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Gender and individual information coded by insect pheromone analogs in the preputial glands in male brandt's voles *Lasiopodomys brandtii* *

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Abstract We analyzed the volatile composition of dichloromethane extracts of preputial glandular secretions (PGS) from intact and castrated male Brandt's voles *Lasiopodomys brandtii* by using gas chromatography-mass spectrometry (GC-MS). Thirty-three volatile compounds were detected and were present in nearly all scent donors; 27 had previously been reported as insect pheromone components. Castration did not eliminate any compounds; however, it significantly suppressed the relative quantity of ten early-eluting, low molecular weight components. These included seven saturated and unsaturated acetates, one hexanoate, and two saturated octanoates. In particular, *E*, *E*-Farnesyl acetate (FA) was the most abundant compound in the PGS. We suggest that FA and other components might be candidates for male-produced pheromones. Two-choice behavioral assays of females revealed that low concentrations of raw PGS (0.056%) and *E*, *E*-farnesyl acetate (FA) (5 parts per million in water v/w) were attractive, whereas high concentrations (0.56% and 50 parts per million, respectively) were repellent. This suggested that FA may act as a male pheromone in a dosage-dependent manner. Extremely high variability of the relative quantities of almost all detected compounds among individuals indicated the inter-individual dissimilarities of PGS and consequent possibilities to communicate individuality to other conspecific members [*Acta Zoologica Sinica* 53 (4): 616 – 624, 2007].

Key words Brandt's vole, *Lasiopodomys brandtii*, Dose-dependency, *E*, *E*-Farnesyl acetate, GC-MS, Pheromone, Preputial gland, Insect pheromone analog

雄性布氏田鼠包皮腺中的昆虫信息素类似物编码的性别和个体信息*

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摘　要　我们利用二氯甲烷抽提和气质联用（GC-MS）比较分析了正常和阉割布氏田鼠的包皮腺分泌物（PGS）成分。我们检测到33个成分，它们几乎在所有的被测布氏田鼠都存在，其中27个成分以前报道为昆虫的信息素组分。睾丸切除不能使任何一个成分完全消失，但是显著降低了10个首先从GC-MS流失出的小分子成分，即7个饱和与不饱和的乙酸酯，一个饱和六酸酯和两个饱和八酸酯，其中，包括PGS含量最高的成分 *E*，*E*-法尼醇乙酸酯。因此，可以认为这些受睾丸调节的成分为雄性信息素的候选成分。对雌鼠的嗅觉双项选择测定说明低

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浓度的 PGS 和法尼醇乙酸酯水溶液对雌性有吸引作用，而高浓度时都具有趋避作用。这说明 PGS 具有剂量依赖的性吸引作用，法尼醇乙酸酯是一种剂量依赖的雄性信息素。进一步的数量比较说明，所有检测到成分的百分组成在个体间表现出巨大的个体变异，说明 PGS 的成分有个体的特异性，可能传递个体的嗅觉信息［动物学报 53（4）：616－624，2007］。

关键词　布氏田鼠　剂量依赖性　*E*，*E*-法尼醇乙酸酯　气质联用　信息素　包皮腺　昆虫信息素类似物

Preputial glands are widely present and vital chemosignal-producing organs in rodents (Muridae and Arvicolinae), and their secretions are discharged into the urine to convey information about species, sex, and dominance to conspecifics (Brinck and Hoffmeyer, 1984; Brown, 1985; Welsh et al., 1988; Harvey et al., 1989; Novotny et al., 1990, 1999; Zhang et al., 2007). The glands are often better developed in males than in females, and are stimulated by androgen and are suppressed by castration. Compounds that vary in relative quantity with endocrine states can be considered as pheromonal candidates subject to further verification of their activities *via* biological assays (Novotny et al., 1999). This methodology for exploring pheromones in mammals has led to successes in the discoveries of a number of pheromones in the house mouse *Mus musculus* (Novotny et al., 1984, 1986, 1999; Jemiolo et al., 1986, 1991; Schwende et al., 1986; Singer et al., 1997; Ma et al., 1999; Zhang et al., 2007). For example, *E*-*β*-farnesene and *E*, *E*-*α*-farnesene from the *M. musculus* preputial gland are androgen-dependent, and biological tests have verified that they are involved in dominance advertisement, signalling attractiveness to females, and induction of estrous cycles of group-caged females (Harvey et al., 1989; Novotny et al., 1990; Jemiolo et al., 1991; Ma et al., 1999).

The Brandt's vole *Lasiopodomys brandtii*, formerly known as *Microtus brandtii*, is mainly distributed in the grasslands of Inner Mongolia in China, Mongolia, and the Russian Far East (Wilson and Reeder, 2005). It lives a gregarious life, and males can establish a stable social hierarchy (Zhong et al., 1999). We had found that preputial glands were larger and always present in male *L. brandtii*, but smaller in and sometimes absent from the females (also termed as clitoral glands in females) (Brown, 1985).

In this study, we used coupled gas chromatograph-mass spectrometry (GC-MS) to analyze the chemical composition of preputial glandular secretions (PGS) from intact and castrated male *L. brandtii*. We also tested the behavioral response of females to the crude extract and to selected chemical components. We hypothesized that if preputial glandular secretions contain information characteristic of gender or individual status, then some of its chemical components might vary in relative abundance with endocrine state or display dissimilarity among individuals. We predicted first, that castration would reduce the amount of some compounds that might be attractive to females, and second, that the variability of the relative quantities of the volatile components would be significantly higher among individuals than within individuals.

1 Methods and materials

1.1 Subjects

Twenty male and 13 female *L. brandtii*, approximately one-year old were selected from a colony maintained in our laboratory. They were first generation progeny of wild-captured parents in Inner Mongolia of China, and their ages were known exactly. Originally, the individuals were caged as male-female pairs. After selection, the voles were housed individually in plastic cages (measuring 37 cm × 26 cm × 17 cm) under a 14 L:10 D light regime (light on at 06:00) and at 21 ± 0.2℃ for one month of acclimation prior to experimental use. Food (Standard rabbit chow, Weilitong-Lihua Laboratory Animal Company, Beijing, China) and water were always provided. The males had fully descended testes and females had perforated vaginae, indicating that they were of reproductive age. All animals had ejaculated successfully. The males were used as scent donors and assigned randomly into two treatment groups (intact and castrated). The females were used as scent detectors.

1.2 Surgical procedure

We performed castration on males through bilateral incisions in the scrotum. The animals were first anesthetized with sodium pentobarbital (45 mg/kg). At the time of castration, the blood vessels were ligated, and the wound was closed with sterile sutures and treated with 75% alcohol solution and 5% tincture of iodine.

1.3 Sampling and extraction

To collect the preputial glands, we killed ten intact and ten castrated males four weeks after surgery dissected out and weighed (accuracy of 0.1 mg) each subgland of the paired preputial gland. The glands were frozen at －20℃ until extraction with dichloromethane (purchased from Beijing Fine Chemical Ltd., Beijing, China, purity >99.5%). To extract the PGS, we gently squeezed the milk-like secretion from a thawing preputial gland into a clear vial. Then, we weighed the material and added dichloromethane into the vial (10 μl solvent/1 mg secretion) and shook the samples. After 24 h at room temperature, we transferred the lower phase into a second vial and then stored it at －20℃ for less than one week for analysis by GC-MS (Zhang et al., 2007). The glands of one castrated male were too small to obtain the

secretion and were therefore excluded from the analysis.

1.4 GC-MS analysis

Analytical GC-MS was performed on an Agilent Technologies Network 6890 N GC system coupled with a 5973 Mass Selective Detector with an NIST2002 library. Agilent software (Windows XP) was used for data acquisition and processing. The GC was equipped with a 60 m glass capillary column (0.25 mm i.d.; film 0.25 μm thickness) coated with DB5MS. Helium was used as the carrier gas (1.0 ml/min flow rate). The temperature of the injector was set at 230℃, and the oven temperature was programmed as follows: initial temperature 100℃ and then increased by 5℃/min up to 180℃ and increased again at 1℃/min up to 220℃ and held for 15 min. Finally, the temperature was increased up to 230℃ at 10℃/min and held for 10 min post run to clean the column. Electron impact ionization was used at 70 eV. Transfer line temperature was 280℃. Scanning mass ranged from 30 to 350 amu. The amount of sample injected was 1 μl every time in split mode (10:1) (Zhang et al., 2007).

1.5 Compound identification

We tentatively identified extract components by comparing the retention times of the GC peaks and through the similarity of their mass spectra with those suggested by the MS library (NIST2002) and the literature. To confirm or to correct each identification, we checked the ions characteristic of each class of compound. For example, m/z 61, m/z 43, and m/z M^+-60 (M^+, Molecular ion) indicative of acetates; m/z 89, m/z 71, and m/z M^+-88 of butyrates; m/z 117, m/z 99, and m/z M^+-116 of hexanoates, and m/z 145, m/z 127, and m/z M^+-144 of octanoates. In addition, the intensity of these diagnostic ions diminishes when the alcohol chains were unsaturated. Fatty acids and alcohols can be diagnosed by fragments at m/z 60, m/z M^+-29, and m/z M^+-43, and m/z 31 and M^+-18, respectively. The branched-chain, unsaturated, low position of double bonds or *trans* isomers of acetates often have a shorter retention time in the gas chromatogram generated via this nonpolar column than did their straight-chain, saturated counterparts. These were helpful criteria to discriminate two isomers with similar mass spectra. The relative intensities of important ions and their ratios [(m_1/z)/(m_2/z)] (41/43, 54/55, 67/68, 81/82, 81/89, and 95/96) were used to further determine agreement of double bond positions between MS detection and library suggestion. Unsaturation of the alcohol chain was determined by the corresponding hydrocarbon's fragments at their ion of m/z (M^+-2). (Horike et al., 1991; Leonhardt and De Vilbiss, 1985; Fang et al., 2005; Zhang et al., 2007)

An examination of the GC retention times and MS spectra from GC-MS analyses of samples of the preputial glands from mice (Harvey et al., 1989; Novotny et al., 1990; Zhang et al., 2007) led to our verification of *E*-β-farnesene in the extracts. Nine other compounds were verified by matching their retention times and mass spectra with synthetic analogs (Table 1, numbered compounds marked by asterisks). The MS data of every GC peak has been presented in detail in another study (unpublished data).

1.6 Quantitative analysis

Relative quantities of each component were calculated by converting the peak area of a particular compound into a percentage of the summed peak areas of all 29 peaks (33 compounds and 4 unresolved peaks) in every sample (Sun and Müller-Schwarze, 1998a, b; Zhang et al., 2003, 2007). In addition, GC peak areas of the undetectable components determined by GC retention time and MS data were taken as zero.

To express the variability of volatile composition among individuals, we calculated relative standard deviation (*RSD*) by using the following formula: $RSD = (SD/mean) \times 100$, where *mean* and *SD* the respective average of each volatile peak area percentage for all intact males and its standard deviation, respectively (Zhang et al., 2003). Inter-individual *RSD* was calculated from data collected from the samples of ten intact males. Intra-individual *RSD* was calculated from data collected from five measurements from one intact male sample.

1.7 Aqueous sample preparation

E, *E*-Farnesyl acetate (FA) (purity: 95%) (purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA) was diluted in deionized water to concentrations of 50 parts per million (v:v) (high concentration) and 5 parts per million (v:v) (low concentration). The approximate concentration of FA in raw male PGS is 0.63% (0.70% v:w) (see Results). PGS from two intact males, whose volatiles had similar relative concentrations to the mean of ten intact males (Table 1), were pooled and diluted in deionized water to 0.56% that is equivalent to 39 parts per million of FA (v:w) (high concentration) and 0.056% that is equivalent to 3.9 parts per million of FA(v:w) (low concentration). These solutions and deionized water (control) were used for in the behavioral assay.

1.8 Behavioral test procedure

Female preference for one of two aqueous samples was tested during the light phase. Three μl of each sample, were presented as a smear simultaneously to experimental animals (2 cm apart and about 5 cm above the bedding) in cages on clean glass rods (3.5 mm in diameter, 25 cm in length) at one side of the cage opposite to where the voles stayed. The test lasted for 3 min after they initially detected the substances by sniffing. (Lai et al., 1996). We used two stopwatches to record the time that the female test subjects spent in sniffing/licking the glass rods by placing their snouts within approximately 1 cm from the treated ends. Every

Table 1 Comparison of relative abundance of preputial gland volatiles between intact and castrated male Brandt's voles *Lasiopodomys brandtii* (Mean ± *SD*)

Peak No.	Retention time (min)	Compounds	Relative abundance (%)			
			Intact males (n = 10)	Castrated males(n = 9)	P	Z/t
1	15.08	9-Decenyl acetate	0.50 ± 0.49	0.08 ± 0.17	0.008	**2.679**
2	15.22	Decyl acetate	0.77 ± 0.85	0.09 ± 0.10	0.005	**2.788**
3	16.16	Isomer of *n*-Heptyl hexanoate	0.32 ± 0.39	0.02 ± 0.03	0.022	**2.428**
4	16.39	*E*-β-Farnesene*	0.11 ± 0.08	0.06 ± 0.05	0.080	1.807
5	19.81	Hexyl octanoate	0.90 ± 1.01	0.12 ± 0.17	0.028	**0.239**
6	19.99	Decyl butyrate	1.02 ± 0.96	0.36 ± 0.28	0.064	1.978
7	20.43	*E*9-Dodecen-1-yl acetate*	0.60 ± 0.44	0.37 ± 0.20	0.178	1.404
8	20.52	*Z*9-Dodecen-1-yl acetae*	2.20 ± 2.00	0.77 ± 0.89	0.035	**2.214**
	20.62	Dodecyl acetate*				
9	21.76	Heptyl octanoate	0.54 ± 0.42	0.18 ± 0.21	0.033	2.322
10	27.30	*Z*9-*E*12-Tetradecadien-1-yl acetate (?)	4.42 ± 2.72	1.57 ± 1.50	0.008	**2.613**
11	27.43	*Z*5-Tetradecen-1-yl acetate*	4.09 ± 1.80	2.26 ± 0.90	0.022	**2.246**
12	27.55	*n*-Dodecyl butyrate	1.14 ± 0.68	1.05 ± 0.46	1.000	**0.000**
13	27.95	9-Tetradecen-1-yl acetate*	4.76 ± 2.00	3.61 ± 2.18	0.248	1.197
14	28.49	*n*-Tetradecyl acetate*	4.33 ± 1.16	4.82 ± 1.22	0.384	0.893
15	29.81	*E*, *E*-Farnesyl acetate*	10.54 ± 7.62	4.00 ± 4.70	0.010	**2.531**
16	36.65	*Z*6-Tetradecen-1-yl butyrate	1.67 ± 1.03	2.32 ± 1.70	0.324	1.015
17	37.09	*Z*7-E11-Hexadecadien-1-yl acetate(?)	3.19 ± 0.67	3.73 ± 0.70	0.103	1.723
18	37.41	*Z*11-Dodecen-1-yl hexanoate	3.57 ± 1.32	5.04 ± 0.92	0.013	2.785
	37.52	*Z*9 (or *E*9)-Tetradecen-1-yl butyrate				
19	37.86	Dodecyl hexanoate	4.37 ± 1.46	5.82 ± 0.99	0.023	2.505
20	38.13	Isomer of *Z*11-Hexadecen-1-yl acetate	7.97 ± 4.15	11.28 ± 2.28	0.049	2.114
	38.22	Tetradecyl isobutyrate (or butyrate)				
21	38.73	*Z*11-Hexadecen -1-yl acetate*	0.38 ± 0.26	0.63 ± 0.29	0.072	1.922
22	39.45	Hexadecyl acetate* (minor), *E*, *E*-Farnesyl butyrate	7.98 ± 2.89	9.56 ± 2.46	0.221	1.271
23	49.15	*Z*6-Tetradecen-1-yl hexanoate	2.40 ± 1.24	3.83 ± 1.66	0.048	2.134
24	50.00	*Z*11-Hexadecen-l-yl butyrate	1.59 ± 1.08	2.14 ± 0.77	0.222	1.267
25	50.17	*Z*9-Tetradecen-1-yl hexanoate	5.46 ± 2.82	8.11 ± 2.52	0.047	2.142
26	50.56	Dodecyl octanoate	3.43 ± 2.67	4.86 ± 1.93	0.202	1.328
27	51.02	Tetradecyl hexanoate	6.02 ± 3.99	8.80 ± 3.43	0.125	1.616
28	51.46	Hexadecyl isobutyrate	5.73 ± 13.83	2.02 ± 0.95	0.315	**1.061**
29	52.35	*E*, *E*-Farnesyl hexanoate	10.05 ± 5.89	12.55 ± 4.08	0.304	1.060

Note: the compound marked by asterisk was verified by synthetic analog; bold printed data indicated Z values and the corresponding data was tested using Mann-Whitney U test, and the other are t values in t test; relative abundance was calculated as described in method section of the text.

vole was used only once every day at a random order. Namely, we randomly assigned the test females into one of three subgroups (four, four, and five individuals, respectively), and then they were subjected to one of three pairs of test samples of raw PGS or FA every test day. Subjects who did not respond within 3 min or those whose time of investigation was less than one sec during the 3 min tests were excluded from the analysis. Thus, sample size ranged from 10 to 13.

All above experimental procedures accorded with the

institutional guidelines for animals use and care at Institute of Zoology, Chinese Academy of Sciences.

1.9 Statistical analyses

Chemical data were first tested for normality by using Kolmogorov-Smirnov test. Then, to test for significant differences between components from intact and castrated males, we used a two-tailed *t*-test to analyze the weights of preputial glands and normal *RSD*s. The Mann-Whitney *U*-test was used for analyzing nonnormal *RSD*s. The Wilcoxon matched-pairs signed-rank test was used to analyze differences in behavioral response by females. Statistical analyses were conducted with SPSS version 10.0. Differences were considered significant at a critical value of $\alpha = 0.05$.

2 Results

2.1 Effect of castration on preputial gland compounds

The preputial gland was significantly lighter in castrated voles than in intact voles (24.68 ± 14.25 mg *vs* 43.53 ± 26.26 mg, $t_{38} = 2.821$, $P = 0.008$), whereas body weights were similar when the two treatment groups were compared (65.23 ± 10.22 g *vs* 62.37 ± 17.14 g, respectively, $t_{18} = 0.453$, $P = 0.656$).

We identified 33 compounds corresponding to 29 GC peaks (Fig.1 and Table 2). Unresolved peaks, 8, 18, 20 and 22 each represented two compounds, which could be discerned by MS analysis. Of 15 GC peaks eluting before 30 min, the relative abundances of nine peaks (1, 2, 3, 5, 8, 9, 10, 11, and 15) were significantly reduced by castration (their *P* values were 0.008, 0.005, 0.022, 0.028, 0.035, 0.033, 0.008, 0.022, and 0.010, respectively) (Table 1, Fig.1). With the exception of peak 28, the 14 GC peaks eluting after 30 min were generally higher in relative abundance in castrated males. Peaks 18, 19, 20, 23 and 25, in particular, attained statistical significance (the *P* values are 0.013, 0.023, 0.049, 0.048, and 0.047). Peak 15 [*E*, *E*-farnesyl acetate (FA)] was not only the most abundant of the 29 GC peaks, but it also accounted for 41.39% of the nine castration-suppressed components. (Table 1, Fig.1) The approximate concentration of FA in the raw PGS was 0.63% (w/w) (equivalent to 0.70% v/w). This was estimated by comparing the GC peak area of peak 15 generated by PGS sample from two vole samples having representative GC peak of FA with FA solution samples prepared in dichloromethane at 2, 20, 200, and 2 000 ng/μl of concentrations, where we had obtained the calibration regression equation ($Y = 1.185X - 15.50$, $R^2 = 0.998$, $F = 825.3$, $P = 0.001$) of logarithm of the FA concentration (Y) versus logarithm of GC peak area (X), and then we obtained the FA concentration (630 ng/μl) in PGS according to the concentration of PGS in dichloromethane (0.1 mg/μl).

2.2 Individual variation in volatile composition

Our quantitative analysis showed that *RSDs* of the relative abundance were higher in inter-individual PGS samples (71.76 ± 41.44) than in the intra-individual samples (13.67 ± 7.24) in intact *L. brandtii* ($t_{28} = 7.371$, $P < 0.001$) (Table 2). This implies that PGS volatiles in male *L. brandtii* were markedly different in relative concentration among individuals (Table 2). In addition, these compounds were almost always present in every vole, and only a few minute components were

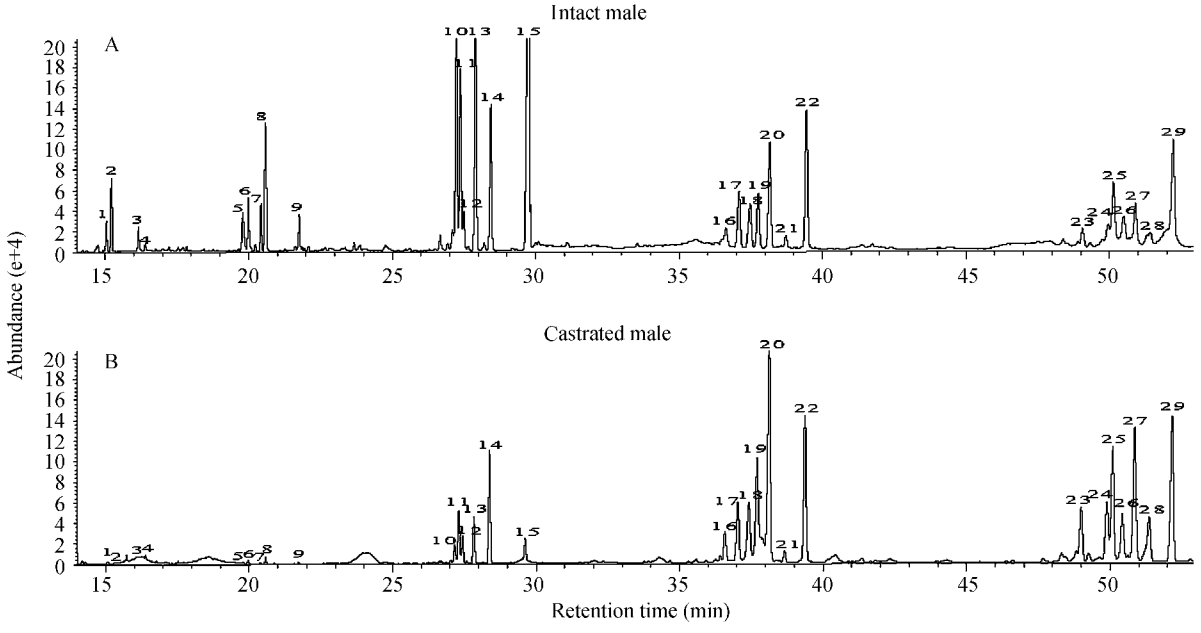

Fig.1 Representative total ion chromatogram from a GC-MS analysis of dichloromethane extracts of preputial gland secretions from (A) intact and (B) castrated male Brandt's voles *Lasiopodomys brandtii*

The numbered peaks correspond to the compounds listed in Table 1.

occasionally undetectable (Table 1).

Table 2 Relative standard deviation (RSD) of relative abundance of inter- and intra-individual preputial gland volatiles in male Brandt's voles *Lasiopodomys brandtii*

Peak No.	Relative abundance of intra-individual samples ($n = 5$) (Mean ± *SD*) (%)	Relative standard deviation (RSD)	
		Inter-individual samples ($n = 10$)	Intra-individual samples ($n = 5$)
1	1.10 ± 0.14	100.20	13.04
2	1.62 ± 0.17	111.32	10.77
3	0.66 ± 0.04	120.75	5.59
4	0.13 ± 0.04	67.86	31.49
5	1.49 ± 0.12	112.44	8.28
6	1.28 ± 0.19	94.09	14.88
7	1.09 ± 0.12	74.67	10.81
8	3.91 ± 0.29	90.56	7.41
9	0.55 ± 0.10	77.79	17.93
10	5.61 ± 0.54	61.49	9.63
11	4.06 ± 0.90	43.89	22.19
12	0.83 ± 0.08	60.11	9.23
13	5.62 ± 0.58	41.86	10.27
14	4.10 ± 0.44	26.85	10.73
15	19.34 ± 1.88	72.34	9.72
16	0.53 ± 0.17	61.62	32.98
17	2.88 ± 0.11	20.98	3.80
18	2.54 ± 0.36	36.91	14.39
19	3.30 ± 0.30	33.40	9.17
20	6.12 ± 0.15	52.03	2.51
21	0.53 ± 0.08	68.17	14.80
22	9.81 ± 1.06	36.25	10.76
23	1.37 ± 0.20	51.58	14.83
24	0.89 ± 0.24	68.23	27.10
25	4.22 ± 0.57	51.62	13.45
26	1.85 ± 0.33	77.94	17.91
27	3.68 ± 0.55	66.26	14.85
28	0.58 ± 0.07	241.24	11.76
29	10.42 ± 1.69	58.64	16.19
	Mean ± *SD*	71.76 ± 41.44	13.67 ± 7.242
	Significance	$t = 7.371$, $df = 28$, $P < 0.001$	

Relative standard deviation (*RSD*) was calculated as described in the method section of the text.

2.3 Responses of females to preputial gland secretion and *E*, *E*-farnesyl acetate

Females significantly reduced the time they spent investigating the high concentration of PGS ($n = 13$, $Z = 2.830$, $P = 0.005$), whereas they significantly increased the time spent investigating the low concentration of PGS ($n = 13$, $Z = 2.551$, $P = 0.011$), both compared to the deionized water control r (Fig.2A). Furthermore, females exhibited a significant preference for the low concentration of PGS over the high concentration of PGS ($n = 10$, $Z = 2.803$, $P = 0.005$).

Female *L. brandtii* significantly reduced the time they spent investigating the high concentration of FA ($n = 13$, $Z = 2.063$, $P = 0.039$), whereas they significantly increased their time spent investigating the low concentration of FA ($n = 13$, $Z = 2.132$, $P = 0.033$) both compared to the deionized water control (Fig.2B). Furthermore, females exhibited significant preferences for the low concentration of FA over the high concentration of FA ($n = 10$, $Z = 2.385$, $P = 0.017$).

3 Discussion

Our results suggest that castration not only suppresses the size of the preputial glands, but also reduces the relative quantities of ten early-eluting low-molecular weight compounds (9-decenyl acetate, *n*-decyl acetate, an isomer of heptyl hexanoate, *n*-hexyl octanoate, *Z*9-dodecen-1-yl acetate, *n*-dodecyl acetate, *n*-heptyl octanoate, a tetradecadien-1-yl acetate, *Z*5-tetradecen-1-yl acetate, and FA) in the PGS of male *L. brandtii*. Thus, it is logical to view these compounds as potential pheromones associated with sex in the voles according to the methodology established through the successful discoveries of several pheromones in house mice (Harvey et al., 1989; Novotny et al., 1986, 1990; 1999; Singer et al., 1997; Jemiolo et al., 1991; Zhang et al., 2007). One of the most abundant components, FA, was attractive to females. With the exception of 9-decenyl acetate and *n*-heptyl octanoate, all other castration-suppressed compounds were previously described as pheromonal components in insects (Byers, 2002; Kelly, 1996). Additionally, castration did not significantly affect the relative abundance of *E*-β-farnesene (minor component) in *L. brandtii*, whereas this compound (characterized previously as a major component) has been proved to be distinctly androgen-dependent in the PGS of house mice (Harvey et al., 1989; Novotny et al., 1990; Zhang et al., 2007). Our data also showed that not all acetates in the PGS were significantly reduced by castration (e.g., early-eluting *Z*9-tetradecen-1-yl acetate and *n*-tetradecyl acetate). On the contrary, the relative amounts of peaks 18, 19, 20, 23, and 25 were enhanced by castration. These compounds might be useful in other aspects of chemosensory communication such as information about genetic background and species status (because they were constant across the reproductive status). Similarly, there are no sex-specific components and only early-eluting low-molecular weight compounds are male-elevated in relative

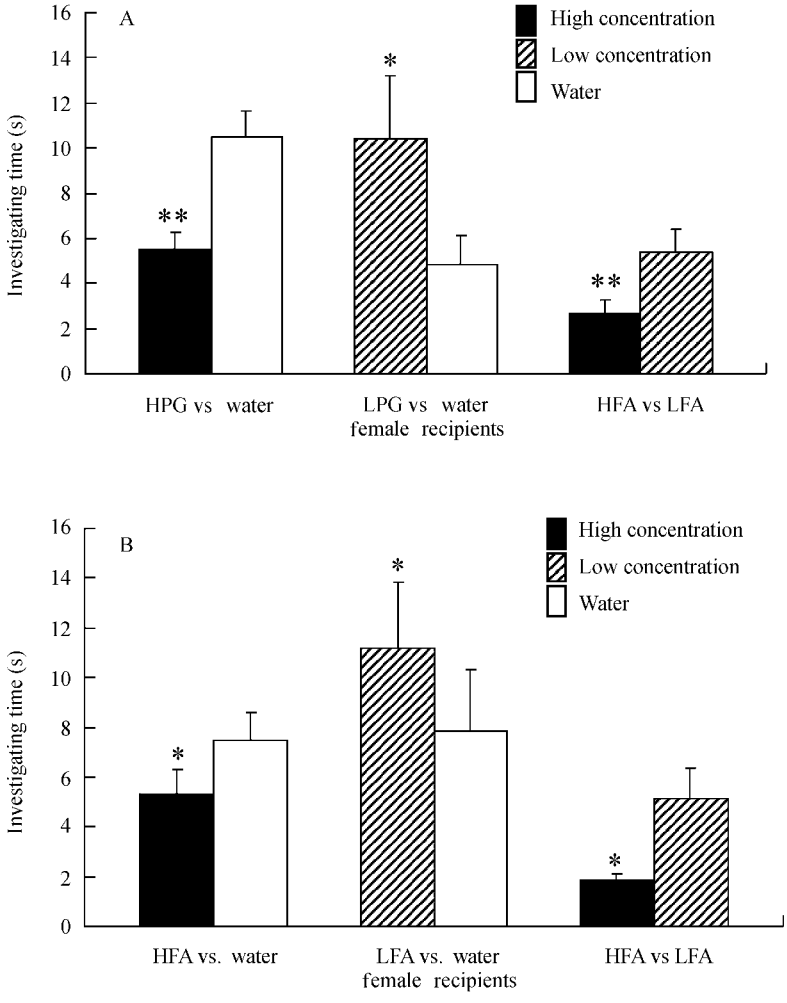

Fig.2 Responses of females to (A) male's preputial gland secretion and (B) *E-E*-farnesyl acetate in Brandt's voles *Lasiopodomys brandtii*

Paired treatments were significant at $^{**}P < 0.01$ and $^{*}P < 0.05$, by using the Wilcoxon matched-pairs, signed-rank test. HPG and LPG referred to high- and low-concentration of preputial gland secretion, respectively; HFA and LFA to high- and low-concentration of *E*, *E*-farnesyl acetate.

quantity in the PGS of house mice (Zhang et al., 2007).

Inter-individual dissimilarities and intra-individual similarities of chemical composition in rodent scents raise the possibilities for these odors to signal individuality (Sun and Müller-Schwarze, 1998a, b; Zhang et al., 2003, 2005). Our results (and specifically the *RSDs*) also showed such inter-individual dissimilarities and intra-individual similarities, suggesting that the PGS might function in inter-individual communication in *L. brandtii*, which accords with the results from the PGS of house mice (Zhang et al., 2007). Since the castration-suppressed compounds also exhibited inter-individual dissimilarities, it was likely for them to serve individual recognition in addition to sex recognition. Thus, one volatile compound might have multiple functions in chemical communication.

Our behavioral data indicate that attractiveness of an extract of the PGS or synthetic FA to females was concentration-dependent. Namely, females' investigatory activities were encouraged by low a concentration of PGS or FA, but discouraged by a high concentration. A recent report also revealed the existence of concentration-dependent pheromones in rabbits, where milk-derived 2-methylbut-2-enal attracted pups through an "effective stimulus intensity" ranging from 0.0025 to 25 parts per million (Coureaud et al., 2004). However, in house mice, two preputial glandular pheromones, *E*-β-farnesene and *E*, *E*-α-farnesene, seem not to be dosage-dependent and attractive to sexually experienced females at concentrations ranging from thousands to several ppm, whereas the natural concentration in voided urine with preputial gland excretion is only several parts per million (Brinck and Hoffmeyer, 1984; Harvey et al., 1989; Novotny et al., 1990, 1999; Jemiolo et al., 1991). Our study is the first to document that the aversion and attraction of female *L. brandtii* might be reversed by differences in concentrations of sex pheromones, for either

a PGS extract or a single primary component. In addition, we found that a high concentration of the PGS extract also caused adults and pups to avoid the scented rod, but a high concentration of FA did not elicit this behavior (Unpublished data). Our speculation that a high concentration of PGS and combination of multiple components might convey alarm cues in *L. brandtii* requires further study.

E, *E*-Farnesyl acetate and the nine putative pheromones identified in *L. brandtii* have not been reported before as chemosignals in mammals. Of all of the compounds detected in the PGS extract from male *L. brandtii*, only *E*-β-farnesene and 16C: Ac (the early minor part of peak 22, which has been recently separated by using a polar column) were previously described as respective pheromones in house mice and bank voles *Clethrionomys glareous*, respectively (Brinck and Hoffmeyer, 1984; Welsh et al., 1988; Boyer et al., 1989; Natynczuk et al., 1995; Zhang et al., 2007). However, these compounds were widely used as pheromonal components in insects (Byers, 2002; El-Sayed, 2005; Roleofs et al., 1995). For example, *E*, *E*-farnesyl acetate is a component of chemosignals in some bee species such as male Scandinavian bumblebees *Bombus pratorum* (Bergman and Bergström, 1997), the rock honeybee *Apis dorsata* (Blum et al., 2000), and the stingless bee *Melipona beecheii* (Cruz-López et al., 2005). Farnesene, a fairly common natural product, has been known to act as an alarm pheromone in aphids (Bowers et al., 1972; Gibson and Pickett, 1983). The straight-chain acetates have been also described as insect pheromone components (Roelofs, 1995; Byers, 2002; El-Sayed, 2005). For example, other than 9-10C: Ac, the acetates found in *L. brandtii* all were reported previously as the pheromones of various species of Lepidoptera with special reference to *Z*9-14C: Ac and *Z*11-16C: Ac, the first and third most common attractants for Lepidoptera (Byers, 2002; Kelly, 1996). *Z*7-12C: Ac, which is structurally-similar to *E* (*Z*) 9-12C: Ac present in *L. brandtii* is the fifth most common lepidopteran attractant, and also has been shown to act as a female pheromone attractive to male African elephants *Loxodonta africana* (Rasmussen et al., 1996). Here, we provide further strong evidence for the convergence of insect and mammal pheromonal compounds.

The idea of digital and analog coding to distinguish the two forms of information coding (presence/absence of chemicals versus varying amounts of shared chemicals) was previously elucidated in the studies of chemical information coding for family membership, relatedness, sexes, and individuals (Sun and Müller-Schwarze, 1998a, b; Zhang et al., 2003). Here, all detected compounds almost always existed in the voles, whether intact or castrated. This suggests that analog coding might be the key to discriminating individuals and sexes in the voles (rather than digital coding). The absence of castration-eliminated compounds might be circumstantial evidence for the existence of dosage-dependent chemosignals associated with sex recognition in the voles like in the PGS of house mice as described recently in mice by Zhang et al. (2007).

In conclusion, our results support our primary hypothesis that volatile components of preputial glandular secretions could release chemical signals coding for sexual recognition and individuality in *L. brandtii*. A large number of behavioral assays will be necessary to sufficiently and definitively decipher the codes with *L. brandtii* in the future.

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