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Flank gland-secreted putative chemosignals pertaining to photoperiod, endocrine states, and sociosexual behavior in golden hamsters

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Abstract Behavioral studies have shown that flank glands are involved in chemical communication in golden hamsters *Mesocricetus auratus* but little chemical analysis has been conducted on volatiles arising from these glands. Using gas chromatography-mass spectrometry, we detected compounds from the flank glands of males, only eight of which were also produced in females. Based on these chemical data we performed a number of further experiments. By manipulating light we found that males exposed to short-photoperiods developed smaller flank glands than those exposed to long-photoperiods. Six flank gland volatiles reduced in relative abundance, which possibly coded for reproductive status of males of this seasonally breeding hamster species. Through dyadic encounters, we were able to induce the formation of dominant-subordinate relationships and show that two glandular compounds became high in relative abundance and may function as dominance pheromones. Castration eliminated all male-specific compounds resulting from flank glands, but bilateral ovariectomies only affected one compound in females. Once these ovariectomized females were treated with testosterone, their glandular compounds resembled those of males, suggesting these compounds are under the main control of androgen. Two female putative pheromones, tetradecanoic acid and hexadecanoic acid, were used in binary choice tests and were both found to attract males over females. Applying a solution of these pheromone compounds to adult males also suppressed their agonistic behavior [*Current Zoology* 56 (6): 800–812, 2010].

Key words Dominance, Flank gland, Gonadectomy, Photoperiod, Putative pheromone, Testosterone

How social information is coded by pheromones is traditionally answered using behavioral and chemical methods (Sun and Müller-Schwarze, 1998a, b). Many behavioral tests have confirmed the role of specialized skin glands in chemical communication in mammals, but corresponding and complimentary chemical studies are seldom attempted. Deciphering the nature of pheromones utilized by mammals requires a greater focus on chemistry and associated analyses (Wyatt, 2003; Brennan and Zufall, 2006; Müller-Schwarze, 2006). Odor-borne information can be coded by unique compounds (qualitative difference) and varying amounts of shared compounds (quantitative difference) within the odor (Sun and Müller-Schwarze, 1998a, b; Singer et al., 1997; Zhang et al., 2007a, 2008a, c). Specialized scent glands are rich sources of pheromones and the site

of most interest to researchers examining pheromonal components in mammals (Wyatt, 2003). For example, it has been shown that the preputial glands of mice and rats are sources of pheromones and secrete information-coding volatiles that vary in quality or quantity with gender, reproductive condition, and between individuals (Zhang et al., 2007a, 2008a, c). The volatile constituents of preputial glands and urine in rats and mice show that either sex-specific compounds (e.g. *S*-2-*sec*-butyl-dihydrothiazole in male mouse urine) or compounds that are found in higher relative concentrations in males (e.g. hexadecyl acetate in mice, squalene in rats) can be male pheromone components involved in the attraction of females (Jemiolo et al., 1986, 1991; Harvey et al., 1989; Zhang et al., 2007a; 2008a, c). However, most scent constituents of rodents are

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sex-common rather than sex-specific (Novotny et al., 1986, 1999a; Harvey et al., 1989; Singer et al., 1997; Zhang et al., 2007, 2008a, b).

In golden hamsters *Mesocricetus auratus*, the function and mechanism of the flank gland in chemical communication has been extensively investigated for more than thirty years. Age, estrous cycle, social rank and photoperiod affect endocrine status and can change morphology, histology and sexual attractiveness of flank glands secretions in golden hamsters (Drickamer et al., 1973; Vandenberg, 1973; Ebling, 1977; Campbell and Church, 1978; Caldwell et al., 2008). Some constituents of preputial glands in mice and rats vary with endocrine states, estrous cycles and social rank (Harvey et al., 1989; Novotny et al., 1990; Liu et al., 2008; Pohorecky et al., 2008; Zhang et al., 2008a, c). Behavioral tests have revealed that flank gland secretions contain rich olfactory information regarding gender, social dominance, reproductive status and individuality (Johnston, 1980, 1985; Huck et al., 1985; Johnston et al., 1993; Novotny et al., 1999b; Johnston and Bullock, 2001; Novotny, 2003; Ballard and Wood, 2007). These hamsters are capable of rubbing their flank glands against objects in their environment, a behavior referred to as “flank marking”, creating scent signatures to advertising social and physiological status. Flank marking is a component of an agonistic response in golden hamsters and is most commonly performed by the winner of a fight (Johnston, 1975, 1985; Ferris et al., 1987). Flank glands are more fully developed in males than females and are dependent on androgen originating from testis and independent of estradiol originating from ovaries, suggesting that flank glands and associated volatiles play a role in females in the assessment of mate quality apart from gender discrimination (Vandenberg, 1973; Brown, 1985; Johnston, 1985; Zhang et al., 2008b).

Chemical investigation of golden hamster odor has focused mostly on vaginal secretions and not on secretions of the flank gland, resulting in the discovery of several putative pheromones such as dimethyl disulfide and aphrodisin (Singer et al., 1977, 1984; Clancy et al., 1984; Singer and Macrides, 1990; Petrulis and Johnston, 1995; Briand et al., 2004). In flank glands, *E5*-dodec-2-one is male-specific, testosterone-dependent and attractive to females. Tetradecanoic and hexadecanoic acids shared by males and females have higher ratios in females than in males and are viewed as putative female pheromones accordingly (Novotny, 2003; Zhang et al., 2008b; W. Ma et al., unpublished data). To date, female glandular compounds associated with coding for olfac-

tory information have neither undergone chemical confirmation nor behavioral validation.

A combined approach using chemical and behavioral analyses has proven successful in the identification of several important pheromones produced by the preputial glands of mice and rats (Jemiolo et al., 1991; Singer et al., 1997; Zhang et al., 2007a, 2008a, b). Here, we conducted chemical analyses using gas chromatography-mass spectrometry (GC-MS) and then related qualitative and/or quantitative differences in especially volatile compounds to corresponding behavioral and physiological characteristics in the golden hamster (Singer et al., 1997; Sun and Müller-Schwarze, 1998a, b; Zhang et al., 2007a, 2008a, c). Furthermore, to verify activity of the two putative female pheromones tetradecanoic acid and hexadecanoic acid (Zhang et al., 2008b), we tested how they affected male and female behavior through behavioral choice tests and agonistic encounters.

1 Materials and Methods

1.1 Experimental animals

Seventy six sex-naïve male and 28 female golden hamsters at eight weeks of age were purchased from Weitong-Lihua Laboratory Animal Company, Beijing, China. They were kept in individual plastic cages (50 cm × 36 cm × 20 cm) in a reversed light:dark regime of 16L:8D (lights on at 17:00 h) (referred herein as normal or long photoperiod-exposed subjects) at 22±2°C for two weeks prior to experimentation. Experiments were conducted at the Institute of Zoology, Chinese Academy of Sciences. Food and water were supplied ad libitum. Wood shavings were used as bedding material and changed once every two weeks. Females with perforated vaginas and scrotal males were classified as potential breeders and used in our experiments.

1.2 Experiment group 1: Chemical analysis of putative pheromone components

The effect of long- and short-photoperiod exposure on flank gland secretions Eight males and eight females housed under 16L:8D (long photoperiod-exposed, also referred to as intact hamsters) for four weeks were randomly selected as flank gland donors. An additional eight males were selected at random from the colony and assigned to a separate room in a reversed light: dark regime of 8L:16D (short photoperiod, lights on at 17:00 h) for four weeks as flank gland donors.

The effect of gonad removal and testosterone treatment (females only) on flank gland secretions Eight males and 16 females were anesthetized using 50 mg/kg

sodium pentobarbital. Male castration was performed through bilateral incisions in the scrotum. Ovariectomy of the females was performed through bilateral incisions in the dorsal area of waist. Testosterone capsules were constructed from 15 mm of silastic tubing (o.d. 2.70 mm, i.d. 2.26 mm, China Medical, Shanghai, China), and packed with 10-mm lengths of crystalline testosterone (Sigma, St. Louis, USA) and sealed with 2.5-mm lengths of Medical Adhesive Silicone Type A (Dow Corning, Midland, USA) at both ends. The capsules were implanted subcutaneously in the dorsal area of the waist of eight of the ovariectomized females via a small incision at the time of ovariectomy. The wound was closed with sterile sutures and treated with 5% tincture of iodine. These methods have been used previously in ratlike hamsters *Tscheskia triton* that have a similar body length and shape to golden hamsters (Zhang et al. 2001).

The effect of dominance-subordination on male flank gland secretions Sixteen breeding adult males were utilized in the social encounter for establishing dominance-subordination. Two males of similar body weight (within 10% difference) were randomly assigned to a pair. All staged dyadic encounters were conducted under dim illumination between 09:00 and 11:00 h of the dark phase over 21 consecutive days. Paired encounters took place in a clear plastic cage with an arena measuring 37 cm × 26 cm × 17 cm and the arena was thoroughly cleaned between trials with both water and 75% ethanol.

For this experiment both paired males were simultaneously removed from their cages to the arena. The frequencies of aggressive behavior and flank marking were continuously recorded for 20 min by hand using a pre-defined data sheet containing a calibrated time scale of 10 s units. Behaviors lasting 10 s or less were allocated one unit, if the duration was greater than 10 s but less than 20 s the act was allocated across two units, and so on (Zhang et al., 2001, 2003). Time was kept using a stopwatch. Recorded agonistic behaviors included attack (pushing with mouth and forepaws, chasing, and sideways postures); defense (fleeing, laying on back and upright postures); and flank marking (Johnston, 1985; Zhang et al., 2001, 2003).

Males from each pair were identified as winner or loser by quantitatively comparing their attack-scores across daily encounters. The individual with the higher attack-score was considered the winner. After some time, the male in each pair displaying more wins than losses was defined as dominant and its opponent as subordinate (Zhang et al., 2001, 2003;

Wang et al., 2006).

To collect the flank glands, we decapitated the donors first and then shaved the top of the fur on the flank glands, and cut off the glands along their outlines on the inner skin and recorded their mass (± 0.1 mg) as previously described by Zhang et al. 2001 and 2003. Flank glands were frozen at -20 °C immediately after weighing for further chemical analyses. Relative flank gland weights are provided as mg of organ / 100 g body weight.

Chemical analyses We used dichloromethane to extract flank gland tissue. First we thawed each pair of flank glands at room temperature, then added dichloromethane into the vial containing the glands according to the ratio 1 mg flank gland material: 5 μ l dichloromethane. 24 hours later, we removed the flank glands, sealed and stored the remaining solution at -20 °C until GC-MS assay within one week. Analytical GC-MS was performed on an Agilent Technologies Network 6890N GC system coupled with 5973 Mass Selective Detector with the library NIST 2002. Xcalibur (Windows XP, Microsoft, USA) was used for data acquisition and processing. The GC was equipped with a DB5WAX glass capillary column (30 m long, i.d. 0.25 mm × 0.25 μ m film). The carrier gas was helium (1.0 ml/min). The injector was at 260 °C. The oven temperature was initially at 100 °C, heated by 5 °C/min to 250 °C. Post-run lasted for 10 min at 250 °C to clean the column. Electron impact ionization was used at 70 eV. Transfer line temperature was 280 °C. Scanning mass ranged from 30 to 350 amu. The amount of sample injected was 1 μ l every time in a split mode (10:1).

Tentative identification was undertaken by matching the mass spectra of GC peaks with those in the MS library (NIST2002) and literature (Novotny, 2003; Zhang et al., 2008b). In particular, the six compounds identified were verified by known standard compounds (saturated acids, viz. compounds 12, 18 and 25 in Table 1) and/or corresponding methyl esters after methylation of samples (unsaturated acids, viz. compounds 19, 26 and 30 in Table 1). The major male-specific compound 1 in Table 1 was identified using the NIST2002 library and literature (Novotny, 2003; Zhang et al., 2008b; W. Ma et al., unpublished data). Finally, we converted the peak area of a particular compound into a percentage (relative abundance) of the summed peak areas of all GC peaks to quantify the relative abundance of relevant compounds.

To determine variability in volatile composition among individuals, we calculated the relative standard

deviation (RSD) using the following formula: $RSD = (SD/mean) \times 100$, where mean and SD are the average of each volatile peak area (percent) for all intact males

and their standard deviation, respectively (Zhang et al., 2007a). The RSD was then compared with the intrasample variation.

Table 1 Comparison of relative abundance of 32 flank gland compounds among LP, SP, dominant and subordinate males and TO females of golden hamsters (mean±SD)

GC peak NO.	Compounds	Percent GC area				
		LP male (n=8)	SP male (n=8)	Dominant male (n=8)	Subordinate male (n=8)	TO females (n=8)
1	E5-dodecen-2-one*	7.19±1.89 ^a	4.77±3.17 ^a	5.09±2.31	5.13±2.56	5.96±3.48
2	5-Isopropyl-2(5H)-furanone	0.53±0.24 ^a	0.35±0.31 ^a	0.38±0.20	0.37±0.18	0.56±0.33
3	Decanoic acid	0.16±0.03	0.15±0.01	0.17±0.04 ^b	0.14±0.04 ^b	0.16±0.06
4	Undecanoic acid	0.22±0.09 ^a	0.14±0.05 ^a	0.19±0.06	0.14±0.04	0.36±0.23
5	10-Undecenoic acid	0.85±0.24	0.67±0.29	0.75±0.34	0.73±0.23	0.92±0.39
6	Dodecanoic acid	0.31±0.09	0.25±0.03	0.28±0.03	0.29±0.04	0.37±0.14
7	Z6-Pentadecen-1-ol	0.19±0.10	0.13±0.08	0.17±0.10	0.15±0.08	0.17±0.11
8	Tridecanoic acid	0.33±0.14 ^d	0.22±0.18	0.20±0.07 ^d	0.23±0.10	0.28±0.16
9	1-Octadecanol	0.68±0.72	0.69±0.48	0.68±0.46	0.69±0.46	0.76±0.73
10	E9-Octadecen-1-ol	0.43±0.28	0.69±0.48	0.33±0.26	0.33±0.28	0.28±0.27
11	E2-Tridecenoic acid	0.30±0.16	0.23±0.14	0.23±0.14	0.25±0.14	0.22±0.14
12	Tetradecanoic acid*	2.97±0.29 ^{c,e}	2.93±0.24	2.87±0.38	2.74±0.19 ^c	3.80±0.77 ^c
13	Z7-Tetradecenoic acid	0.51±0.10 ^a	0.40±0.14 ^a	0.49±0.05	0.45±0.14	0.53±0.19
14	E9-Tetradecenoic acid	0.52±0.08	0.61±0.10	0.53±0.06	0.54±0.15	0.65±0.21
15	A pentadecanoic acid	0.96±0.47 ^a	0.64±0.19 ^a	0.58±0.17	0.67±0.26	1.09±0.48
16	A pentadecanoic acid	0.65±0.12	0.65±0.15	0.66±0.09	0.66±0.19	0.78±0.22
17	Z14-Pentadecenoic acid	6.33±1.88	5.04±2.28	5.43±1.31	5.19±1.82	7.40±2.66
18	Hexadecanoic acid*	15.47±1.56 ^{a,e}	17.66±1.95 ^a	16.73±1.81 ^b	17.81±1.79 ^{b,e}	17.50±2.15
19	Z9-Hexadecenoic acid*	13.35±2.22	15.11±2.15	13.69±1.56	13.59±2.30	14.17±2.60
20	Hexadecane-1,2-diol	2.39±1.70	1.80±1.01	1.42±0.84	1.71±1.32	2.44±1.93
21	Heptadecanoic acid (branched?)	0.48±0.27 ^d	0.37±0.11	0.29±0.09 ^d	0.38±0.20	0.70±0.26
22	A hexadecanediol	0.67±0.67	0.41±0.16	0.29±0.17	0.43±0.52	0.94±0.90
23	Heptadecanoic acid	0.34±0.12	0.29±0.08	0.28±0.08	0.26±0.14	0.44±0.16
24	A heptadecenoic acid	2.07±0.59 ^{a,d,e}	1.36±0.55 ^a	1.66±0.27 ^{b,d}	1.34±0.31 ^{b,e}	1.67±0.41
25	Octadecanoic acid*	4.52±1.45	3.88±0.36	3.80±1.06	3.76±0.70	5.08±0.81
26	Z9-Octadecenoic acid*	19.81±2.69 ^{d,e}	22.22±2.81	22.62±2.17 ^d	22.50±2.56 ^c	17.68±4.25
27	Z11-Octadecenoic acid	1.60±0.51	1.44±0.23	2.11±1.19	1.67±0.23	2.01±0.44
28	A nonadecanediol	0.63±0.67	0.41±0.34	0.45±0.50	0.16±0.08	0.34±0.41
29	Nonadecanoic acid	0.23±0.16	0.18±0.06	0.16±0.08	0.22±0.11	0.20±0.12
30	Z9,Z12-Octadecadienoic acid*	12.76±2.74 ^d	14.20±2.37	15.50±3.42 ^d	15.05±2.52	10.83±4.36
31	A octadecatrienoic acid	0.72±0.29 ^c	0.84±0.09	0.83±0.17	0.81±0.14	0.45±0.19 ^c
32	Eicosanoic acid	1.83±0.61 ^{c,d,e}	1.64±0.70	1.19±0.54 ^d	1.22±0.76 ^c	1.24±0.59 ^c

Note: The means in a row marked by the same superscript letters (a–e) are significantly different at the 0.05 level. LP=long photoperiod; SP=short photoperiod; TO=testosterone-treated ovariectomized. The compounds marked by asterisks are definitively identified.

1.3 Experiment group 2: Behavioral validation of putative female pheromones

Quantitative measurement of tetradecanoic and hexadecanoic acids in flank glands We determined the amounts of tetradecanoic and hexadecanoic acids in dichloromethane extracted from flank glands by comparing their GC areas in the samples with the established standard curve of GC area versus concentration. Likewise, the concentration of tetradecanoic acid (Myristic acid, hereafter as MA) (2 ppm) and hexadecanoic acids (Palmitic acid, hereafter PA) (5 ppm) dissolved in water were determined by directly injecting 0.5 μ l of the aqueous samples into the GC-MS and comparing their GC peak areas with the standard curve.

Behavioral two choice tests of male and female attraction to MA and PA We used respective 2 μ l dichloromethane extracts from male and female flank gland tissue to evenly paint the tip (approximately 12 mm²) of two glass rods (length: 20 cm, diameter: 4 mm) and vaporized the solvent for 2 min (Lai et al. 1996; Zhang et al. 2008a, c). Similarly, we used the same method to test the preferences of male and female hamsters between the mixture [MA (100 ppm) and PA (200 ppm)] dissolved in dichloromethane and dichloromethane itself. We used one glass capillary (length: 10 cm, inner diameter: 1.8 mm) to absorb 10 μ l MA (2 ppm) and/or PA (5 ppm) dissolved in distilled water in comparison with the other glass capillary containing equal amount of plain water (Zhang et al., 2008c). The other end of the glass rod or capillary was held by the tester (blind to the sample). Two samples were simultaneously presented to the subject for 3 min after its first sniff or lick. We measured the time that one hamster spent sniffing and licking each tip. Hamsters showing no responses to either rod or capillary over the 3 min test period were excluded for the day. Intact hamsters (housed in long photoperiod and no surgery) of the same sex were randomly assigned into two groups everyday and used to test their responses to different odor substances. One hamster was used at random only once a day following Zhang et al. 2008a and 2008c.

The effect of female putative pheromones on male agonistic behavior The effects of pheromonal analogs on agonistic behavior in staged dyadic encounters were used to further assess pheromonal activity. We placed two subjects of similar body mass ($\pm 10\%$) in two respective plastic cages (32 cm \times 21.5 cm \times 17 cm) and sprayed 2 ml aqueous solution or water (the control) onto their bodies before placing them in the arena for a

10 min encounter. To test the behavioral effect of MA (200 ng) and PA (400 ng) during dyadic encounters of intact hamsters, two hamsters of the same sex and of similar body mass were randomly assigned to a pair once each day across four consecutive days. Then half were sprayed with water and the other with aqueous solution of either MA or PA.

Statistical tests We first explored data normality. For paired samples, we used a Wilcoxon signed-rank tests for non-normal data and paired t-tests for normal data. For independent samples, we used a Mann-Whitney test for non-normal data and independent t-test for normal data. P was set at < 0.05 and SPSS (v13.0) was used for all analyses (SPSS Inc., Chicago, USA).

Ethical Note Animal care and usage was approved by the Animal Use Committee of the Institute of Zoology, Chinese Academy of Sciences. Our protocol (no. 2006-001) required that if an animal was injured during staged dyadic encounter, it would be removed from the study and received immediate medical care; however, no animals were visibly or obviously injured during encounters in our study.

2 Results

2.1 Experiment group 1: Flank gland-secreted volatiles covarying with physiological and social status

For long-photoperiod animals, we found that flank glands were larger in males than females (Fig. 1A) and the GC-MS assay unveiled a greater number of compounds (δ : 32 compounds, ♀ : 8) (Table 1 and 2, Fig. 2). Of these, compounds 12, 18, 19, 20, 25, 26, 27 and 30 were found in the flank glands of both sexes and remaining compounds were unique to males (Table 3). Of the shared compounds, compounds 12 and 18 were higher in females than males and compounds 19 and 20 were higher in males than females (Table 2).

Flank glands were significantly heavier in long-photoperiod males than in short-photoperiod males (Fig. 1A). The exact same 32 compounds were detected in flank glands from both groups of males, however the relative abundance of compounds 1, 2, 4, 13, 15 and 24 were higher and that of compound 18 lower in long-photoperiod males (Table 1).

Flank gland sizes did not differ between dominant and subordinate males (Fig. 1A) and the same number of compounds (32) was detected. However, compounds 3 and 24 were of a higher relative abundance and compound 18 of lower relative abundance in dominant males compared to subordinate males (Table 1). Com-

pared to normal males, a few compounds detected in the flank glands of subordinate and dominant males differed in relative abundance (see Table 1 for a compound by compound analysis).

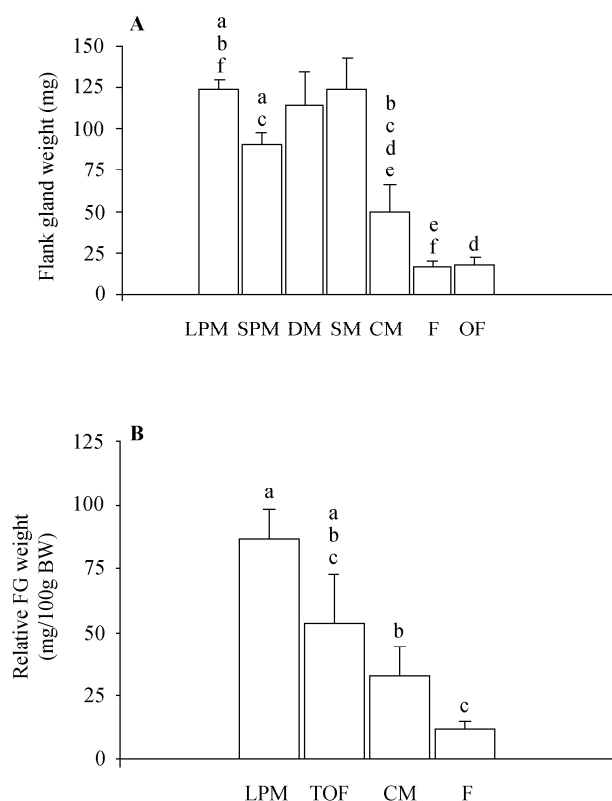


Fig. 1 Comparison of flank gland weights (Panel A) and relative weights (Panel B) in golden hamsters subjected to different treatments

Data is expressed as mean \pm SD. Bars with the same letters are significant at 0.05. LPM=long-photoperiod-exposed males; SPM=short-photoperiod-exposed males; DM=dominant males; SM=subordinate males; CM=castrated males; F=females; OF=ovariectomized females; TOF=testosterone-treated OF.

In males, castration led to the development of smaller flank glands compared to all other classes of male (Fig. 1A). However, whether or not an ovariectomy was performed on females did not affect the size of the flank gland (Fig. 1A). As shown in Figure 3 and Table 2, of the glandular compounds shared by both sexes compounds 12 and 18 in females and compounds 19 and 20 in males were present at a higher relative abundance than those in opposite-sex conspecifics. Castration not only eliminated all male-specific compounds, but also quantitatively affected the compounds common to both sexes in males: compounds 12, 19 and 20 were reduced but compounds 18, 26 and 30 were enhanced in castrated males as compared to normal males. However, in females, ovariectomy only increased the relative abundance of compound 20 (Table 2 and Fig. 2). Differences in the kinds of glandular compounds were not present between castrated males and either normal or ovariectomized females. However, relative to castrated males, compounds 12 and 25 were increased and compounds 19, 26 and 30 were reduced in both normal females and ovariectomized females; compound 20 and 27 were only increased in ovariectomized females (Table 2 and Fig. 2).

The relative weight of the flank glands in testosterone-treated and spayed females was higher than castrated males and normal females, but lower than those of normal males (Fig. 1B). Administering overdose testosterone to spayed females made their flank glands secrete all compounds unique to males in addition to those shared by both sexes. According to the GC percentage of each compound, most compounds were found to be quantitatively equal between testosterone-

Table 2 Comparison of relative abundance of the eight flank gland compounds shared by both sexes among golden hamsters subjected to different treatments (mean \pm SD)

GC peak NO.	Compounds	Percentages of GC area				
		LP (intact) male (n=8)	LP (intact) female (n=8)	Castrated males (n=8)	Ovariectomized females (n=8)	TO females (n=8)
12	Tetradecanoic acid*	4.09 \pm 0.54 ^{a,b,f}	7.58 \pm 2.66 ^{a,c,e}	1.74 \pm 0.29 ^{b,c,g}	6.98 \pm 2.81 ^g	5.21 \pm 1.07 ^{e,f}
18	Hexadecanoic acid*	21.21 \pm 1.31 ^{a,b,f}	29.89 \pm 4.21 ^{a,c}	26.92 \pm 2.53 ^b	28.70 \pm 1.48	23.83 \pm 1.80 ^{c,f}
19	Z9-Hexadecenoic acid*	18.39 \pm 3.35 ^{a,b}	8.47 \pm 1.52 ^{a,c,c}	10.95 \pm 1.91 ^{b,c,g}	8.26 \pm 0.80 ^g	19.55 \pm 4.52 ^c
20	Hexadecane-1,2-diol	3.31 \pm 2.47 ^{a,b}	1.33 \pm 0.37 ^{a,d,e}	0.83 \pm 0.29 ^{b,g}	2.26 \pm 1.05 ^{d,g}	3.50 \pm 2.94 ^e
25	Octadecanoic acid*	6.25 \pm 2.12	7.30 \pm 1.54 ^c	5.53 \pm 0.67 ^{c,g}	7.16 \pm 1.07 ^g	6.95 \pm 1.07
26	Z9-Octadecenoic acid*	27.13 \pm 2.43 ^{b,f}	25.44 \pm 2.59 ^c	30.38 \pm 2.19 ^{b,c,g}	26.02 \pm 3.15 ^e	23.78 \pm 3.04 ^f
27	Z11-Octadecenoic acid	2.21 \pm 0.77	2.10 \pm 0.59	1.81 \pm 0.47 ^g	2.45 \pm 0.57 ^g	2.81 \pm 0.80
30	Z9,Z12-Octadecadienoic acid*	17.41 \pm 2.98 ^b	17.89 \pm 2.80 ^c	21.84 \pm 2.31 ^{b,c,g}	18.16 \pm 2.04 ^g	14.39 \pm 4.03

Note: The means in a row marked by the same superscript letters (a–g) are significant different at the 0.05 level. LP=long photoperiod; TO=testosterone-treated ovariectomized. The compounds marked by asterisks are definitively identified.

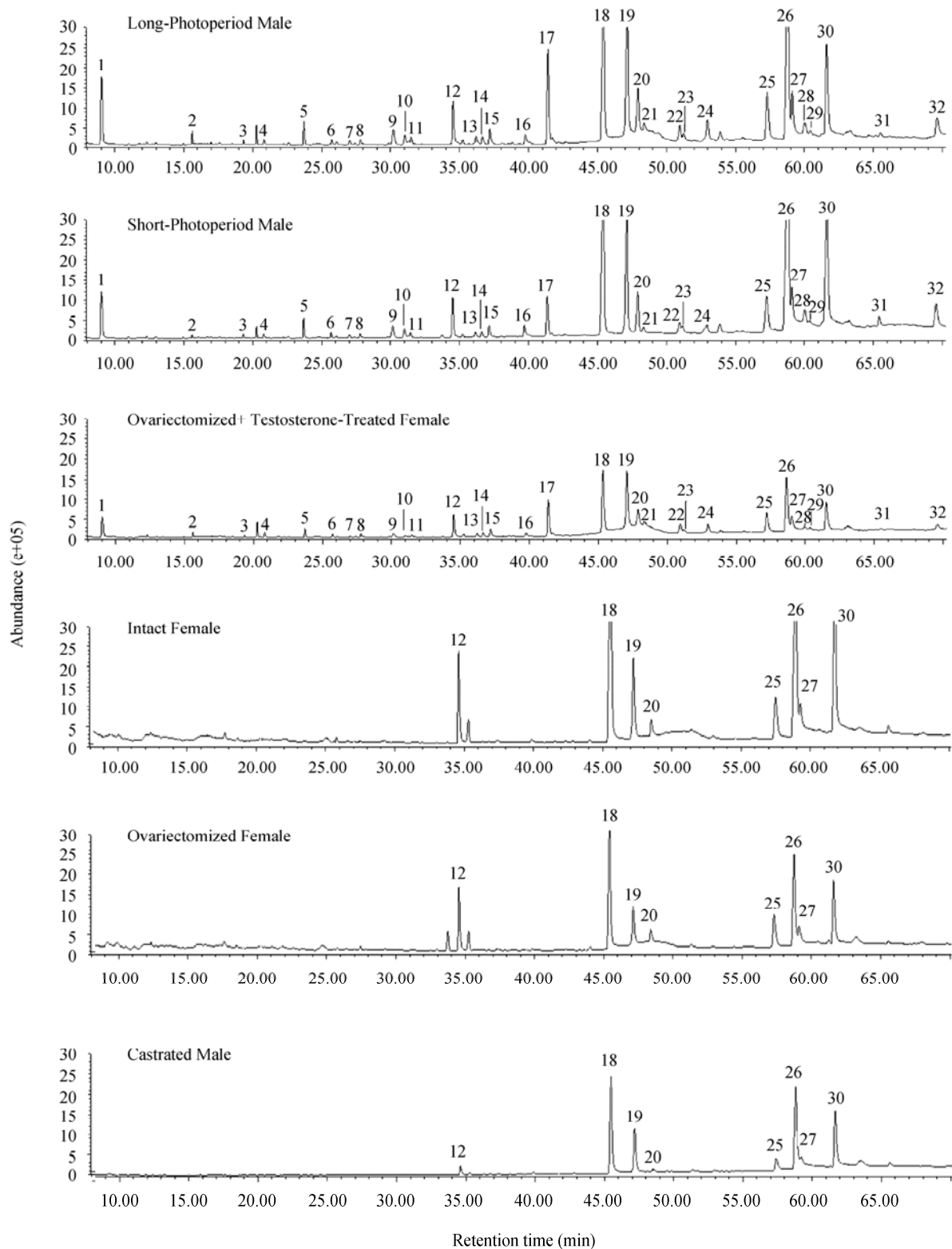


Fig. 2 Representative gas chromatogram of volatile compounds from the flank glands of long-photoperiod-exposed males (top panel), short-photoperiod-exposed males (second panel) and Testosterone-treated ovariectomized females (third panel), intact females (fourth panel), ovariectomized females (fifth panel), and castrated male (bottom panel) golden hamsters. The numbers of GC peaks correspond with peak numbers in Table 1 and 2.

treated spayed females and normal males but the relative abundance of a number of compounds differed between these groups (Table 1 and Fig. 2).

All volatile compounds from the flanks glands of either males or females showed higher interindividual RSDs than intraindividual RSDs (Table 3).

Table 3 Individual variation in relative abundance of flank gland constituents among individuals and within individuals in golden hamsters (mean±SD)

GC peak NO.	Compounds	32 GC peaks all present in males			8 GC peaks shared by both sexes		
		LP males	Intra-individuals (Six replicates of a LP male)		LP Females	Intra-individuals (six replicates of a LP female)	
		RSD	RSD	Relative abundance	RSD	RSD	Relative abundance
1	<i>E5</i> -dodecen-2-one*	26.29	11.72	5.80±0.68			
2	5-Isopropyl-2(5H)-furanone	45.28	16.00	0.50±0.08			
3	Decanoic acid	18.75	5.88	0.17±0.01			
4	Undecanoic acid	40.36	12.50	0.24±0.03			
5	10-Undecenoic acid	28.24	5.22	1.15±0.06			
6	Dodecanoic acid	29.03	7.14	0.28±0.02			
7	<i>Z6</i> -Pentadecen-1-ol	52.63	6.90	0.29±0.02			
8	Tridecanoic acid	42.42	11.11	0.27±0.03			
9	1-Octadecanol	105.88	12.93	1.47±0.19			
10	<i>E9</i> -Octadecen-1-ol	65.12	22.41	0.58±0.13			
11	<i>E2</i> -Tridecenoic acid	53.33	4.65	0.43±0.02			
12	Tetradecanoic acid*	9.76	3.27	3.06±0.10	35.09	2.97	4.38±0.13
13	<i>Z7</i> -Tetradecenoic acid	19.61	12.70	0.63±0.08			
14	<i>E9</i> -Tetradecenoic acid	15.38	4.62	0.65±0.03			
15	A pentadecanoic acid	48.96	7.14	1.12±0.08			
16	A pentadecanoic acid	18.46	4.35	0.69±0.03			
17	14-Pentadecenoic acid	29.70	10.84	8.12±0.88			
18	Hexadecanoic acid*	10.08	1.69	15.34±0.26	14.08	1.09	21.93±0.24
19	<i>Z9</i> -Hexadecenoic acid*	16.63	10.68	13.48±1.44	17.95	10.23	19.26±1.97
20	Hexadecane-1,2-diol	71.13	16.37	3.36±0.55	27.82	16.67	4.80±0.80
21	Heptadecanoic acid (branched?)	56.25	7.55	0.53±0.04			
22	A hexadecanediol	100.00	60.27	0.73±0.44			
23	Heptadecanoic acid	35.29	26.09	0.46±0.12			
24	A heptadecenoic acid	28.50	11.01	2.18±0.24			
25	Octadecanoic acid*	32.08	4.84	5.58±0.27	21.10	5.14	7.98±0.41
26	<i>Z9</i> -Octadecenoic acid*	13.58	2.34	17.49±0.41	10.18	2.76	25.00±0.69
27	<i>Z11</i> -Octadecenoic acid	26.29	11.72	2.43±0.13	28.10	5.17	3.48±0.18
28	A nonadecanediol	45.28	16.00	0.92±0.38			
29	Nonadecanoic acid	18.75	5.88	0.27±0.04			
30	<i>Z9,Z12</i> -Octadecadienoic acid*	40.36	12.50	9.22±0.45	15.65	5.16	13.18±0.68
31	A octadecatrienoic acid	28.24	5.22	0.40±0.08			
32	Eicosanoic acid	29.03	7.14	2.16±0.33			
	Mean±SD	41.11±26.46	12.56±11.88		21.25±8.433	6.140±5.034	
	Significance	Z=4.937, P=0.000			Z=2.521, P=0.012		

Note: RSD refers to relative standard deviation, which was calculated using the formula $RSD = (SD/mean) \times 100$, where mean and SD are the average of each compound peak area (in percentage) and their standard deviation, respectively. The compounds marked by asterisks are definitively identified.

2.2 Experiment group 2: Behavioral effects of the putative female pheromones

Quantitative analysis showed that MA and PA in one microlitre of the extract (equivalent to 0.2 mg of flank gland tissue) roughly attained respective 40 ng and 100 ng in males and 100 ng and 200 ng in females.

Through binary choice tests we found that male hamsters spent more time investigating the crude dichloromethane extract of the flank glands (1 mg tissue in 5 μ l solvent) of female hamsters over males (23.46 s \pm 4.510 s vs. 15.07 s \pm 3.098 s; $t = 2.365$, $df = 11$, $P < 0.05$). We also applied 200 ng MA and 400 ng PA dissolved in dichloromethane to the rod tip at a ratio approximating natural in females as contrasted with the control (vaporized dichloromethane) and found that sexually inexperienced males showed a preference for the mixture of MA and PA ($Z = 2.197$, $n = 12$, $P < 0.05$), whereas females responded equally (Fig 3). Furthermore, two glass capillary tests revealed that adding a mixture of MA (2 ppm) and PA (5 ppm) ($Z = 2.401$, $P < 0.05$), MA ($t = 2.260$, $P < 0.05$) or PA ($t = 2.338$, $P < 0.05$) to water significantly enhanced the attraction of males to the water (Fig 4a). Adding PA to water enhanced the attrac-

tion of females ($t = 2.900$, $P < 0.01$), but adding MA or a mixture of MA and PA to water failed to provoke a differential response in females (Fig 4b).

Spraying MA (2 ppm) or PA (5 ppm) dissolved in water onto paired males significantly suppressed their flank marking (MA: $Z = 2.196$, $P < 0.05$; PA: $Z = 2.344$, $P < 0.05$) although this treatment did not influence aggression compared to the control (Fig 5a, b).

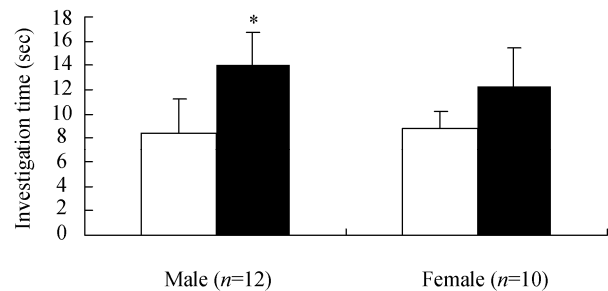


Fig. 3 Response (mean \pm SE, s) of male and female hamsters to a mixture (black bar) of tetradecanoic (200 ng) (MA) and hexadecanoic acid (400 ng) (PA), quantified by dissolving them in dichloromethane, versus dichloromethane control (white bar)

*: $P < 0.05$, using Wilcoxon test or paired t test.

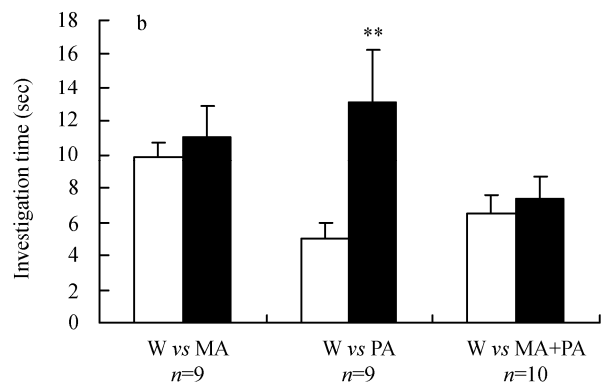
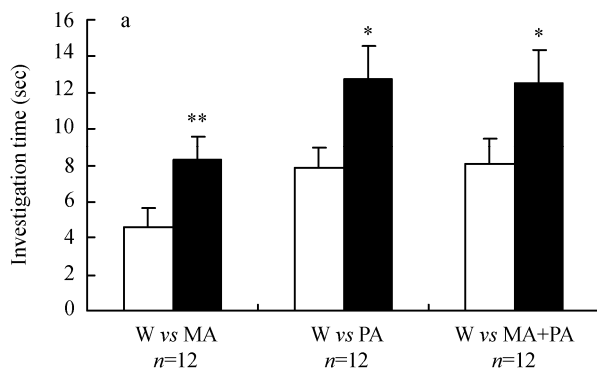


Fig. 4 Response (mean \pm SE, s) of male (panel a) and female (panel b) hamsters to aqueous samples (black bar) of tetradecanoic (2 ppm) (MA), hexadecanoic acid (5 ppm) (PA) or their mixture (MA+PA) versus distill water control (W) (white bar)

*: $P < 0.05$ and **: $P < 0.01$, using Wilcoxon test or paired t test.

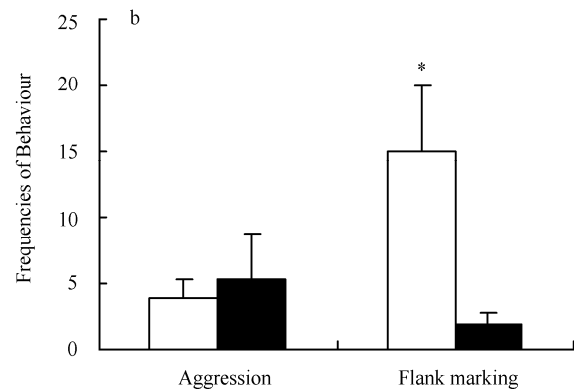
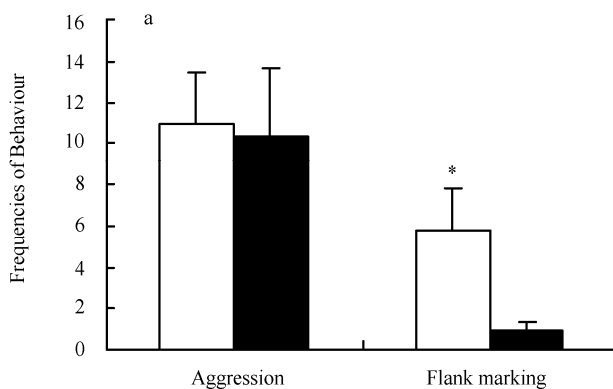


Fig. 5 Effects of spraying aqueous samples of tetradecanoic (2 ppm) (MA) or hexadecanoic acid (5 ppm) (PA) (black bar) and water (white bar) on agonistic behavior (i.e. aggression and flank marking) of males (panel a for MA and b for PA, $n = 10$)

*: $P < 0.05$, using Mann-Whitney test.

3 Discussion

3.1 Putative chemical signals

We have identified male and female pheromone candidates based on differences in the relative abundance of four compounds (12 and 18 in females, 19 and 20 in males) from our sample (see also Zhang et al., 2008b). In addition, for the first time we are aware we have detected more male-specific compounds that were traditionally viewed as male pheromone candidates using GC-MS utilizing a polar capillary column (Zhang et al., 2008b).

Compounds 1, 2, 4, 13 and 15 are male pheromone candidates and compounds 12 and 18 are female pheromone candidates (Zhang et al., 2008b). The five male pheromone candidates decreased, and only compound 18 increased in short-photoperiod exposed male golden hamsters, suggesting that short photoperiods exert a deleterious effect on maleness. This is consistent with our finding that flank glands in short-photoperiod males are smaller. Previous work has shown that photoperiodically induced refractoriness to hormones in this species appears more limited to the male (Morin et al., 1977) and almost all short-photoperiod females can conceive normally (Cherry, 1987). Although short-photoperiod males exhibit reductions in endogenous testosterone levels, sexual behavior and flank gland sizes, short-photoperiod males are still behaviorally and physiologically capable of reproducing (Luderschmidt et al., 1984; Matochik et al., 1986; Miernicki et al., 1990; Caldwell et al., 2008). Conversely, exposure of wild-caught rat-like hamsters (which are similar and related to golden hamsters) to short-photoperiods completely disables reproduction (Zhang, 1997¹). Therefore, a short-photoperiod imparted less impact on the volatiles and the size of the flank glands of male golden hamsters than castration.

Consistent with previous research (Frost et al., 1973; Vandenberg, 1973), our data shows that gonadectomy resulted in a sharp decrease in the size of the flank gland in males, but not in females. We further observed that gonadectomies profoundly affected the constituents of flank glands in males only. All male-specific compounds disappeared in castrated males despite the increase of a putative female pheromone, whereas an ovariectomy only heightened a putative male pheromone component (compound 20) in females. The presence of all male-specific compounds in testosterone-administrated

spayed females, indicate that these compounds are largely dependent on androgens. It has been previously found that ovariectomies only have a weak effect on preputial gland volatiles in rats (Zhang et al., 2008c). We still observed that testosterone-treated ovariectomized females had a female pheromone candidate (compound 12) showing a higher ratio and two male pheromone candidates (compound 31–32) showing lower ratios than normal male golden hamsters, suggesting that testosterone therapy could not completely upregulate the glandular volatiles to male levels. In addition, ovariectomy eliminated the four day rhythm of estrous cycles and significantly reduced the total amount of flank marking (Albers and Rowland, 1989), suggesting that estrogen might also affect flank marking in addition to the glandular morphology and volatile components.

It has been well documented in golden hamsters that dominant males flank mark more frequently and have larger flank glands than subordinates and therefore are more attractive to females (Drickamer et al., 1973; Johnston, 1975, 1977; Huck et al., 1985; Ferris et al., 1987). Both dominant and subordinate hamsters appear to utilize their flank glands to communicate social status in order to inhibit overt aggression during encounters (Ferris et al., 1987). Here, we failed to observe differences in flank gland masses between dominant and subordinate males, possibly because of different experiment methods of dominance-subordination establishment (see Drickamer et al., 1973). However, we found that dominant males had heightened male pheromone candidate compounds 3 and 24 and lowered female pheromone candidate compound 18, suggesting that dominant males had more predominance than subordinate ones. These three compounds might contribute to the higher female attraction to dominant males. Thus far, we know that *E*, *E*- α -farnesene and *E*- β -farnesene of male pheromones secreted by preputial glands are enhanced in dominant males and consequently increase female attraction only in mice (Harvey et al., 1989; Novotny et al., 1990). Recent work shows that dominant male rats have some heightened preputial volatile compounds (Pohorecky et al., 2008).

Intriguingly, when compared to normal male hamsters, we found that some male-specific pheromone candidates were lower in short-photoperiod exposed males, while castration-stimulated compounds (26 and 30) and a female pheromone candidate (compound 18)

¹ Zhang JX, 1997. Chemical communication of ratlike hamsters. PhD thesis. Institute of Zoology, Chinese Academy of Sciences, Beijing, China.

were heightened in dominant or/and subordinate males. This suggests that chronic dyadic encounters and consequent social status might attenuate the characteristics of males, regardless of the dominant or subordinate hamsters.

In addition, large individual variation (reflected by higher inter-individual RSDs) of shared compounds detected in flank glands in our study support the behavioral results that the volatiles might code for information regarding individuality in the analog form in the golden hamster (Johnston et al., 1993; Johnston and Bullock, 2001). A similar coding method seems to be used in mice and rats, where most differences in the chemical constituents of preputial glands are quantitative (Zhang et al., 2007a, 2008a, c).

The odorous constituents unique to, or covarying in quantity with, certain biological characteristics can be viewed as pheromone candidates and most likely to convey information (Singer et al., 1997; Novotny et al., 1999b; Zhang et al., 2007a, 2008a, b). Therefore, the above-mentioned variable compounds shared by both sexes, and male-specific ones from the flank glands, may be various pheromone candidates. Further, they might communicate information about gender, endocrine, social status and individuality to conspecific golden hamsters.

3.2 Female pheromones

Although it has been known that flank glands play various roles in sexual communication (Johnston, 1985; Laurent et al., 1988), we provide the first evidence that male golden hamsters are capable of distinguishing females from males using glandular secretions. This indicates an existence of sexually dimorphic constituents for female signaling. As above-mentioned, in females having no unique glandular volatiles, but chemical conclusion showed compounds MA (12) and PA (18) with higher ratios in females were female pheromone candidates (Zhang et al., 2008b).

Furthermore, the blend of MA and PA, whether pure or dissolved in water, was attractive to males, but not to females. PA alone showed attractiveness to both males and females. Such olfactory attractiveness to males suggests that the blend of MA and PA comprised a two-component female pheromone. In mammals, two-component pheromones have been validated from the urine (2-*sec*butyl-4,5-dihydrothiazole and dehydro-*exo*-brevicomine) and preputial glands (hexadecanol and hexadecanol acetate) of mice (Novotny et al., 1985; Jemiolo et al., 1986; Zhang et al., 2008a).

Encounters between adult golden hamsters are almost

always agnostic, except when between males and estrous females (Johnston, 1975, 1985). Flank marking as a component of agonistic behavior in male golden hamsters tends to be inhibited by exposure to female scent (Johnston, 1980, 1985). Our data showed that MA or PA, putative female pheromone components, inhibited agonistic behavior (reflected by flank marking) between males, providing further evidences for MA and PA to be female pheromone components.

In conclusion, our chemical inference that flank gland volatiles may communicate information about gender, endocrine state, social rank and individuality provide insight into the role of flank glands in chemical communication. In particular, the pheromonal activity of two putative female pheromone components was validated by bioassay, which in turn illustrates the feasibility of this method for future studies that involve both chemical and behavioral analyses.

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