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Mice Recognize Recent Urine Scent Marks by the Molecular Composition

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Abstract

Male mice mark the territory with urine scent marks that are frequently renewed to maintain the territory ownership. We measured the response of male mice to small spots of urine deposed either 0, 5, 11, 22, 45, 90 min, or 24 h before testing and show that mice loose interest in sniffing scent marks as they become older and older. We asked what scent features tell a mouse how recent a scent mark is, and therefore, we studied the molecule-to-behavior relationship by correlating 6 behavioral variables—the number of sniffing acts, the latency to the first sniff, the number of urine marks, the latency to the first mark, the area of the marks, and the number of fecal pellets—to 2,4-dehydro-*exo*-brevicomin, linalool, 2-*sec*-butyl-4,5-dihydrothiazole, 2,4-dimethylphenol, 4-ethylphenol, and 6,10-dimethyl-5,9-undecadien-2-one released from urine spots over the time, identified, and quantified by gas chromatography and mass spectrometry. Canonical correlation between the molecular and the behavioral principal components was strong ($R_{(1)} = 0.96$, $P = 0.026$). The principal component based on 2,4-dehydro-*exo*-brevicomin, linalool, and 2-*sec*-butyl-4,5-dihydrothiazole correlated negatively with countermarking and positively with the sniffing behavior, suggesting a semantic feature of fresh male mouse urine.

Key words: behavior, mouse, olfaction, scent, time

Introduction

Most mammals have a highly developed olfactory sense which they employ in social communication using chemical signals originating in urine, faeces, or skin scent glands (Bossert and Wilson 1963; Thiessen and Rice 1976; Burger 2005). A large share of the mammalian chemical advertising behavior centers around the active deposition of organic chemicals; this implies specialized motor patterns and delivery systems to deposit the chemical signals on environmental objects or other animals of the same species (Ralls 1971; Mucignat-Caretta et al. 2004).

Nocturnal habits and dark living environments have led to the evolution of olfaction as a major method of communication among mice. Their olfactory signals originate mainly from urine. Male mice very frequently scent mark the environment with spots of urine to prove their ability to maintain ownership over an area; they also countermark spots released by other mice invading their territory, to make sure that their scent marks are the most recent in that area. Males which fail to countermark any competing scent marks can no longer ensure that their own marks are the most recent and, thus, indicate their inability to prevent or overcome challenges to their dominance (Hurst 1990; Rich and Hurst 1998). Hence, the olfactory communication by means of recent scent marks is supposed to give an advantage and enforce the position of a male mouse in a ''scent war'' (Hurst and Beynon 2004). Recent studies have focussed on the role of mammalian scent molecules on social, sexual, maternal behaviors as to the relevance of olfactory communication (Burger 2005). To a large extent, the studies did not take into consideration the ''ageing'' process of the scent marks brought about by evaporation, diffusion, and possibly chemical changes of the urine molecular components, when the marks are left in the field exposed to the air.

The volatile molecules of urine confer a characteristic scent. Several chemical scent molecules have been identified in the urine of adult male mice (Holland et al. 1984; Novotny et al. 1985; Ma et al. 1999). Moreover, a protein family abundant in male mouse urine, the major urinary protein complex (MUP), binds and hence concentrates scent molecules in urine and slowly releases them into the air, thus prolonging the lifetime of the scent (Bacchini et al. 1991; Böcskei et al. 1992; Robertson et al. 1996; Jemiolo et al. 2002; Sharrow et al. 2002; Armstrong et al. 2005). The scent molecules dissociate from MUP and are released in the air with a different time course (Cavaggioni et al. 2006).

The scent molecules released from urine induce a sequence of behavioral responses in mice consisting in an early exploration by repetitively sniffing and contacting the urine

followed by countermarking (Novotny et al. 1990; Jemiolo et al. 1992; Mucignat-Caretta and Baldini 1998; Ma et al. 1999; Mucignat-Caretta 2002). The scent molecules bound and released by MUP are a stimulus for behavioral responses to explore and countermark (Hurst et al. 1998; Mucignat-Caretta and Caretta 1999a, 1999b; Mucignat-Caretta 2002; Cavaggioni et al. 2003). Interestingly, mice prefer to explore and countermark recent scent marks (Hurst 1990; Cavaggioni et al. 2003). Thus, the timely release of scent molecules from MUP could work as a clock for mice, to "tell the time" of the spot release. Also, golden hamsters (Wilcox and Johnston 1995) are able to time scent marks and this ability may be more widespread among mammals than presently appreciated.

The study of the diffusion rates of scent molecules in the air and the extent to which these differences influence the outcome of behavioral tests in mammals is a neglected area due to the difficulty of analyzing the diffusion rate of scent molecules. Solid phase microextraction (SPME), however, is an extraction method that can be used with confidence to study the diffusion of molecules in the air (Arthur and Pawliszyn 1990). SPME from the headspace of scent marks was first used by Burger et al. (1997) to analyze the diffusion rate of volatile pheromone molecules from the preorbital secretion of the South African antelope, *Oreotragus oreotragus*. We recently adopted SPME to identify 9 scent molecules and analyze their diffusion from spots of mouse urine (Cavaggioni et al. 2006).

In addition, a number of molecules diffusing from scent marks in the air may be perceived holistically as a complex unity that influences a number of behaviors. We here use the release in the air of 6 molecules from urine spots of different "age" and canonical correlation to describe a multivariate relationship with the mouse behavior (Dillon and Goldstein 1984).

Materials and methods

Subjects

All procedures were authorized by a decree of the Italian Ministry of Health (154/2003-B) and were performed according to the European laws on animal experimentation and handling. Twelve adult male Swiss mice, *Mus musculus*, between 24 and 28 weeks old (body weight 38.25–47.00 g) were born and reared with both parents and littermates in the animal facility of the department in standard polycarbonate cages (42 × 26 × 15 cm) with water and food pellets (Altromin, Riper Co., Bolzano Italy) ad libitum. The mice were weaned at 21 days of age and housed 6 per cage with same sex and age cage mates up to beginning of the experiment. Cage bedding was made of wood shavings changed twice a week according to standard animals' room procedure. The animals' room temperature was 25 ± 1 °C, with relative humidity 70% and 6 air renewals per hour. The light cycle, with 12-h light on, started at 0500 hours. Each mouse was housed isolated in the polycarbonate cages for at least 30 days before the experiments. Mice were isolated in order to increase the excretion of pheromones under androgen control (Lombardi et al. 1976) and hence to reduce the number of mice employed in the tests. Isolated mice are not more stressed than group-housed mice (Hunt and Hambly 2006). On completion of the experiment, the mice were sacrificed with excess anesthesia, 3 times the surgical dose (ketamine 225 mg/kg body weight and xylazine 60 mg/kg body weight) given with an intraperitoneal injection according to the suggestions of the ethical committee and legal requirements.

Urine collection and spot deposition time

Urine was collected daily from the 12 mice for 14 days. A mouse was placed for 2 h in a cage with a mesh grid fixed 1 inch (2.54 cm) above a clean polycarbonate floor, and the urine was collected with a plastic pipette as soon as it was spontaneously voided on the floor. The urine was kept over ice during the collection and was stored at –20 °C. Mice were randomly assigned to 2 groups, A and B, balanced for litter and body weight. The urine samples of group-A mice were then pooled and so were the urine samples of group-B mice in order to minimize individual variability, up to a volume of 7.5 ml from group-A mice and 6.0 ml from group-B mice, a volume sufficient for the chemical analysis of the molecules and the behavioral tests. The 2 pools were spun down with a centrifuge (1130 × g, 10 min, 25 °C), split into 0.3-ml aliquots, and stored at –20 °C. The behavioral tests and the chemical analysis were then carried out within 6 months. In order to avoid self-odors, in the behavioral tests, group-A mice were exposed to the group-B urine and group-B mice were exposed to the group-A urine.

A glass tile (10 × 10 × 1 cm) covered with filter paper was placed 1 cm from the short walls of a polycarbonate cage (42 × 26 × 15 cm), and a 15 µl spot of urine (15.2 ± 0.5 µl) was laid down in the center of the tile in a dedicated room with no air drifts (temperature 20 ± 2 °C and relative humidity 72%). It was then left exposed to the air for 0, 5, 11, 22, 45, or 90 min or, as an end point, for 24 h, to obtain a 0-min-old spot, a 5-min-old spot, and so on.

Behavioral tests

The urine was presented to mice to mimic progressively older spots. A repeated measures experimental design was used. The mice were taken from their home cage and tested in a transparent polycarbonate cage (42 × 26 × 15 cm) whose floor was lined with Bench Guard (Bibby Sterilin, Staffordshire, UK) and equipped with 2 glass tiles (10 × 10 × 1 cm) covered with filter paper placed 1 cm from the short walls of the cage. The mice were tested with urine aged for a different time (0-, 5-, 11-, 22-, 45-, 90-min-, and 24-h-old spots) in random order, different for each mouse, once a day and between 0800 hours and 1100 hours. Two tests were made. In double

choice tests, a tile was spotted with 15 µl of male urine and the other tile with water or both the tiles were spotted with the same urine. In a blank experiment, the 12 mice were tested with both tiles spotted with 15 µl of water in order to exclude a side preference. The 180 tests (2 tests × 12 mice × 7 urine spots, a blank test × 12 mice) were done in a testing room under dim light. The mice were observed for 10 min by the same observer. During the test, the observer recorded 5 variables for each tile: The latency to the first sniffing act (sniffing) within 1 cm of the spot and lasting at least 2 s and the number of the sniffings as indexes of exploratory behavior; the latency to the first urine voiding and the number of the voidings on the tile (1 drop or streak of urine) as indexes of countermarking behavior, and the number of fecal pellets on the cage floor as an index of autonomic activation. Subsequently, the area of the spots was measured: a digital record of the fluorescence of the spots under UV light was taken and the area was determined on a personal computer using the software Corel Photo Paint 12 (www.corel.com) and Scion Image 4.02 software (www.scioncorp.com). In the end, 6 behavioral variables were evaluated.

Data analysis

Analysis of behavior

Each behavioral variable was separately analyzed for each time of exposure of urine to the air with the Wilcoxon test. The trend of responses in the spot-age groups was analyzed with regression analysis. The value for statistical significance was $P \leq 0.05$.

The data sets

The 6 behavioral variables observed on the $K = 7$ experimental groups (spot age), with each observation being repeated on 12 mice, formed the behavioral data set. The K experimental groups were defined by the postdeposition time (0, 5, 11, 22, 45, 90 min, or 24 h) of the test urine, $K = K(t)$. The observations were the number of sniffing acts (y_{i}), the latency to the first sniff (y_{ii}), the number of urine marks (y_{iii}), the latency (y_{iv}), the area (y_{v}) of the marks, and the number of fecal pellets (y_{vi}). The data set of the chemical variables was the measure of the release $x_{\mathbf{I}}$ of 2,4-dehydro-*exo*-brevicomin (**I**), $x_{\mathbf{III}}$ of linalool (**III**), $x_{\mathbf{IV}}$ of 2-*sec*-butyl-4,5-dihydrothiazole (**IV**), $x_{\mathbf{V}}$ of 2,4-dimethylphenol (**V**), $x_{\mathbf{VI}}$ of 4-ethylphenol (**VI**), and $x_{\mathbf{XI}}$ of 6,10-dimethyl-5,9-undecadien-2-one (**XI**), from $K = 7$ samples of the test urine defined by the postdeposition time, with each measure being made on the urine pool A and on the urine pool B. The measurements were made by gas chromatography and mass spectrometry of the headspace of 15 µl spots of urine and the compounds are numbered according the gas chromatographic retention time as described in Cavaggioni et al. (2006).

Factor analysis

Canonical correlation analysis proceeded in sequential stages. A first step reduced the dimensions of the behavioral and the chemical data sets by extracting the principal components. A second step analyzed the canonical correlation between the behavioral and the chemical principal components. The statistical approach is outlined by the boxes and arrows of Figure 3 of the Results.

Principal components

Principal component (**PC**) analysis is based on the equation

$$(\mathbf{R} - \boldsymbol{\lambda}_{(\mathbf{i})}\mathbf{I})\boldsymbol{\gamma}_{(\mathbf{i})} = 0, \quad \mathrm{i} = 1, 2, \ldots,$$

where **R** is the correlation matrix of the vector of the data, $\boldsymbol{\lambda}_{(\mathbf{i})}$ are the roots of **R** from the greatest to the smallest, **I** is the identity matrix, and $\boldsymbol{\gamma}_{(\mathbf{i})}$ are the characteristic vectors expressing the coefficients of the variables in the **PC** (Dillon and Goldstein 1984). Hence, the first **PC** of the vector of the behavioral data $\mathbf{Y} = \mathbf{Y}(t)$ is b-$\mathbf{PC}_{(\mathbf{1})} = \boldsymbol{\gamma}_{(\mathbf{1})}\,\mathbf{Y}$, the second is b-$\mathbf{PC}_{(\mathbf{2})} = \boldsymbol{\gamma}_{(\mathbf{2})}\,\mathbf{Y}$, and so on, where $\boldsymbol{\gamma}_{(\mathbf{i})}$ are the characteristic vectors. A similar analysis with the vector $\mathbf{X} = \mathbf{X}(t)$ of the data of the chemical variables gives a first chemical c-$\mathbf{PC}_{(\mathbf{1})} = \boldsymbol{\delta}_{(\mathbf{1})}\,\mathbf{X}$, a second c-$\mathbf{PC}_{(\mathbf{2})} = \boldsymbol{\delta}_{(\mathbf{2})}\,\mathbf{X}$, and so on, where $\boldsymbol{\delta}_{(\mathbf{i})}$ are the characteristic vectors.

Canonical correlation analysis

Canonical correlation here analyses the relationship between the b-**PC**s and the c-**PC**s by finding 2 linear combinations (canonical variates or **CV**s), one for the c-**PC**s with coefficients (weights) $\boldsymbol{b}_{(\mathbf{i})}$ and one for the b-**PC**s with coefficients $\boldsymbol{a}_{(\mathbf{i})}$ (weights), such that their ordinary correlation is as large as possible. The first canonical root is

$$\sum\nolimits_{(\mathrm{i})=1,2,\ldots} \boldsymbol{a}_{(\mathbf{i})}\,\mathrm{b}\text{-}\mathbf{PC}_{(\mathrm{i})} = \sum\nolimits_{(\mathrm{i})=1,2,\ldots} \boldsymbol{b}_{(\mathbf{i})}\,\mathrm{c}\text{-}\mathbf{PC}_{(\mathrm{i})},$$

where the left term is the behavioral canonical variate b-$\mathbf{CV}_{(\mathbf{1})}$ and the right term the chemical canonical variate c-$\mathbf{CV}_{(\mathbf{1})}$; a second linear combination characterized by the greatest correlation and independent on the first linear correlation is the second canonical root; and so on. Canonical correlation analysis is here used for descriptive purposes without distributional assumptions.

The programs Statistica 5.1 (StatSoft, Tulsa, OK) and SPSS 12.0 (SPSS, Chicago, IL) were used and the varimax orthogonal rotation was applied to the principal components.

Results

Behavioral data

The mice did not prefer a particular tile when both tiles were scented with urine or both were blank tiles. During the test, the mice sniffed the tile scented with a spot of urine more

than the control tile spotted with water (Wilcoxon test, $P < 0.05$), Figure 1 (sniffing number and time to the first sniff, means, and standard error of the mean [SEM]). The number of sniffing decreased monotonically with the spot age, 0, 5, 11, 22, 45, 90 min, and 24 h. The $t(1/2)$ of the sniffing rate was approximately 2 h. The Pearson's product-moment correlation of the sniffs with the trace age was $r = -0.27$ (95% confidence interval –0.47 to –0.039) and the correlation was significant ($P < 0.05$).

The outcomes of the countermarking are shown in Figure 1 (urine deposition number, time to the first deposition, area of the urine trace, means, and SEM). Analysis of the differences in response within the groups showed that, relative to water, the mice countermarked significantly faster and with a larger area the 22-min-old spots; in addition they released more fecal pellets (Wilcoxon test, $P < 0.05$), Figure 1.

In conclusion, exploration depends on the age of the urine declining over time as urine becomes older.

Pearson's correlation

The release in the air of molecules **I**, **III**, **IV**, **V**, **VI**, and **XI** measured as a function of the age of the urine is shown in the

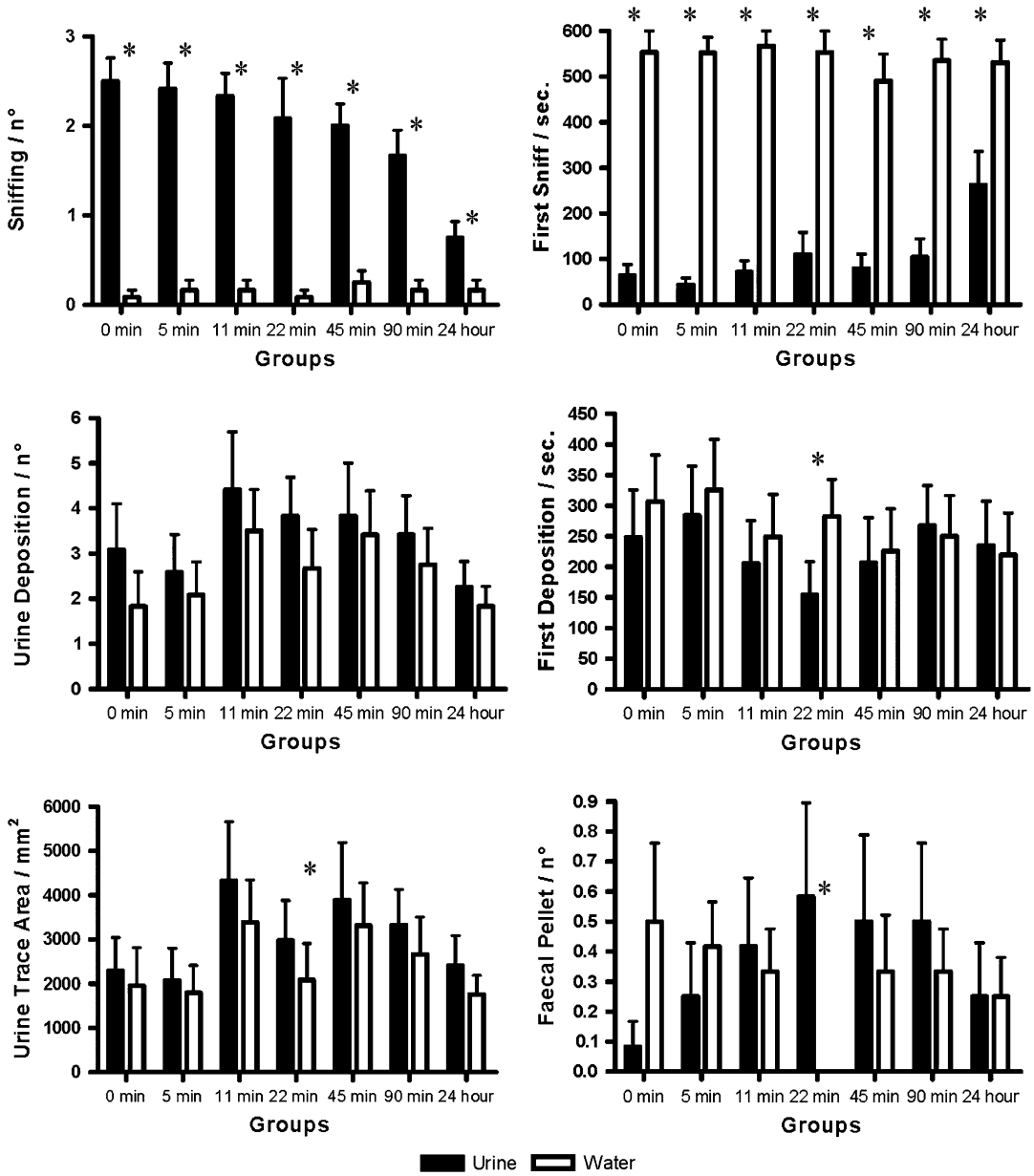

Figure 1 Mouse response to urine at different postdeposition times. A spot of male mouse urine (15 µl), exposed to the air for different time periods, was presented to male mice and the response was recorded for 10 min (mean ± SEM, $n = 12$). The responses significantly different (Wilcoxon test, $P \leq 0.05$) from the controls (water) are shown by asterisks.

Figure 2. The figure shows that **I** and **III** were released from urine in the air only for a short period after spot deposition (early molecules), whereas the other molecules were released for a longer time. The ordinary product-moment correlation coefficient r of the data shown in the Figures 1 and 2 described the one-to-one relationship between molecular and behavioral variables. The Pearson's r between the number of sniffs and **V** was 0.84, between the number of sniffs and **XI** was 0.81, and 0.75 between the number of pellets and **IV** ($P < 0.05$). The correlations prompted an analysis of the relationship between sets of molecules and types of behaviors.

Principal components of the chemical data set

We studied the relationship between **I**, **III**, **IV**, **V**, **VI**, and **XI** and the behavioral variables by analyzing sets of variables rather than the single variables. Given the great number of variables, a reduction of the dimensions was made by principal component analysis while retaining as much as possible of the original information. The analysis extracted a first principal component c-$\mathbf{PC_{(1)}}$ based on $x_{\mathbf{I}} - x_{\mathbf{III}} - x_{\mathbf{IV}}$. The Figure 2 shows that **I** and **III** were characterized by an early release. The analysis extracted also a second c-$\mathbf{PC_{(2)}}$ based on $x_{\mathbf{V}} - x_{\mathbf{VI}} - x_{\mathbf{XI}}$. The Figure 2 shows that **V**, **VI**, and **XI** had a longer lasting release. The respective coefficients and loadings (italics) in the c-**PC**s are shown in the caption of Figure 3. The positive signs on top of the arrows indicated that the variables contributed positively to the size of the **PC**s. The loadings give the ordinary correlation of the original variable and the respective **PC**. The negative loading of $x_{\mathbf{VI}}$ indicates an inverse relationship. The loadings showed that the molecules had correlations greater than 0.7 (absolute value). The c-**PC(1)** explained 42% of the original variability, the c-**PC(2)** about 41%, and together they explained 83% of the original variability.

To extract the behavioral **PC**s (b-**PCs**), we used the observations on the test tile from y_{i} to y_{vi}. The analysis extracted a first b-$\mathbf{PC_{(1)}}$ based on the set $y_{\mathrm{iii}} - y_{\mathrm{iv}} - y_{\mathrm{v}} - y_{\mathrm{vi}}$ that describes the countermarking behavior and a second b-$\mathbf{PC_{(2)}}$ based on $y_{\mathrm{i}} - y_{\mathrm{ii}}$ that describes the sniffing behavior. The respective coefficients and the loadings (italics) in the b-**PCs** are shown near the lower arrows in the scheme of Figure 3. The positive signs on top of the arrows indicate that the variables contributed positively to the size of the **PC**s. The loading of the latencies is negative indicating that an increase of the b-**PC** corresponds to a decrease of the latencies. The loadings showed that the behavioral variables had correlations greater than 0.7 (absolute value). The b-$\mathbf{PC_{(1)}}$ explained about 48%, the b-$\mathbf{PC_{(2)}}$ about 36%, and together they explained 84% of the original variability.

The data of the Figures 1 and 2 are now represented by the **PC**s with a contraction of the dimensions from 6 + 6 to 2 + 2 and with least information loss. The scores of the **PC**s in the $K = 7$ groups that describe the unfolding in time of the **PC**s are reported in Table 1. In order to detect a relationship between the scores, the correlation between c-**PC**s and b-**PC**s was analyzed by canonical correlation.

Canonical correlation

Canonical correlation between the c-**PC**s and the b-**PC**s extracted a first canonical root with correlation $\boldsymbol{R_{(1)}} = 0.959$ between the chemical canonical variate c-$\mathbf{CV_{(1)}}$ and the behavioral canonical variate b-$\mathbf{CV_{(1)}}$. The coefficients of the **CV**s are reported near the arrows in the upper part of the scheme of Figure 3. The **CV**s values of the 0-min-old and 5-min-old spots clustered far apart from the older spots, Figure 4. The index of goodness of fit was $P = 0.026$. The first root extracted 46% of the variance shared by the c-**PC**s and the b-**PC**s. The canonical weight of the **PC**s, expressing the importance of a **PC** for obtaining a maximum correlation between **CV**s, is reported near the arrow of Figure 3. The canonical weight of c-$\mathbf{PC_{(2)}}$ in c-$\mathbf{CV_{(1)}}$ was 1.000 and that of c-$\mathbf{PC_{(1)}}$ was –0.017. Thus, the canonical weight of c-$\mathbf{PC_{(2)}}$ gave a great contribution increasing the c-$\mathbf{CVs_{(1)}}$, whereas the canonical weight of c-$\mathbf{PC_{(1)}}$ gave a very small contribution decreasing the c-$\mathbf{CVs_{(1)}}$. The canonical correlation between the **CV**s showed that an increase of the **CV**s corresponded to decreasing the b-$\mathbf{PC_{(1)}}$ of the countermarking behavior (canonical weight –0.871) and to increasing the b-$\mathbf{PC_{(2)}}$ of the sniffing behavior (canonical weight 0.489). The coefficients and the loadings (italics) of $x_{\mathbf{I}} - x_{\mathbf{III}} - x_{\mathbf{IV}}$ in c-$\mathbf{PC_{(2)}}$, reported in the lower part of the scheme of Figure 3, did not differ substantially suggesting that each molecule was similarly important.

The second canonical root extracted 23.5% of the residual variance shared by the c-**PC**s and the b-**PC**s and had a correlation $\boldsymbol{R_{(2)}} = 0.684$. The index of goodness of fit $P = 0.137$ indicated a poor fit. The smaller fraction of variance shared by the $\mathbf{CVs_{(2)}}$, the lower value of $\boldsymbol{R_{(2)}}$ and the worse fit, showed that the second canonical root was less relevant than the first one.

In order to test the specificity of the molecules used in the analysis, we repeated the analysis with the set of molecules **IV, V, VI**, **VIII**, **X**, and **XI,** thus excluding **I** and **III** and including **VIII** and **X**. The Figure 2 shows that the new molecules **VIII** (indole) and **X** (2-butyl-1-octanol) are characterized by a longer lasting release than **I** and **III**. The canonical correlation was lower and the fit was worse than with the original set ($\boldsymbol{R_{(1)}} = 0.886$, $P = 0.139$).

Discussion

The aim of the present study is to determine whether the scent molecules of the urine help a mouse in timing scent marks. The present relationship between the scent molecules and the mouse behavior lends credit to this possibility.

The behavior of the male mice observed in the presence of scent marks confirms that male mice strongly respond to

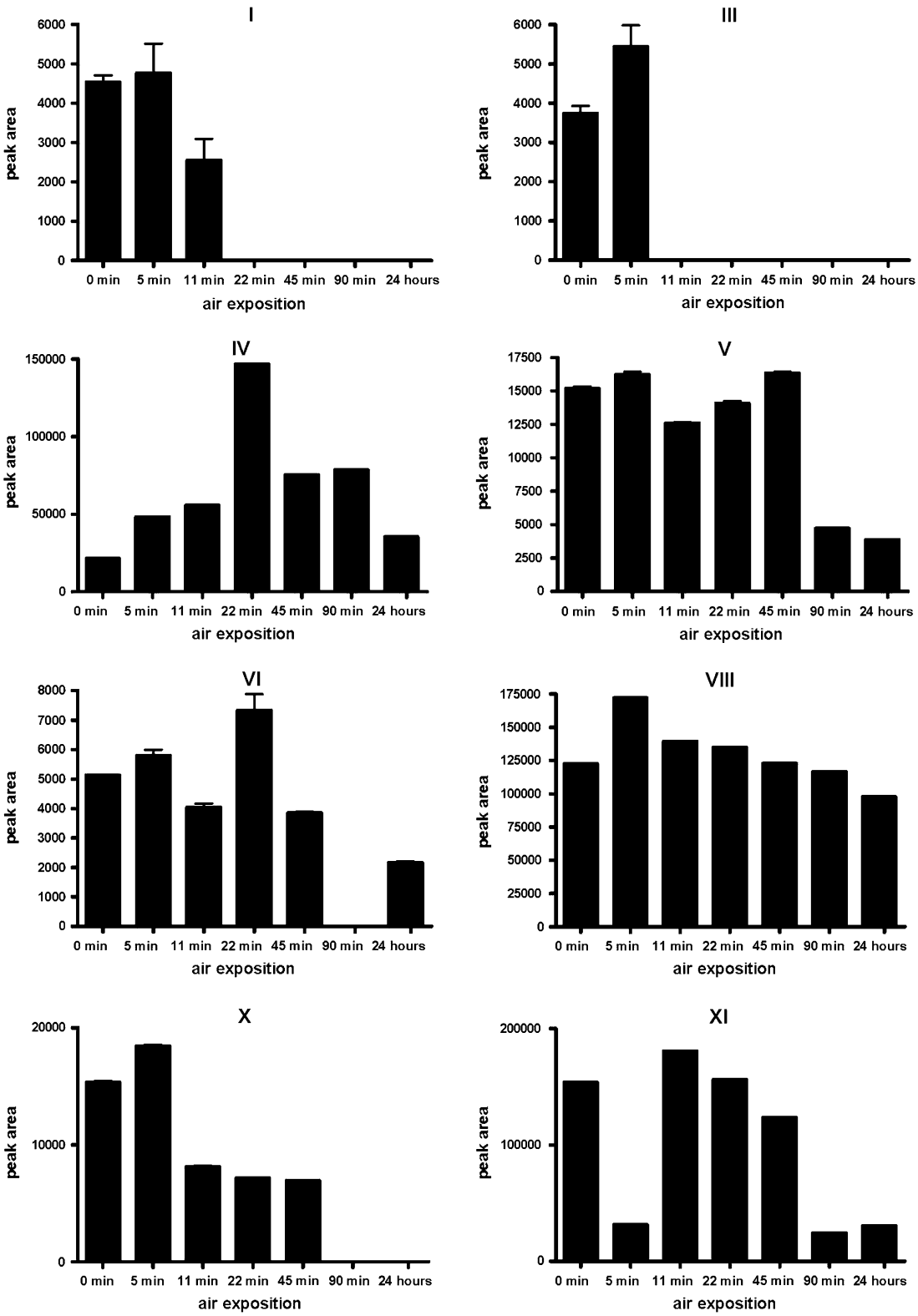

Figure 2 Release of scent molecules from a spot of urine (15 μl) in the air, as a function of time. The headspace of a spot of the male mouse urine (15 μl) was sampled by SPME and analyzed by gas chromatography. On the *y* axis is the gas chromatographic peak area (mean ± SEM, $n = 2$). 2,4-dehydro-*exo*-brevicomin (**I**), linalool (**III**), 2-*sec*-butyl-4,5-dihydrothiazole (**IV**), 2,4-dimethylphenol (**V**), 4-ethylphenol (**VI**), indole (**VIII**), 2-butyl-1-octanol (**X**), and 6,10-dimethyl-5,9-undecadien-2-one (**XI**). The signal-to-noise ratio was always greater than 20.

scent marks with exploratory behavior and by countermarking (Hurst 1990). The rate of countermarking is less than reported by other authors (Maruniak et al. 1974) although it is within the range of previous experiments in our laboratory. The reason of the difference may be that we scored the scent marks on the surface of the tiles excluding those on the cage floor outside of the tiles. The effect of the age of scent marks on the behavior of mice is not new. Cavaggioni et al. (2003) compared countermarking of recent and old scent marks. At variance with the previous studies, the present data focus on the behavior elicited by traces in the minutes after urine deposition.

Firstly, we show that the sniffing rate decreases with the age of the scent mark, up to 90 min old.

Secondly, we here show the effect on the mouse behavior of a set of 6 scent molecules that comprises 2 early molecules that diffuse rapidly and for a short time from urine spots, 2,4-dehydro-*exo*-brevicomin (**I**) and linalool (**III**), and 4 molecules with a slower diffusion, 2-*sec*-butyl-4,5-dihydrothiazole (**IV**), 2,4-dimethylphenol (**V**), 4-ethylphenol (**VI**), and 6,10-dimethyl-5,9-undecadien-2-one (**XI**). The aggressive behavior in response to **I** and **IV** (Novotny et al. 1985, 1990) and the countermarking behavior in response to **IV** stereoisomers (Cavaggioni et al. 2003) were investigated in earlier studies. The present investigation confirms the activity of these molecules and considers also **III**, **V**, and **XI**. Ordinary correlation analysis between the behavioral variables and the molecules, however, gives low correlation indexes and suggests that a statistical analysis that treats the variables simultaneously rather than one by one should be used to unravel a possible relationship. Hence, we first extracted the

Table 1 Scores of the principal components as a function of the postdeposition time

	Behavior		Molecules	
Postdeposition time	Countermarking, b-$\mathbf{PC_{(1)}}$	Sniffing, b-$\mathbf{PC_{(2)}}$	**V** − **VI** − **XI**, c-$\mathbf{PC_{(1)}}$	**I** − **III** − **IV**, c-$\mathbf{PC_{(2)}}$
0 min	−1.05696	0.84001	0.45324	1.18365
5 min	−1.26694	0.81580	0.18094	1.53768
11 min	1.03116	0.61344	0.39605	−0.13870
22 min	1.01784	−0.19896	1.32155	−1.24028
45 min	0.83525	0.14394	0.37461	−0.55249
90 min	0.12218	0.17417	−1.39084	−0.56789
24 h	−0.68253	−2.04007	−1.33556	−0.22197

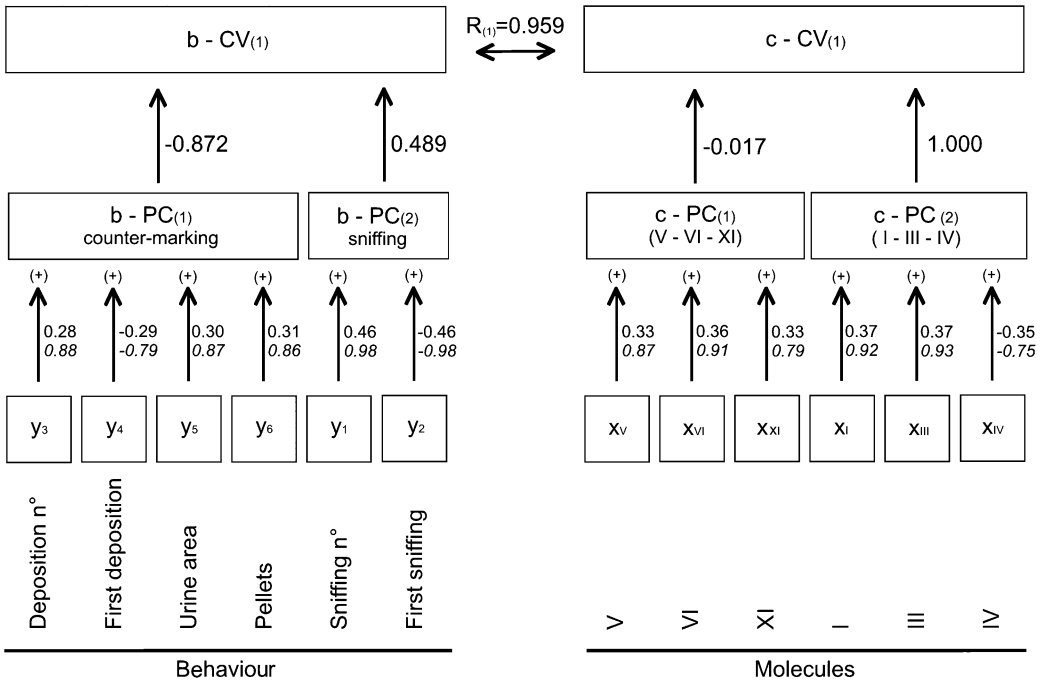

Figure 3 Scheme of the canonical correlation analysis. In the lower boxes are the symbols of the behavioral and chemical variables. Intermediate boxes represent the principal components, namely, linear composites of the variables that reduce the dimensions while retaining as much as possible of the original information. The numbers near the arrows are the coefficients of the variables in the principal components and (italics) the correlations of the variables with the respective principal component. The plus signs indicate a positive effect of the variables on the principal components. The upper boxes represent the canonical variates of the first canonical root, namely, the first linear composites that maximally correlate between them. The numbers near the joining arrows are the coefficients of the principal components in the canonical variates. The arrow between the canonical variates represents the canonical correlation. Molecules: 2,4-dehydro-*exo*-brevicomin (**I**), linalool (**III**), 2-*sec*-butyl-4,5-dihydrothiazole (**IV**), 2,4-dimethylphenol (**V**), 4-ethylphenol (**VI**), and 6,10-dimethyl-5,9-undecadien-2-one (**XI**).

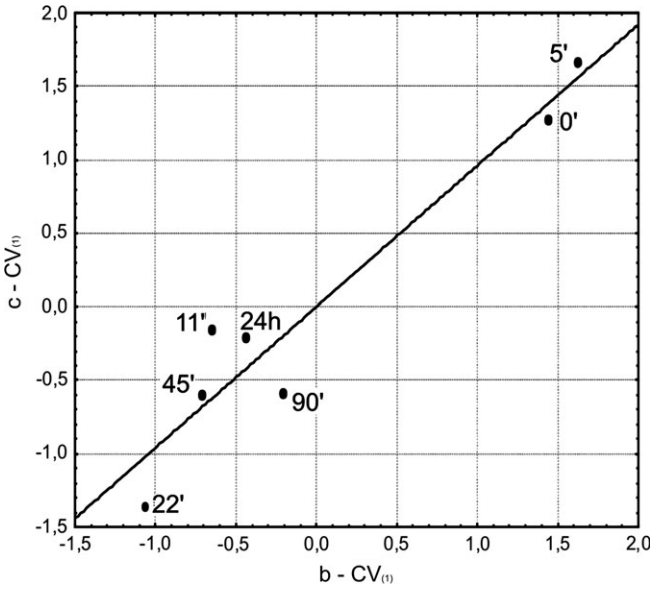

Figure 4 Scatterplot of the canonical variates. On the *x* axis is the canonical variate expressing the mouse behavior and on the *y* axis the canonical variate expressing the release of scent molecules from spots of mouse urine. The numbers near the points are the postdeposition time, namely, the age of the urine spots. The continuous line represents the function $y = 7.3E - 17 + 0.959x$. Note the cluster of the 0- and 5-min points.

molecular and behavioral principal components that describe features and maintain as much of the original information as possible while reducing the number of variables. We then found by canonical correlation analysis, the linear composites of the principal components that maximally correlate between them. The canonical correlation obtained is higher than the ordinary correlations determined between the variables. Canonical correlation associates the principal component based on **I**, **III**, and **IV** to reducing the countermarking component and increasing the sniffing component. None of the molecules is more important than the others in the principal component. It is very interesting that both **I** and **III** are chemically related to insect pheromones. **I** is a dehydrogenated form of (+)-*endo*-brevicomin that is an aggregating pheromone of the southern pine bark beetle *Dendroctonus frontalis* (Vité et al. 1985; Sullivan et al. 2007) and S-(+)-linalool, a mate attractant pheromone of the solitary bee *Colletes cunicularius* (Borg-Karlson et al. 2003), is an isomer of **III**. The principal component based on **V**, **VI**, and **XI** seems less relevant for inducing behavioral modifications in the recipient mouse. The substantial amount of variability, that is, information, shared by the behavioral and scent principal components and accounted for by the behavioral and scent variables, shows a fair description. Thus, the canonical correlation describes a relationship between the urine scent and the mouse behavior giving insights into the mechanisms that mice use to recognize recent scent marks.

Canonical correlation analysis as a way of guiding feature selection toward the underlying semantics was never applied to olfaction before. The present study, however, has a number of limitations. The behavioral variables did not meet the requirements of multivariate normality and homogeneity of the variance for testing the significance of the relationships. Hence, the canonical correlation is here used for descriptive purposes only. Moreover, the number of the groups investigated reduced the number of molecules that could be analyzed with statistical consistency to 6. The 6 molecules considered are a small subset of the urinary molecules that a mouse may conceivably perceive. The SPME extracted some molecules more efficiently than others from the urine headspace depending on the polarity of the molecules. As an example, farnesene isomers were below our detection threshold although their wide-ranging role in the mouse behavior is well demonstrated (Ma et al. 1999). Finally, mice approaching a scent mark often contacted the urine to investigate nonvolatile molecules (Hurst 1990; Nevison et al. 2000); thus, we do not know whether the countermarking behavior is a response to molecules of different volatility.

We here combine behavior, chemistry, and canonical correlation analysis to provide some insight into the specialized perceptual and motivational mechanisms that mice use to respond to the molecules that unfold from scent marks over time—the scent of time.

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