urine (12.2 ± 2.7 vs. 5.9 ± 1.5, $P < 0.05$). However, there were no significant differences in the relative sniffing times of the 5W plus 4-MP urine and the 5W plus hexane urine (6.4 ± 2.5 vs. 0.5 ± 1.8, NS; Figure 3B).

Discussion

The present study shows that puberty-related changes in male urine odor are attractive to female rats and that this attraction is due, in part, to changes in the levels of specific volatile compounds in male urine. Our chemical analysis revealed that the levels of 2-HP, 4-MP, and 4-EP all increase in the urine of adult (over 8 weeks of age) male rats compared with their levels in prepubescent (5-week-old) male rats. To our knowledge, no previous studies are available to understand how the levels of these chemicals change as male rats mature sexually. Thus, this is the first investigation to show that puberty elevates the urinary levels of 2-HP, 4-MP, and 4-EP in male rats (Table 1, Figure 2). In addition, our behavioral experiments indicate that after puberty, male rat urine develops a distinctive odor that is attractive to mature female rats in an experimental setting (Experiment 1, in Figure 3). Together, these observations suggest that this increased attraction is due to an increase in the absolute amounts of particular volatile compounds in the urine, namely, 2-HP, 4-MP, and 4-EP (Table 1).

In adjunct behavioral experiments, we demonstrated that when prepubescent male rat urine is supplemented with 2-HP, 4-MP, and 4-EP, this creates an olfactory signal that is attractive to female DN rats (Experiment 2, in Figure 3). This strongly supports the hypothesis that 2-HP, 4-MP, and 4-EP play a behaviorally significant role in mediating female attraction to adult male rat urine odor.

Previous studies have shown that 4-MP and 4-EP are also volatile chemicals in mouse urine (Singer et al. 1997; Achiraman and Archunan 2002; Osada et al. 2003, Osada, Curran, et al. 2008). 2-HP has been reported to be a female mouse pheromone that serves to accelerate the onset of puberty and to facilitate estrus synchronization (Novotny et al. 1986; Jemiolo et al. 1989). However, it has not been shown previously that these 3 chemicals increase the attractiveness of males for females in any other mammalian system, including in mice. Therefore, it seems to be unique to rats that 4-MP, 4-EP, and 2-HP in male rat urine act as female attractants.

One recent study has shown that normal SD male rat (20W) urine has higher levels of certain chemicals, including 2-HP and 4-EP, than conspecific female or castrated male rat urine and that these chemicals have been shown to act as female attractants (Zhang et al. 2008). Our study clearly supports these observations. Moreover, we showed that it was possible to almost fully restore the female rat's preference for adult DN male rat urine by supplementing prepubescent male rat urine with 4-MP, 4-EP, and 2-HP to the levels that these chemicals occur in adult male rat urine. This supports

the notion that these 3 chemicals play a crucial role in female attraction to male rats (Figure 3). Notably, Zhang et al. (2008) could not make castrated male urine more attractive to female rats until the level of normal rat urine by supplementing it with the putative pheromones squalene, 4-EP, and 2-HP. However, they did not examine the effect of adding 4-MP. This inconsistency in the urinary putative pheromone levels may be attributable to differences between the strains of the rats used in the present study and those used in the previous study by Zhang et al. The most dominant urinary volatile of male 5–14W DN rat was 4-MP (Figure 1 and Table 1). In 20W male SD rat urine, however, 4-MP was present at a lower level than either 2-HP or 4-EP. Clearly, further experimental work is required to identify the specific male urinary pheromones to each strains of rat. We also found that 3-hepten-2-one (peak #11) was present at higher levels in 8W urine than in 5W urine (Table 1). However, because supplementation of 5W urine with only 2-HP, 4-EP, and 4-MP induced almost the same level of female attraction as 8W urine, it may be that 3-hepten-2-one only plays a small role, if any, in eliciting a female response.

Previous studies of mice have suggested that DB, BT, α-farnesene, and β-farnesene are androgen-dependent mouse pheromones that activate female attraction (Jemiolo et al. 1985, 1991) or intermale aggressive behavior (Novotny et al. 1985; Morgan et al. 2004), accelerate the onset of puberty, and promote estrus synchronization (Jemiolo et al. 1986; Novotny et al. 1999). It is therefore conceivable that 2-HP, 4-MP, and 4-EP could have similar endocrinological effects on female and male rats. Further research is needed to determine whether these chemicals can indeed promote intermale aggressive behavior and promote the onset of puberty or estrus synchronization in female rats.

The exposure to urine preparations excreted from young male (10W) rats that had no prior sexual experience did not induce remarkable expression of Fos-immunoreactive (Fos-ir) cells in the accessory olfactory bulb (AOB), whereas exposure to urine from sexually experienced males (12W) did induce remarkable expression of Fos-ir cells (Kashiwayanagi 2005). In the present study, we did not observe significant differences in volatile concentrations, including those of 2-HP, 4-MP and 4-EP, between the urine of sexually experienced and the urine of sexually inexperienced males, suggesting that sexual experience does not affect the concentrations of volatiles in urine.

It is not yet known which of the olfactory systems, namely, the main olfactory system or the vomeronasal system, mainly detects the urinary volatiles in male rat urine and evokes female attraction. In a previous study, in vitro electrophysiology using the mouse vomeronasal organ (VNO) has shown that 6 volatile pheromones, including DB and BT, induce excitatory responses in single vomeronasal neurons that generate action potential and elevate calcium entry (Leinders-Zufall et al. 2000). However, other studies have found that DB and BT do not induce c-fos mRNA expression in the mouse AOB (Guo et al. 1997) or increase Fos-ir cells in the mouse VNO (Kimoto et al. 2005). Therefore, it remains unclear whether the volatile chemosignals in male mouse urine solely stimulate the murine AOB. However, exposure of the female rat VNO to urine from sexually experienced males has been shown to remarkably elevate the number of Fos-ir cells in this organ. As described in the previous report, both the low (<500 Da) and high (>500 Da) molecular weight substances in sexually experienced male rat urine are needed for this increase in Fos-ir cells in the AOB of female rats (Yamaguchi et al. 2000; Kashiwayanagi 2005). Therefore, it is conceivable that 2-HP, 4-MP, and 4-EP are at least some of the primary low molecular weight substances (if they are not ligands of high molecular protein) that play a role in activating the AOB. However, in our pilot study, exposure to 5W male rat urine spiked with 2-HP, 4-MP, and 4-EP did not remarkably elevate the number of Fos-ir cells in the AOB of female rats (data not shown). This suggests that the urine spiked with 2-HP, 4-MP, and 4-EP is mainly detected by the main olfactory system of the female rat. However, this result does not preclude the possibility that the combination of these volatile chemosignals and the high molecular weight substances that are contained in sexually experienced rat urine can also evoke responses from the female rat AOB.

It has been shown that both male and female rats are attracted to the odors of the preputial gland from the opposite sex (Brown 1985). Gawienowski et al. (1976) suggested that a number of aliphatic acetates were the active components of the male preputial gland. More recently, the preputial gland extract components dodecyl propionate (Brouette-Lahlou et al. 1999), squalene (Zhang et al. 2008), were found to be putative rat pheromones. Unfortunately, we could not identify them by the extraction methods employed, namely, HS-SPME or organic solvent extraction. Actually, these 2 sampling approaches are complementary, and both are important in obtaining a comprehensive profile of volatile, gas chromatographable chemicals in samples (Gallagher et al. 2008).

In conclusion, we showed that the urine odor of mature male rats is attractive to female rats and that certain attractants are associated with puberty. Female attraction to male rat urine may therefore be induced by several chemicals, including the attractive semiochemicals we identified in this study, namely, 2-HP, 4-MP, and 4-EP.

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References

Achiraman S, Archunan G. 2002. Characterization of urinary volatiles in Swiss male mice (*Mus musculus*): bioassay of identified compounds. J Biosci. 27:679–686.

Beauchamp GK, Katahira K, Yamazaki K, Mennella JA, Bard J, Boyse EA. 1995. Evidence suggesting that the odortypes of pregnant women are a compound of maternal and fetal odortypes. Proc Natl Acad Sci USA. 92:2617–2621.

Beauchamp GK, Yamazaki K, Curran M, Bard J, Boyse EA. 1994. Fetal H-2 odortypes are evident in the urine of pregnant female mice. Immunogenetics. 39:109–113.

Brouette-Lahlou I, Godinot F, Vernet-Maury E. 1999. The mother rat's vomeronasal organ is involved in detection of dodecyl propionate, the pup's preputial gland pheromone. Physiol Behav. 66:427–436.

Brown RE. 1977. Odor preference and urine-marking scales in male and female rats: effects of gonadectomy and sexual experience on responses to conspecific odors. J Comp Physiol Psychol. 91:1190–1206.

Brown RE. 1985. The rodents. II. Suborder Myomorpha. In: Brown RE, Macdonald DW, editors. Social odours in mammals. Oxford: Clarendon Press. p. 345–417.

Brown RE, Roser B, Singh PB. 1989. Class I and class II regions of the major histocompatibility complex both contribute to individual odors in congenic inbred strains of rats. Behav Genet. 19:659–674.

Bruce HM. 1960. A block to pregnancy in the mouse caused by proximity of strange males. J Reprod Fertil. 1:96–103.

Bruce HM, Parrott DM. 1960. Role of olfactory sense in pregnancy block by strange males. Science. 131:1526.

Gallagher M, Wysocki CJ, Leyden JJ, Spielman AI, Sun X, Preti G. 2008. Analyses of volatile organic compounds from human skin. Br J Dermatol. 159:780–791.

Gawienowski AM, DeNicola DB, Stacewicz-Sapuntzakis M. 1976. Androgen dependence of a marking pheromone in rat urine. Horm Behav. 7: 401–405.

Guo J, Zhou A, Moss RL. 1997. Urine and urine-derived compounds induce c-fos mRNA expression in accessory olfactory bulb. Neuroreport. 8: 1679–1683.

Gutierrez-Garcia AG, Contreras CM, Mendoza-Lopez MR, Garcia-Barradas O, Cruz-Sanchez JS. 2007. Urine from stressed rats increases immobility in receptor rats forced to swim: role of 2-heptanone. Physiol Behav. 91:166–172.

Harvey S, Jemiolo B, Novotny M. 1989. Pattern of volatile compounds in dominant and subordinate male mouse urine. J Chem Ecol. 15:2061–2072.

Holland M, Rhodes G, DalleAve M, Wiesler D, Novotny M. 1983. Urinary profiles of volatile and acid metabolites in germfree and conventional rats. Life Sci. 32:787–794.

Inagaki H, Kiyokawa Y, Kikusui T, Takeuchi Y, Mori Y. 2008. Enhancement of the acoustic startle reflex by an alarm pheromone in male rats. Physiol Behav. 93:606–611.

Jemiolo B, Alberts J, Sochinski-Wiggins S, Harvey S, Novotny M. 1985. Behavioral and endocrine responses of female mice to synthetic analogues of volatile compounds in male urine. Anim Behav. 33:1114–1118.

Jemiolo B, Andreolini F, Xie TM, Wiesler D, Novotny M. 1989. Puberty-affecting synthetic analogs of urinary chemosignals in the house mouse, *Mus domesticus*. Physiol Behav. 46:293–298.

Jemiolo B, Harvey S, Novotny M. 1986. Promotion of the Whitten effect in female mice by synthetic analogs of male urinary constituents. Proc Natl Acad Sci USA. 83:4576–4579.

Jemiolo B, Xie TM, Novotny M. 1991. Socio-sexual olfactory preference in female mice: attractiveness of synthetic chemosignals. Physiol Behav. 50:1119–1122.

Kashiwayanagi M. 2005. Augmentation of sensitivity to urinary pheromone and excreting of urinary pheromone by sexual experiences. Chem Senses. 30(Suppl 1):i138–i139.

Kimoto H, Haga S, Sato K, Touhara K. 2005. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. Nature. 437:898–901.

Kiyokawa Y, Kikusui T, Takeuchi Y, Mori Y. 2005. Alarm pheromone that aggravates stress-induced hyperthermia is soluble in water. Chem Senses. 30:513–519.

Leinders-Zufall T, Lane AP, Puche AC, Ma W, Novotny MV, Shipley MT, Zufall F. 2000. Ultrasensitive pheromone detection by mammalian vomeronasal neurons. Nature. 405:792–796.

Lucas PD, Donohoe SM, Thody AJ. 1982. The role of estrogen and progesterone in the control of preputial gland sex attractant odors in the female rat. Physiol Behav. 28:601–607.

Morgan C, Thomas RE, Ma W, Novotny MV, Cone RD. 2004. Melanocortin-5 receptor deficiency reduces a pheromonal signal for aggression in male mice. Chem Senses. 29:111–115.

Novotny M, Harvey S, Jemiolo B, Alberts J. 1985. Synthetic pheromones that promote inter-male aggression in mice. Proc Natl Acad Sci USA. 82: 2059–2061.

Novotny M, Jemiolo B, Harvey S, Wiesler D, Marchlewska-Koj A. 1986. Adrenal-mediated endogenous metabolites inhibit puberty in female mice. Science. 231:722–725.

Novotny MV. 2003. Pheromones, binding proteins and receptor responses in rodents. Biochem Soc Trans. 31:117–122.

Novotny MV, Ma W, Wiesler D, Zidek L. 1999. Positive identification of the puberty-accelerating pheromone of the house mouse: the volatile ligands associating with the major urinary protein. Proc R Soc Lond B Biol Sci. 266:2017–2022.

Osada K, Curran M, Kurachi M, Tsunoda K, Yamazaki K. 2008. Effect of an orally ingested mugwort and mushroom extract mixture on urine odor from aged mice. Biosci Biotechnol Biochem. 72:1249–1256.

Osada K, Tashiro T, Mori K, Izumi H. 2008. The identification of attractive volatiles in aged male mouse urine. Chem Senses. 33:815–823.

Osada K, Yamazaki K, Curran M, Bard J, Smith BP, Beauchamp GK. 2003. The scent of age. Proc R Soc Lond B Biol Sci. 270:929–933.

Rhodes G, Holland ML, Wiesler D, Novotny M, Moore SA, Peterson RG, Felten DL. 1982. Excretion of urinary volatile metabolites in response to alloxan induced diabetes of short duration in rats. J Chromatogr. 228: 33–42.

Singer AG, Beauchamp GK, Yamazaki K. 1997. Volatile signals of the major histocompatibility complex in male mouse urine. Proc Natl Acad Sci USA. 94:2210–2214.

Tashiro T, Osada K, Mori K. 2008. Syntheses of 2-isopropyl-4, 5-dihydrothiazole and 6-hydroxy-6-methyl-3-heptanone, pheromone components of the male mouse, *Mus musculus*. Biosci Biotechnol Biochem. 72:2398–2402.

Van Der Lee S, Boot LM. 1955. Spontaneous pseudopregnancy in mice. Acta Physiol Pharmacol Neerl. 4:442–444.

Van Der Lee S, Boot LM. 1956. Spontaneous pseudopregnancy in mice. II. Acta Physiol Pharmacol Neerl. 5:213–215.

Vandenbergh JG. 1967. Effect of the presence of a male on the sexual maturation of female mice. Endocrinology. 81:345–349.

Whitten WK, Bronson FH, Greenstein JA. 1968. Estrus-inducing pheromone of male mice: transport by movement of air. Science. 161:584–585.

Yamaguchi M, Yamazaki K, Beauchamp GK, Bard J, Thomas L, Boyse EA. 1981. Distinctive urinary odors governed by the major histocompatibility locus of the mouse. Proc Natl Acad Sci USA. 78:5817–5820.

Yamaguchi T, Inamura K, Kashiwayanagi M. 2000. Increases in fos-immunoreactivity after exposure to a combination of two male urinary components in the accessory olfactory bulb of the female rat. Brain Res. 876:211–214.

Yamazaki K, Beauchamp GK, Bard J, Boyse EA. 1989. Sex-chromosomal odor types influence the maintenance of early pregnancy in mice. Proc Natl Acad Sci USA. 86:9399–9401.

Yamazaki K, Beauchamp GK, Shen FW, Bard J, Boyse EA. 1994. Discrimination of odortypes determined by the major histocompatibility complex among outbred mice. Proc Natl Acad Sci USA. 91:3735–3738.

Yamazaki K, Boyse EA, Bard J, Curran M, Kim D, Ross SR, Beauchamp GK. 2002. Presence of mouse mammary tumor virus specifically alters the body odor of mice. Proc Natl Acad Sci USA. 99:5612–5615.

Zhang JX, Sun L, Zhang JH, Feng ZY. 2008. Sex- and gonad-affecting scent compounds and 3 male pheromones in the rat. Chem Senses. 33:611–621.

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