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The Identification of Attractive Volatiles in Aged Male Mouse Urine

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Abstract

In many species, older males are often preferred mates because they carry "good" genes that account for their viability. In some animals, including mice, which rely heavily on chemical communication, there is some indication that an animal's age can be determined by its scent. In order to identify the attractants in aged male mouse urine, chemical and behavioral studies were performed. We herein show that aged mice have higher levels of 3,4-dehydro-*exo*- brevicomin (DB), 2-*sec*-butyl-4, 5-dihydrothiazole (BT), and 2-isopropyl-4,5-dihydrothiazole (IT) and a lower level of 6-hydroxy-6-methyl-3-heptanone relative to adult male mice. We also demonstrate that the attraction of females to the odor of male mouse urine is greater when the urine is from aged males. However, the attraction of aged urine odor was offset by the ultrafiltration of adult and aged mouse urine. When DB, BT, and IT were added to adult urine, the attraction of the urine was enhanced. Our results suggest that inbred aged male mice develop an aging odor that is attractive to female mice in an experimental setting and that this attraction is due to increased mouse pheromone signaling.

Key words: age discrimination, mating, mouse, pheromones, urinary volatiles, urine odor

Introduction

In many species, older males may often be preferred mates because they have demonstrated longevity and greater survival ability under a variety of environmental conditions than younger males (Trivers 1972; Halliday 1978, 1983; Manning 1985; Kokko and Lindstrom 1996; Brooks and Kemp 2001). This is a specific example of the more general ''good genes'' hypothesis or indicator mechanism for choosy behavior in females (Anderson 1994). For such a process to occur, some male phenotypic traits must both reliably indicate male genetic quality and influence the pattern of mate choice by females.

The ability of females to discern a male's age is a matter of question and most likely involves a range of sensory and behavioral signals. For animals that rely on chemical communication for the regulation of social and sexual interactions, there is some indication that an animal's age can be determined by its scent. For example, Ferkin (Ferkin 1999) showed that both male and female meadow voles are attracted to the odors of older conspecifics. An age-related odor change is suggested by the research of Robinson et al. who showed that the composition of mouse urine varies with age (Robinson et al. 1976; Miyashita and Robinson 1980). Similarly, Muller-Schwarze (Muller-Schwarze 1971) demonstrated that the volatile composition of black-tailed deer (*Odocoileus hemionus columbianus*) tarsal glands varies between juvenile and adult males, and Goodrich and Mykytowycz (Goodrich and Mykytowycz 1972) showed that the chromatographic profile and odor (as perceived by humans) of rabbit anal gland secretions varies with age.

In a recent study, we used a Y-maze olfactometer to demonstrate that the age-related changes in urine odor allow mice to discriminate age (Osada et al. 2003). This study was the first experimental confirmation that age-related odor changes in inbred mice could be distinguished by the mice themselves.

In order to understand how aging produces characteristic and attractive odors, we sought to identify the semiochemicals in mouse urine that are the source of the olfactory attractability of urine from aged male mice. The ability of the olfactory signal to travel in a slow stream of air in the Y-maze olfactometer allows us to infer that the discrimination is most probably based on the chemosensory detection of volatile compounds. The obvious approach to the analysis of complex mixtures of volatile compounds is to use gas

chromatography (GC). Earlier attempts to apply this technique to the analysis of age-related differences in mouse urine samples prepared by traditional extraction methods (e.g., ether extraction or cold trap) suggested differences in certain urinary compounds depending on age (Robinson et al. 1976; Osada et al. 2003). However, the chemicals that are attractive to female mice in aged male mouse urine still remain elusive.

For several reasons, we explored the volatile content of the gas in the headspace above neutral mouse urine using the headspace solid phase microextraction method (HS-SPME).

First, HS-SPME is ideal for the analysis of urine and other biological specimens because interference from high–molecular weight components (e.g., protein) in the matrix is reduced, yielding cleaner extracts. In addition, the extract conditions (e.g., temperature, pH, etc.) of HS-SPME for the odor source can be controlled depending on the conditions. HS-SPME can therefore analyze components from biological specimens in more natural conditions. Second, the combination of HS-SPME and flame ionization detector-GC (FID-GC) and GC–mass spectrometry (GC–MS) enabled us to conduct a simple and high-performance analysis to identify the volatile chemicals specifically found in mouse urine (Kayali-Sayadi et al. 2003; Lin et al. 2005; Osada 2006). Third, although the most abundant chemicals in the headspace gas above mouse urine are neutral volatiles, including ketone, aldehydes, alcohols, hydrocarbons, etc., (Miyashita and Robinson 1980), some of which have a very strong smell (Kwak et al. 2005), little is known about the characteristics of the age-related changes in these volatiles in the mouse urine. Fourth, some of these volatiles are known as mouse pheromones (Jemiolo et al. 1985; Novotny et al. 1985; Jemiolo et al. 1986; Novotny, Jemiolo et al. 1999; Novotny, Ma, et al. 1999; Lin et al. 2005), which suggests that female mice plausibly could be attracted to aged mouse urinary odor if they are present in higher concentrations in aged mouse urine.

We are not aware of any other prior behavioral studies of female scent preferences in inbred animals or analysis of the chemical basis of female mouse attractants. Therefore, in this study, we examined the responses of female mice to the odor of aged males and younger adults. A 2-choice preference test was used to quantify the relative attractiveness of the whole, intact urine from aged male mice to that of adult male mice. In addition, in an attempt to ascertain whether the urinary volatiles conveyed by major urinary proteins (MUPs) are involved in the formation of the odor of aged mouse urine, we conducted a behavioral and chemical study using adult and aged mouse urine from which these urinary proteins had been removed by means of ultrafiltration (10 kDa cutoff). Moreover, we also examined the response of female mice to the odor of whole urine versus whole urine spiked with odorants that increase or decrease with age.

In this study, we investigated male mouse urinary volatiles and the urine odor of adult (3–8 months [M]) and aged (15–20 M) groups. Additionally, in a chemical experiment, we also analyzed old (28 M) mouse urine. The age groups roughly correspond to the adult (17–33 years old), aged (55–71 years old), and old (over 90 years old) age groups of human, as determined from the proportional relationship between puberty and maximal life span in mice and humans (Arking 2000).

Materials and methods

Animals

The mice were of the inbred strain C57BL6J (B6) and were bred at the Health Sciences University of Hokkaido. They were maintained at 22 °C with a photoperiod of 12:12 light: dark and were provided with continuous food (Lab Chow, MF, Oriental Yeast, Tokyo, Japan) and water. The animals were cared for in accordance with the Guidelines for the Care and Use of Laboratory Animals, and the experimental protocols were previously approved by the Animal Ethics and Research Committee in the Health Sciences University of Hokkaido.

Collection of urine samples

Urine samples were collected from adult (3–8 M), aged (15–20 M), and old (28 M) male mice by gentle abdominal pressure. A panel of 6–16 healthy donors provided urine samples used for the study (see the paragraph of Sample preparation for GC analyses with HS-SPME). To avoid diurnal fluctuation, urine collection was performed at 1700–1800 h. The urine samples were stored in sterile tubes at –80 °C. Due to the small amount of urine typically produced, test samples consisted of pooled urine collected from 2 to 6 individual animals from the same age group.

Ultrafiltration treatment of mouse urine samples

In order to clarify whether the urinary volatiles that are conveyed by urinary proteins are involved in the formation of aging odor, the ultrafiltration procedure was conducted on some mouse urine samples. Urine samples were pretreated with Amicon Ultra-4 Ultracel-10k (10 KDa cutoff) at 5000 × g at 5 °C to remove the volatile ligands and urinary proteins including MUPs that are thought to contribute to chemical signaling of body odor by acting as carriers for certain volatile compounds (Hurst et al. 2001).

Sample preparation for GC analyses with HS-SPME

Sample preparation was performed by HS-SPME. To concentrate the volatiles from the urine, an SPME fiber (2 cm to 50/30 μm DVD/Carboxen/PDMS StableFlex, Supelco, Bellefonte, PA) was inserted for 30 min into the headspace of a 4-ml vial with a Teflon septum (Supelco) containing

150 µl of mouse urine that was saturated with NaCl, mildly heated to 37–40 °C, and constantly stirred. In this study, we explored aging chemicals by means of the following approach. An analysis of peak areas (as a percentage of the total) was conducted for 38 representative compounds present in both adult (3–8 M: $n = 16$) and aged (15–20 M: $n = 10$) urine using the area normalization method. This kind of analysis is commonly used to compare entire chromatograms of one group with those of different groups in order to determine the relative changes in the number of volatile constituents at a time (Singer et al. 1997; Osada et al. 2003). This approach was conducted to identify the compounds, which are significantly greater in aged mouse urine samples, from 38 representatives. Second, in order to demonstrate the detailed time course changes in the putative aging odor chemicals, which were identified from 38 representatives by the first approach, analyses were performed on urine specimens from various age groups (3 M: $n = 15$, 8 M: $n = 16$, 15 M: $n = 14$, 20 M: $n = 12$, and 28 M: $n = 6$). We analyzed aging odor candidates, of which the relative value was significantly greater in the aged mouse urine samples than in the adult mouse urine samples, by using 500 ng of 7-tridecanone (dissolved in methyl acetate) as an internal standard (IS). To avoid IS interference in the binding affinity between urinary proteins and volatiles, we performed an ultrafiltration urine study without the IS.

A total of 79 samples were prepared for GC analysis: 12 from each of the aged and adult groups without IS, 7 from each of the 5 groups with IS, and 5 from each of the aged and adult groups without IS that were subjected or not to centrifugal ultrafiltration.

Chemical analysis performed with FID-GC and GC–MS

The chemical analysis was carried out on a Hewlett Packard 5890 GC equipped with a FID (Agilent technologies, Santa Clara, CA). The GC was fitted with a Restek Stabilwax column (30 m × 0.32 × 0.5 mm) (Restek, Bellefonte, PA). 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 and was then increased by 10 °C/min to 200 °C and then 5 °C/min to 240 °C. The injector temperature was held constant at 230 °C.

The identification of odor compounds was performed by GC–quadrupole MS (GC–MS) QP5000 ver. 2 (Shimadzu, Tokyo, Japan). The column and oven conditions were identical to those described for the chemical analyses. The identification of structures of representative peaks was performed using both the NIST92 library and a manual interpretation of mass spectra based on a comparison with those reported in the literature. In addition, a comparison of relative retention times and mass spectra of authentic chemicals was performed by employing samples of 3,4-dehydro-*exo*-brevicomin (DB), 2-*sec*-butyl-4,5-dihydrothiazole (BT) (Tashiro and Mori 1999), and 6-hydroxy-6-methyl-3-heptanone (HMH) (Tashiro, Osada, Mori, in preparation). These authentic chemicals were used as a hexane solution and were preserved in a deep freezer (–80 °C).

Behavioral study

Eighty-one virgin, 2- to 5-M-old B6 female mice were used as test animals because previous reports demonstrated that young virgin females have an ability to distinguish between different male mouse pheromones and different male mouse odors (Jemiolo et al. 1985; Moncho-Bogani et al. 2002). Three or 4 mice were housed per home cage in our sterile animal facility. The preference tests were conducted in a rectangular, open bottomed arena (27 × 17 cm). Two circular ports (1 cm in diameter) located 3 cm above the base of the wall in the centers of the 2 short sides of the arena were used for odor presentations. A glass vial (2 × 4.5 cm), containing a 0.5 ml urine sample, was fitted into each port so that an animal could sniff the opening but could not access its contents. The animal was considered to be sniffing at a port when its snout was oriented toward the opening and held within 1 cm of it. The female mice were tested individually once per day. Prior to each test, a 3-min period was provided for habituation. In this habituation procedure, the average sniffing time was 10.5 s. Following habituation, vials containing adult or aged mice urine were mounted behind each port. The test period was 3-min long, and an observer used separate timers to record the amount of time spent sniffing each of the 2 ports. All the behavioral studies were performed as blind tests. If the test mice did not reach the total sniffing time of 11 s between the test periods, we considered that the session had failed to provide enough habituation and omitted it from the data. Each of the adult and aged urine samples was composed of a mixture of 2 or 3 aliquots of urine sample harvested on different days.

Each test was conducted between 0900 and 1200 h. 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 mouse.

In this study, we conducted 3 types of odor preference tests. Experiment 1 was designed to determine whether the age of B6 male mice affects female attraction to the urine from these mice and whether the urinary volatiles, which are conveyed by MUPs, play a role in the formation of the aging odor. Experiment 1 was conducted to compare the degree of female attraction to aged male intact urine with that to adult male intact urine as well as the degree of female attraction to aged male ultrafiltered (10 kD cutoff) urine with that to adult male ultrafiltered urine. In experiment 2, adult urine plus DB, BT, and IT and adult urine spiked with equal amounts of hexane were used as odor sources. This experiment was conducted to investigate whether the addition of DB, BT, and IT, which are greater in aged urine (see Table 1), to adult urine up to the level of that in aged mouse urine results in the same activity

Table 1 Results of Friedman nonparametric analysis of variance (ANOVA) on the average peak area (as percentage of total) for identified urinary chemicals

Peak number	Peak identity	Adult interquartile		Aged interquartile		Friedman nonparametric ANOVA adult versus aged
		Median	Range	Median	Range	
1	Trimethylamine[a]	0.83	0.16–4.14	0.48	0.07–1.97	
2	Methyl mercaptan[a]	0.07	0.05–0.50	0.10	0.07–0.41	
3	Acetone[a]	0.43	0.24–1.76	0.85	0.19–2.70	
4	6H-6M-3H derivative[a]	47.5	39.1–52.2	37.9	32.4–41.8	$P < 0.001$
6	6H-6M-3H derivative[a]	15.4	12.7–16.9	12.0	10.4–13.4	$P < 0.001$
10	2,4 Dimethylcyclopentanone[b]	0.06	0.05–0.19	0.08	0.06–0.35	
12	*p*-Xylene[a]	0.12	0.05–0.72	0.17	0.06–0.52	
13	6H-6M-3H derivative[a]	6.09	5.14–6.83	4.78	4.14–5.37	$P < 0.001$
14	Nitromethame[a]	0.19	0.09–0.59	0.10	0.07–0.30	$P < 0.01$
16	2-Heptanone[a]	0.16	0.05–0.45	0.09	0.07–0.16	
18	2-Penten-1-ol acetate[b]	0.15	0.09–0.35	0.10	0.06–0.20	$P < 0.05$
20	5-Hepten-2-one[b]	0.16	0.07–0.34	0.08	0.06–0.11	
21	4-Hepten-2-one[b]	0.55	0.26–1.24	0.31	0.23–0.56	$P < 0.05$
23	4-Methyl-6-hepten-3-one[b]	0.11	0.05–0.28	0.37	0.10–0.48	$\underline{P < 0.01}$
24	3-Hepten-2-one[a]	0.44	0.08–0.55	0.34	0.12–0.53	
26	6-Methyl-5-hepten-3-one[b]	0.42	0.31–0.94	0.34	0.18–0.41	
28	IT[a]	0.32	0.21–0.47	0.59	0.44–0.67	$\underline{P < 0.001}$
29	DB[a]	6.05	3.57–8.94	10.4	7.68–14.0	$\underline{P < 0.001}$
30	BT[a]	11.0	9.20–13.1	20.7	18.4–24.7	$\underline{P < 0.001}$
32	Benzaldehyde[a]	0.27	0.14–1.03	0.30	0.23–0.50	
33	β-Farnesene[a]	0.84	0.53–3.37	0.72	0.19–1.15	
36	*o*-Toluidine[a]	1.58	0.93–2.55	1.17	0.78–2.69	
38	Dimethylsulfone[a]	0.21	0.07–0.64	0.22	0.06–0.61	

Median height from 12 urine samples. Underline signifies an increase in peak area with age, and no line signifies a decrease.
[a]Identified by mass spectrum (quadrupole) and by retention time matching their retention times against authentic chemicals.
[b]Tentatively identified by mass spectrum (quadrupole).

in the behavioral paradigm. DB, BT, and IT were spiked into adult urine at concentrations simulating the content in aged mouse urine. The difference between adult and aged mice was roughly 2 ppm v/v for DB and BT and 0.2 ppm v/v for IT.

Finally, in order to determine whether decreases in the amount of HMH (see Table 1) caused greater attraction to aged urine samples, aged urine plus HMH and aged urine spiked with equal amounts of hexane were used as the odor sources (Experiment 3). HMH was spiked into aged urine at concentrations simulating the content observed in adult mouse urine (roughly 200 ppm v/v).

During these experiments, for each case, we prepared 3 sets of urine samples for each case and then used one set of these samples at random to evaluate the mice.

Results

Chemical studies

Typical examples of FID-GC chromatograms of adult and aged mouse urine extracts are shown in Figure 1. The FID-GC analysis demonstrated no absolute differences in the composition of the extracts, although differences were observed in the ratio of these components. Among 38 representative peaks (Figure 1), which were chosen for comparison purposes based on our ability to reliably measure them, 23 were identified by GC–MS (Table 1). Of these compounds, 10 peak areas differed significantly between the adult and aged urine (Table 1). The most prominent differences involved significantly greater amounts of DB, BT, and

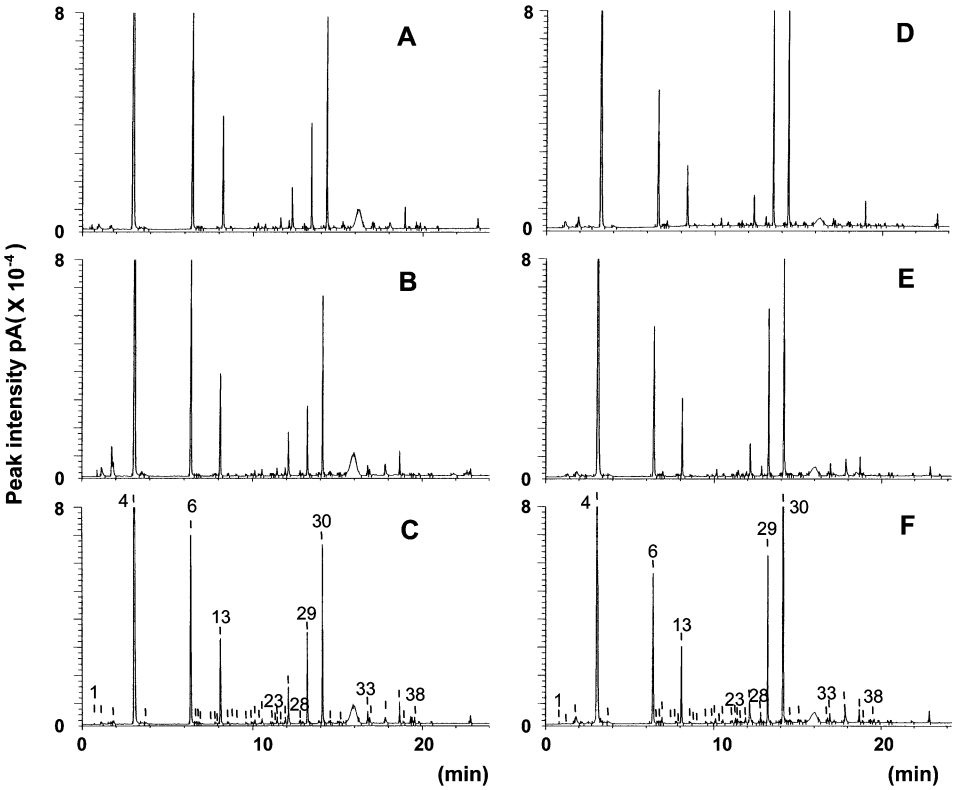

Figure 1 Chromatograms from the GC analysis of HS-SPME of 3 different urine samples from B6 mice (adult: 4–8 M old: **A**, **B**, and **C**), and (aged: 15–20 M old: **D**, **E**, **F**). The 38 peaks, which were chosen for comparison, are indicated with tick marks and sequential numbers.

2-isopropyl-4,5-dihydrothiazole (IT) and significantly lower amounts of 3 HMH derivatives in the aged samples relative to the adult samples.

In order to determine the time course changes of the urinary levels of DB, BT, IT, and HMH, some urinary samples were analyzed with 500 ng of 7-tridecanone as an IS. The urinary BT levels increased from 3 M to 15 M, and the higher level was maintained beyond 20 M. We also observed that the urinary level of DB and IT significantly increased until at least 15 M and decreased thereafter with age, although the urinary levels of HMH, another male mouse pheromone (Novotny and Jemiolo et al. 1999), simply decreased with age (Figure 2).

A comparison of the chemical components in mouse urine with and without the ultrafiltration procedure is shown in Figure 3. The ultrafiltration treatment mostly removed urinary proteins as well as DB, BT, and IT, and the significant difference in the concentrations of DB, BT, and IT between the adult and aged urine samples disappeared after this procedure. In contrast, the concentrations of the 3 HMH derivatives were decreased by only 10–20% after ultrafiltration, and the difference in the concentrations of these chemicals between the age and adult urine samples was unchanged by this procedure (Figure 3). There were no significant differences in the amount of 4-methyl-6-hepten-3-one (which was present in the higher age groups) between the whole urine samples and the filtered urine samples (data not shown).

Behavioral studies

Experiment 1

The average total time spent engaged in sniffing during the 3-min test was 39.1 ± 2.7 s (SED) for whole urine and 36.8 ± 2.2 s (SED) for ultrafiltered urine. There were no significant differences in the total sniffing time between the whole urine study and the ultrafiltered urine study (Wilcoxon test). The distribution of "sniffing investigations" between aged and adult intact urine indicated preference for the aged male urine (62%, $P < 0.0001$; Wilcoxon test: Figure 4, Experiment 1). In contrast, there were no significant differences between the sniffing times of adult and aged urine samples using ultrafiltered urine (Figure 4, Experiment 1). These results indicate that the filtration procedure excluded the aging odor that attracted the female mice.

Experiment 2

The average total time spent engaged in sniffing during the 3-min test was 34.3 ± 2.8 s (SED) for adult urine with or

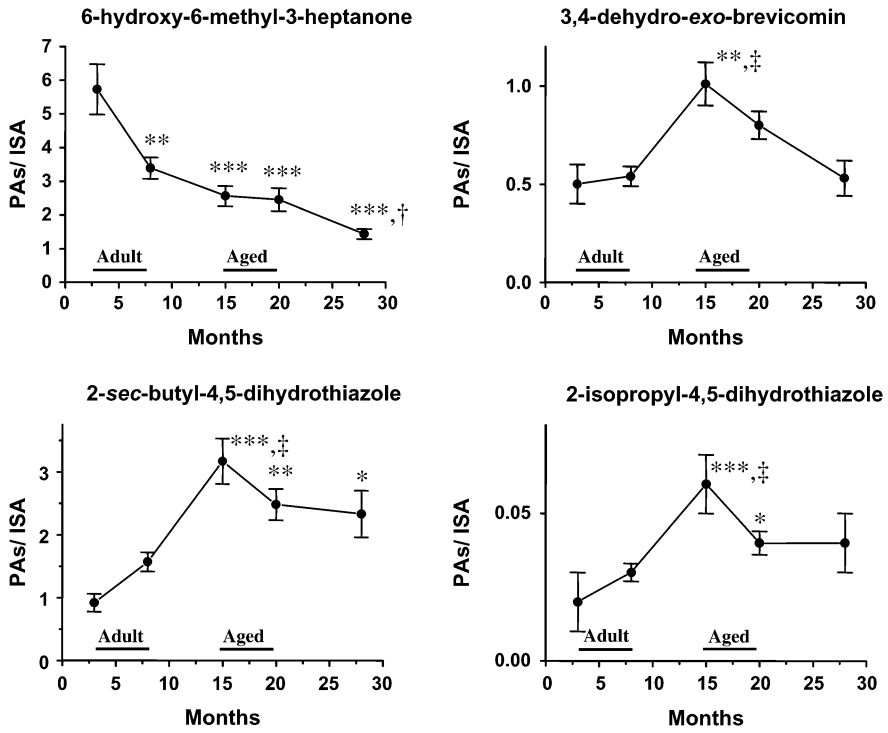

Figure 2 The change in urinary levels of DB, BT, IT, and HMH (peak #4) over time in male mouse urine. Urine samples were collected from 5 different aged mouse groups, namely, 3 M, 8 M, 15 M, 20 M, and 28 M. Each piece of data was analyzed from 7 urine samples. A panel of 8–16 donors provided the urine samples used for the chemical analysis. The statistical significance of the differences in the 3 or 8 M group was assessed by analysis of variance followed by a post hoc test (Tukey honest significant difference [HSD]) (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$ for 3 M group: †$P < 0.05$ and ‡$P < 0.01$ for 8 M group).

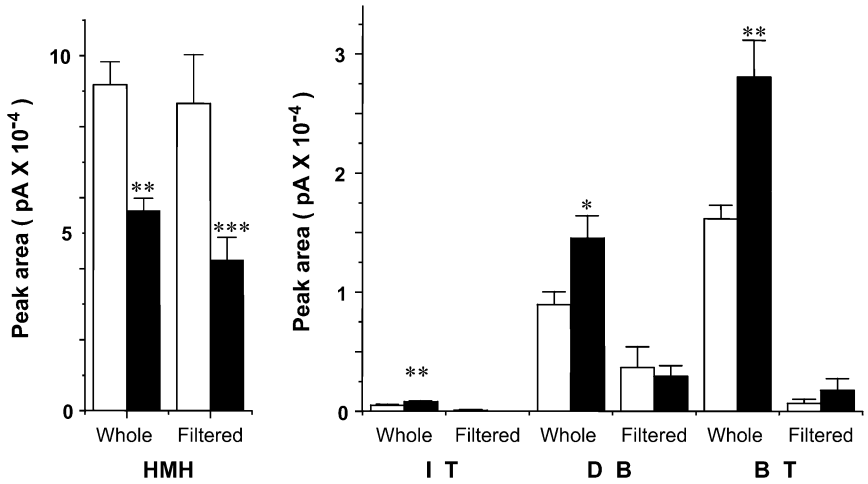

Figure 3 The effect of the ultrafiltration procedure (MW 10.000 cutoff) on adult ($n = 5$; open bar) and aged ($n = 5$; filled bar) mouse urine. FID-GC analysis data are shown without the IS. The urinary levels of IT, DB, and BT were decreased, and no significant differences were present between adult and aged urine samples after the ultrafiltration procedure. Ultrafiltration resulted in a very small effect on HMH (peak #4). The statistical significance of the differences between adult and aged mice were assessed by analysis of variance followed by a post hoc test (Tukey HSD) (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

without DB, BT, and IT. The distribution of sniffing investigations between urine samples indicated preferences for the DB, BT, and IT spiked adult male samples (59%, $P < 0.01$; Wilcoxon test: Figure 4).

Experiment 3

The average total time spent engaged in sniffing during the 3-min tests was 41.4 ± 2.5 s (SED) for the aged urine with or without HMH. There were no significant differences in the

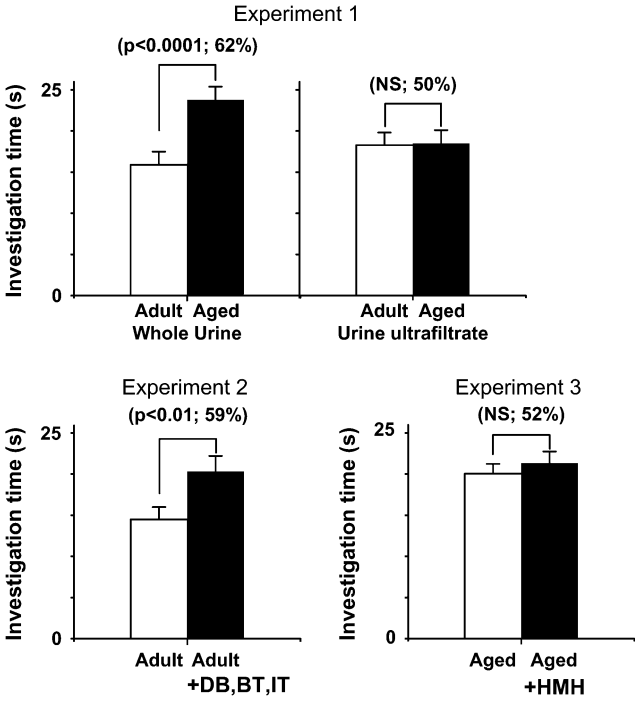

Figure 4 Olfactory preference of female mice during investigations of 2 stimulus samples. The mean (±standard error of the mean) amount of time that mice investigated the scent from the odor port of 1) aged urine or adult urine (with or without ultrafiltration: $n = 31$), 2) adult urine or adult urine + DB, BT, and IT ($n = 25$), and 3) aged urine or aged urine + HMH ($n = 25$). The statistical significance of the differences between each of the samples was assessed by Wilcoxon test. Each value is expressed as the percentage of sniffing time of 1) aged urine, 2) adult urine + DB, BT, and IT, and 3) aged urine + HMH to the total sniffing time.

sniffing times between these urine samples (52%, not significant; Wilcoxon test: Figure 4).

Discussion

Our chemical analysis demonstrated that DB, BT, and IT all increase in male mouse urine with age (Table 1, Figure 2). DB and BT are reported to be androgen-dependent mouse pheromones which promote both estrus synchronization and puberty acceleration (Jemiolo et al. 1986; Novotny, Ma, et al. 1999) while also activating intermale aggressive behavior and female attraction (Jemiolo et al. 1985; Novotny et al. 1985). However, little or no experimental evidence has been reported on the age-related changes of these chemicals. To our knowledge, this study is the first investigation indicating that DB, BT, and IT are present at higher levels in the urine of aged male mice (15–20 M old) than in the urine of adult mice (3–8 M old: Table 1, Figure 2). In addition, behavioral experiments using whole urine indicate that inbred aged male mice develop an aging odor that is attractive to female mice in an experimental setting (Figure 4, Experiment 1). Therefore, these results suggest that this attraction is at least partly due to an increase in the absolute amounts of specific volatile compounds in the urine, namely, DB, BT, and IT (Figure 2).

Three synthetic compounds, DB, BT, and IT, when spiked into adult urine, created an olfactory signal that was attractive to female B6 mice (Figure 4, Experiment 2). The results of this preference test strongly support the hypothesis that DB, BT, and IT play a behaviorally significant role in mediating female attraction to aged mouse urine odor. Jemiolo et al. demonstrated that when DB and BT were added to the urine obtained from castrated mice (castrate urine), the attractiveness of the urine was enhanced and the urine from the castrated mice became as attractive to the females as the whole, intact male urine (Jemiolo et al. 1985). Therefore, the results of our study are in support of this previous behavioral observation.

There have been very few previous reports about IT; however, one recent report investigating the chemical component of the strong odor in mouse urine addressed the presence of IT (Kwak et al. 2005). The results of our behavioral study show that IT could be a component of the aging odor that attracts female mice. Further research is needed to determine whether IT contributes to the attraction of female mice to aged mouse urine odor.

It has been postulated that HMH, whose level significantly decreases with age, could make aged urine more attractive because it provokes aversive action among female mice. In our behavioral experiment 3, however, we found no significant differences between the sniffing times of aged mice urine samples with or without HMH. We do not therefore consider the age-related decrease of HMH to contribute to the attractiveness of the aged mouse urine odor.

DB and BT are known to be tight ligands of MUPs (Sharrow et al. 2002), which are small monomeric proteins with a molecular weight of about 18 kDa (Hurst et al. 2001). We therefore expected that the ultrafiltration of mouse urine (>10 kDa cutoff) would selectively exclude these volatiles. In fact, the chemical analysis indicated that the filtration procedure effectively removed MUPs as well as MUP-bound volatile molecules, namely, DB, BT, and also IT. Therefore, the significant differences in the concentrations of DB, BT, and IT between adult and aged urine samples disappeared after this procedure (Figure 3). However, there was no significant effect of this procedure on the concentrations of other chemicals (e.g., HMH, Figure 3). Moreover, we found that the ultrafiltration of mouse urine offsets the difference in female attraction between adult and aged mouse urine (Figure 4, Experiment 1). The behavioral and chemical data obtained using ultrafiltered male mouse urine support the possibility that DB, BT, and IT, either individually or in combination, may be the heretofore unidentified female attractants of aged male mouse urine.

We also found 4-methyl-6-hepten-3-one (Peak #23) to be present at higher levels in aged mouse urine than in normal adult mouse urine (Table 1). However, its concentration did not change in mouse urine after ultrafiltration (data not

shown), suggesting that it does not contribute to the attractiveness of aged male urine.

It has been postulated that MUPs could be the pheromones that attract the female mouse (Hurst et al. 2001), suggesting that the elimination of MUPs by ultrafiltration could offset the difference in the attractiveness of adult and aged male mouse urine to female mice. In our study, however, we conducted an odor preference test on female mice that could only sniff the volatiles developed from adult and aged male urine but could not access the urine itself (see Materials and methods). In addition, previous studies have indicated that, without MUPs, volatile pheromones can still evoke excitatory responses in single vomeronasal neurons, leading to both action potential generation and elevated calcium entry (Leinders-Zufall et al. 2000), as well as the promotion of female attraction (Jemiolo et al. 1985). Therefore, we do not consider MUPs to be the essential attractants of aged male mouse urine that attract female mice.

There are several other known mouse pheromones, namely, 2-heptanone and β-farnesene (Harvey et al. 1989; Jemiolo et al. 1989), which are also present in male mouse urine. Among these chemicals, β-farnesene is known to be the major volatile constituent of the male mouse preputial gland and to act not only as a primer pheromone but also as a signaling pheromone for attracting females (Jemiolo et al. 1991). However, as shown in Table 1, there is no evidence that β-farnesene increases with age in male mouse urine, suggesting that β-farnesene does not contribute to the attractiveness of aged mouse urine odor to female mice.

In contrast to DB, BT, and IT, the HMH concentrations significantly decreased with age. HMH is an androgen-dependent, puberty-accelerating pheromone that is found in male mice (Novotny, Jemiolo, et al. 1999; Novotny, Ma, et al. 1999). HMH can also bind to MUPs in mouse urine (Sharrow et al. 2002), and therefore, it should display characteristics similar to those of DB, BT, and IT. Nevertheless, the age-related dynamics of HMH were different from those of the other 3 MUP ligands, which were present at higher concentrations in aged mouse urine (Table 1, Figure 2). As shown in Figure 3, despite the elimination of most urinary DB, BT, and IT content by ultrafiltration, a considerable amount of urinary HMH still remained in the ultrafiltered urine after this procedure. Moreover, unlike DB, BT, and IT concentrations, there was a significant difference in HMH concentration between the aged and the adult male mouse urine ultrafiltrates. HMH is one of the most abundant volatiles in the urine of male mice (around 1000 ppm), and it exists not only in the MUPs fraction but also as a ubiquitous constituent of other fractions when mouse urine is fractionated by Sephadex chromatography (Novotny, Jemiolo, et al. 1999). Moreover, the binding affinity of HMH to MUPs is distinctly weaker than that of DB and BT (Sharrow et al. 2002). Therefore, it is possible that a portion of the HMH content may remain free in the urine or bind to an unknown carrier of less than 10 kDa. We still do not know the precise reason why the amount of HMH in mouse urine decreased with age: however, the difference in the urinary distribution between HMH and DB, BT, and IT could be correlated with differences in the age-related dynamics between HMH (decreased with age) and the other 3 chemicals (increased with age). Female mice may therefore be able to discern the age of male mice by sensing age-related changes in androgenic pheromones in male mouse urine.

In our previous study (Osada et al. 2003), we found that B6 mice could distinguish aged male urine from adult male urine by sensing changes in the relative amounts of specific volatile compounds, including 2-phenylacetamide and indole. However, we did not identify DB, BT, and IT as aging odor chemicals because the mouse urine was pretreated by ultrafiltration to remove the abundant mouse urinary proteins that interfere with the subsequent ether extraction procedure. Although this experimental procedure is useful for the analysis of the free acidic components of mouse urine (Singer et al. 1997), it was not suitable for the analysis of volatiles bound to MUPs.

The results of behavioral experiment 3 suggest that HMH does not play a role as a female "repellent" in adult mouse urine; however, this does not preclude its possible role as a chemosignal affecting female behavior. In fact, previous behavioral studies of mouse pheromones have been performed using a simpler solvent such as water or castrate urine (Jemiolo et al. 1985; Lin et al. 2005) rather than normal adult urine. Further is needed to clarify the pheromonal effect of castrate urine or water spiked with HMH by using the same behavioral paradigm that we used in this study or the Y-maze olfactometer which is considered to be a more suitable and accurate evaluation method for the elucidation of the characteristics of HMH (Yamaguchi et al. 1981; Yamazaki et al. 2000; Lin et al. 2005).

The mechanisms that cause the age-related changes in the concentrations of these compounds are unknown. One previous study reported that, although mouse testosterone levels do not differ between age groups, there are significant differences between the intermediate steroid secretion profiles of young and old mice (Chubb and Desjardins 1984). These changes could be responsible for the different amounts of androgen-dependent pheromones detected among the different age groups. An alternative explanation is that an age-related increase in the urinary high–molecular weight protein level could increase the concentration of attractants in urine (Pashko et al. 1986; Lehman-McKeeman and Caudill 1991). Clearly, further experimental work is needed to clarify the mechanism of age-related changes in urinary volatiles in mice.

In conclusion, we have provided evidence that certain attractants are associated with aging and suggest that the urine odor of inbred aged male mice is attractive to female mice. The "scent of age" may therefore be created by several chemicals, including the attractive semiochemicals that have been identified in this study as DB, BT, and possibly IT.

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