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Dual role of preputial gland secretion and its major components in sex recognition of mice

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ABSTRACT

This study was aimed at validating the sexual attractiveness of hexadecanol and hexadecyl acetate, two putative pheromone compounds, from preputial gland secretion of mice. These two compounds have been reported to be among the major components of preputial gland secretion in both sexes but higher in quantity in males than females. In this study, we show that castration suppressed the production of the two compounds, further suggesting their association with maleness. Adding preputial gland secretion and the synthetic analogs of the two compounds to castrated male urine at their physiological levels in intact males increased the attractiveness of castrated male urine to female mice, showing that the two compounds were indeed male pheromones. Furthermore, their sexual attractiveness disappeared upon removing the vomeronasal organs (VNOs) from female recipients. Replenishing castrated male urine with preputial gland secretion and the two compounds at their physiological levels in females increased the attractiveness of castrated male urine to males. Such a reversal of sexual attractiveness for hexadecanol and hexadecyl acetate suggests that they had opposing dual effects in sexual attractiveness in a dosage-dependent manner.
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1. Introduction

Pheromones are molecular signals, which are secreted to the outside by animals and used for communication with other conspecific members, which react by changing their behavior or developmental process [36,44]. Generally, less is known about chemical aspects of mammalian pheromones as compared with those of insect pheromones [2,25,36,42,44]. However, the house mouse (*Mus musculus*) is among the best studied species for mammalian pheromones [2,25,43]. Most of known mouse pheromones are found in voided urine including some volatiles (e.g. 2,5-dimethylpyrazine, 2-heptanone, 6-hydroxy-6-methyl-3-heptanone, *R*,*R*-dehydro-*exo*-brevicomin, *S*-2-*sec*-butyl-dihydrothiazole) and major urinary proteins [9,17,23,25,26,31,33]. Some peptides from exocrine (lacrimal) glands have been characterized as male pheromones in mice, too [13]. Two male pheromones (i.e. *E*-α-farnesene and *E*, *E*-β-farnesene) have been found from the preputial glands, which are discharged into voided urine to signal conspecific membership [7,20]. Because specialized scent glands are rich sources of pheromones in mammals as well as in insects, it is highly likely that the preputial glands of mice may have more pheromone components that are yet to be found [7,20,36,43]. This suspicion is encouraged by our recent findings of two new major compounds, hexadecanol and hexadecyl acetate, from preputial gland secretion [40]. Similar to farnesenes, hexadecanol and hexadecyl acetate are also more abundant in males than in females, indicative of potential male pheromones [40]. Moreover, these two compounds have been documented to be pheromonal components in many insects and the bank vole (*Clethrionomys glareolus*) [1,36]. Such convergent uses of the same compounds in vastly different species show that these compounds possess some typical chemical properties of pheromones, one of which might be that they are able to convey airborne cues over a distance. Therefore, their pheromonal activity is worthy of further verification by bioassay [13,17,21–23,33].

It has become a general approach to validate pheromonal activity by adding synthetic analogs of urinary pheromone candidates to castrated or adrenalectomized urine and then examining the recurrence of the activity of intact urine [10,11,17,21–23,42]. In the current study, we examined the recurrence of the attractiveness of castrated male urine to females by adding hexadecanol and hexadecyl acetate at the concentrations typical of those in intact males.

An increasing body of neural evidence has shown that the main olfactory system and vomeronasal organ (VNO) can overlap in detecting pheromonal compounds, but chemosensory sex preference other than sex discrimination is found to be VNO-dependent in rodents. Surgical lesion of the VNO can result in a range of deficits in social and sexual behavior in their response to some pheromones [4,15,16,19,27–29,34,35,38]. However, there are noted exceptions to

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VNO-dependent functional responses to pheromones in other mammals [6]. For example, the nipple-search pheromone is still effective for rabbit pups with VNO ablation [8]. Also, VNO lesion does not affect the effects of male pheromones on the sexual behavior of estrous sows [5]. Hence, VNO-dependency in detecting pheromonal molecules can be used as a secondary criterion in screening for pheromones in rodents.

In the current study, we explored VNO-dependency of pheromonal activities to further the knowledge of the relationship between pheromonal compounds and the VNO in addition to confirming their identity as male pheromones. Furthermore, since preputial glands are larger in size and these two compounds are greater in amount in males than in females, we also attempted to probe whether the compounds could attract males at lower concentrations typical of females. We measured the contents of preputial gland secretion and the amounts of hexadecanol and hexadecyl acetate discharged into male urine, and examined the effect of castration on the production of the compounds. Then, we examined the attractiveness of preputial gland secretion, hexadecanol and hexadecyl acetate to female mice including VNO-dependency of such attractiveness. Finally, we examined how males respond to lower dosages of preputial gland secretion, hexadecanol and hexadecyl acetate.

2. Materials and methods

2.1. Subjects

We used 68 male and 70 female ICR/albino mice that were sexually inexperienced and at 8–16 weeks of age (Weitong-Lihua Experimental Animal Company, Beijing, China). Males were housed individually whereas females were housed in groups of four in plastic cages (31.8×20.2×31.5 cm for each). The room had a reversed 14L:10D light/dark photoperiod (lights on at 19:00) and 23±2 °C in temperature. Food and water were provided *ad libitum*. The procedure of animal handling complied with the institutional guidelines for animal use and care at the Institute of Zoology, the Chinese Academy of Sciences.

2.2. Castration and VNO lesion

Nine males underwent bilateral castration via a single midline incision following the sodium pentobarbital (40 mg/kg) anesthesia procedure. Four weeks later, these castrated males, together with 9 intact males, were used for urine collection and then killed to dissect out preputial glands for collection of their secretion as stated below.

To lesion the VNO (VNOx), anesthetized females (n=12) were placed supine with head down in a sloping small table with holders. We made a midline incision on the roof of the mouth, gently opened the lower jaw, and exposed and cut the parts (about 2–3 mm long) of the underlying vomer bones. We then removed them by forceps, and closed the incision with medical super-glue (VNOx) [37,38]. To control for the surgery, a group of females (n=12) went through the similar procedure other than the removal of the vomer bones (VNOi). All animals were allowed two weeks for recovery prior to behavioral trials. Surgical removal of the VNO was tentatively validated by testing the subjects for their olfactory preference for male vs. female urine before and after behavioral trials. In comparison with the VNOi subjects, the VNOx animals showed no preference between the two types of urine. After all behavioral trials, we sacrificed all VNOx females and anatomized the nasal cavity to confirm the disconnection of the VNO under microscope.

2.3. Scent collection and dichloromethane extraction

To collect urine, we placed each intact or castrated male donor in a clean mouse cage with a wire grid floor. Upon animal's urination, the urine was immediately absorbed and transferred to a vial by a disposable glass capillary (i.d. 1.8 mm, 15 cm long) for behavioral and chemical assays. To quantify hexadecanol in urine samples, we mixed 250 μl dichloromethane with 250 μl urine, let it sit for 24 h at 0 °C, and then used the bottom phase (i.e., the layer with dichloromethane) for chemical analysis as described previously [40].

We dissected out preputial glands immediately after sacrificing the mice by neck displacement and then collected the yellowish preputial gland secretion by squeezing the glands and loading their secretion into a vial. The collected secretion was kept at −20 °C until behavioral tests (only intact males were used) and 24-h solvent extraction (both intact and castrated male preputial glands were used).

Prior to extraction, we weighed the secretion and added dichloromethane into the vial in the proportion of 1-mg secretion in 10 μl dichloromethane (purity >99.5%) and kept it at 0 °C for 12 h. Then, we removed the supernatant and stored the remaining solution at −20 °C until GC–MS analysis.

2.4. GC–MS assay

Analysis was performed on an Agilent Technologies Network 6890N GC system coupled with 5973 Mass Selective Detector (NIST 2002 Library). The GC was equipped with a HP5MS glass capillary column (30 m long, i.d. 0.25 mm×0.25 μm film). The carrier gas is helium (1.0 ml/min). The injector temperature was set at 230 °C. The oven temperature was set initially at 100 °C (for preputial gland secretion) or 50 °C (for urine), heated by 5 °C/min to 180 °C and then ramped by 1 °C/min until 220 °C. Electron impact ionization was at 70 eV. Transfer line temperature was set at 280 °C. Scanning mass ranged from 30 to 350 amu. 1-μl sample was injected in a split mode (10:1) for preputial gland secretion and a splitless mode for urine. The purities of purchased hexadecanol (>98%) and synthesized hexadecyl acetate (>95%) were determined by GC–MS. The hexadecanol and hexadecyl acetate in the preputial gland secretion were confirmed by authentic analogs. We quantified them in the preputial gland secretion by comparing their GC areas in the samples with the established standard curve of GC area vs. concentration.

2.5. Quantification of the preputial gland secretion, hexadecanol and hexadecyl acetate in voided urine

To measure the contents of the preputial gland secretion discharged into voided urine of intact males, we added graded concentrations of the preputial gland secretion, which was mixed equally from nine intact males, into bladder urine samples, which were collected fresh mixed equally from five sacrificed male donors. Then, we conducted parallel dichloromethane extraction and GC–MS analysis of voided urine and bladder urine that had been supplemented with the secretion at known concentrations. Finally, we matched the GC area of hexadecanol in the voided urine with the counterpart in the bladder urine to obtain the content of the secretion in voided urine of intact males. Furthermore, we corroborated our calculation of the content of hexadecyl acetate in the voided urine of males based on the ratio of hexadecanol to hexadecyl acetate in male preputial gland secretion.

On the other hand, based on known sexual differences in preputial gland size (10 times larger in males) and relative abundances of hexadecanol and hexadecyl acetate of preputial gland secretion (2.5 times higher in males) [40], the contents of hexadecanol and hexadecyl acetate discharged into voided urine should be roughly 25 times higher in males than in females. Thus, we could estimate the concentrations of the two pheromonal compounds accordingly.

The preparation of the saturated water solution of hexadecanol and hexadecyl acetate was described below. We determined the saturated concentrations of hexadecanol and hexadecyl acetate in water by directly injecting 0.5 μl of the solution into GC–MS, and then matching their GC areas with established curve of GC area vs. quantity.

2.6. Sample preparation for behavioral test

To prepare scented castrated male urine with the preputial gland secretion and two synthetic analogs for behavioral assays, we first mixed preputial gland secretions equally from nine intact males and then diluted the secretion mixture, hexadecanol, or hexadecyl acetate with dichloromethane to manageable concentrations. We added proportionally each of the prepared solutions into a vial and allowed the vial uncovered for 5 min to vaporize the solvent and leave the compounds. Then, we added the castrated male urine, which was mixed equally from nine castrated donors, into the vial to obtain the castrated male urine that had been added with the preputial gland secretion or pheromonal analogs at the desired concentrations.

In addition, the concentrations of the preputial gland secretion, hexadecanol and hexadecyl acetate added into castrated male urine to test the responses of females were roughly estimated by the method described above (e.g. 400 ppm preputial gland secretion, 1 ppm hexadecanol and 30 ppm hexadecyl acetate in castrated male urine) or chosen from pilot experiments that revealed the threshold concentration of hexadecanol (20 ppm) or hexadecyl acetate (2000 ppm) alone in castrated male urine and the threshold concentration (0.012 ppm hexadecanol and 4 ppm hexadecyl acetate for female recipients, and 0.006 ppm hexadecanol and 2 ppm hexadecyl acetate for male recipients) in water. The concentrations of male preputial gland secretion (16 ppm and 4 ppm) and a mixture of hexadecanol (0.3 ppm) and hexadecyl acetate (7 ppm) added into castrated male urine used for simulating female urine were roughly estimated through the aforementioned way of calculation.

To prepare for the saturated water solutions of hexadecanol (0.06 ppm) and hexadecyl acetate (4 ppm), we added sufficient synthetic hexadecanol and hexadecyl acetate to redistilled water, stored the solution for 12 h at room temperature (25 °C) and drew the lower parts of the solution with a syringe. We then diluted the saturated concentrations to different concentrations of hexadecanol and hexadecyl acetate and tested the response of mice to diluted aqueous samples to screen for the effective concentrations of hexadecanol and hexadecyl acetate in water.

Pure hexadecanol and hexadecyl acetate were presented to mouse recipients through painting their dichloromethane solutions with the concentrations of 0.1% (w/v), 800 ppm (w/v) and 19,200 ppm (w/v), respectively, on the tips of glass rods. We laid the rods aside for 5 min to vaporize the solvents prior to use.

2.7. Behavioral assay

The responses of mouse recipients to two scented glass rods (20 cm long and 4 mm in diameter for each) were tested in their home cages in a separate dim room in the dark phase as described previously [14,41]. Briefly, 2 μl of sample was painted evenly on one tip (about 12 mm^2) while the other end was held by the tester (blind to sample treatments). Two scented glass rods were simultaneously presented to a subject and recorded for 3 min after the subject showed first sniffing or licking the tips of the rods. We measured the time each mouse spent in sniffing and licking each tip. The durations that the test mouse sniffed within the 1-cm distance from the tip and licked the end of the rods were recorded with two hand-held stopwatches. Any subject that did not respond to the two rods over the first 3 min was excluded for the day. We randomly assigned 48 males and 48 females to the respective four groups with 12 individuals of the same sex in each for odor preference test everyday for 28 consecutive days. Each group was chosen randomly and used in test only once a day. We made sure that these intact male and female recipients were capable of showing a preference for the urine of the opposite sex over same-sex urine or castrated male urine.

2.8. Data test

We first examined the distribution of the raw data by the Kolmogorov–Smirnov test in SPSS for Windows and then used *t* test for normal data and non-parametric test for non-normal data. In particular, Wilcoxon signed-rank test and paired *t* test were used for non-normal and normal behavioral data, respectively. The level of significance (α) was set at 0.05 for all tests.

3. Results

3.1. Contents of preputial gland secretion, hexadecanol and hexadecyl acetate in voided urine

Our data obtained from dichloromethane extraction and GC–MS analysis showed that intact male mice had more abundant hexadecanol (intact vs. castrated males: 7.357±11.88 μg vs. 0.025±22.50 μg, $Z=3.490$, $N=18$, $P<0.001$, Mann–Whitney test) and hexadecyl acetate (intact vs. castrated males: 160.6±54.40 μg vs. 84.96±56.65 μg, $Z=2.563$, $N=18$, $P<0.01$, Mann–Whitney test) in the preputial gland secretion (per mg) than castrated males did (Fig. 1). Also, intact males had significantly larger preputial glands than castrated males did (149.5±18.99 vs. 62.32±29.35 mg, $t=7.884$, $df=18$, $P<0.001$, independent *t* test). On the other hand, the GC area of hexadecanol in 1 μl of dichloromethane extraction of voided urine was 2.36±1.10 ($n=8$) million, which corresponded to that of 400 ppm (w/v) male preputial gland secretion dissolved in voided urine of intact males, suggesting that intact male urine roughly contained 3 ppm hexadecanol and 70 ppm hexadecyl acetate.

For females, we inferred by calculation that intact female urine contained 40 ppm female preputial gland secretion, 0.12 ppm hexadecanol and 2.8 ppm hexadecyl acetate, which was equivalent to 16 ppm male preputial gland secretion.

3.2. Attractiveness of preputial gland secretion, hexadecanol and hexadecyl acetate to females

Binary choice tests revealed that sexually naïve females were more attracted to castrated male urine spiked with 400 ppm preputial gland secretion ($t=3.483$, $P<0.01$), 16 ppm preputial gland secretion ($t=6.208$, $P<0.001$), a mixture of 1 ppm hexadecanol and 30 ppm hexadecyl acetate ($t=3.443$, $P<0.01$), 20 ppm hexadecanol ($Z=2.599$, $P<0.01$), and 2000 ppm hexadecyl acetate ($t=3.365$, $P<0.01$) than to castrated male urine (Fig. 2a, b). Females responded equally to castrated male urine before and after being spiked with lower concentrations of hexadecanol (2 ppm) (2.837±0.527 vs. 2.579±0.658 s) or hexadecyl acetate (60 ppm) (5.145±1.720 vs. 3.010±0.434 s). Furthermore, scenting the water with either 0.012 ppm hexadecanol ($Z=2.981$, $P<0.01$) or 4 ppm hexadecyl acetate ($Z=2.973$, $P<0.01$) significantly increased the attractiveness of water to females but not to males (Fig. 2c).

In addition, females showed no preference between castrated male urine and castrated male urine added with 4 ppm preputial gland secretion (Fig. 2a) nor did they show preference between castrated male urine and castrated male urine added with a low concentration of hexadecanol (0.3 ppm) or hexadecyl acetate (7 ppm) (Fig. 2b). Also, females showed no preference between plain water and water scented with a low concentration of hexadecanol (0.006 ppm) or hexadecyl acetate (2 ppm) (Fig. 2c).

3.3. VNO-dependent preference of female mice for preputial gland secretion, hexadecanol and hexadecyl acetate

VNOi females displayed a preference for castrated male urine spiked with 400 ppm preputial gland secretion ($t=2.460$, $P<0.05$) or a mixture of hexadecanol (1 ppm) and hexadecyl acetate (30 ppm)

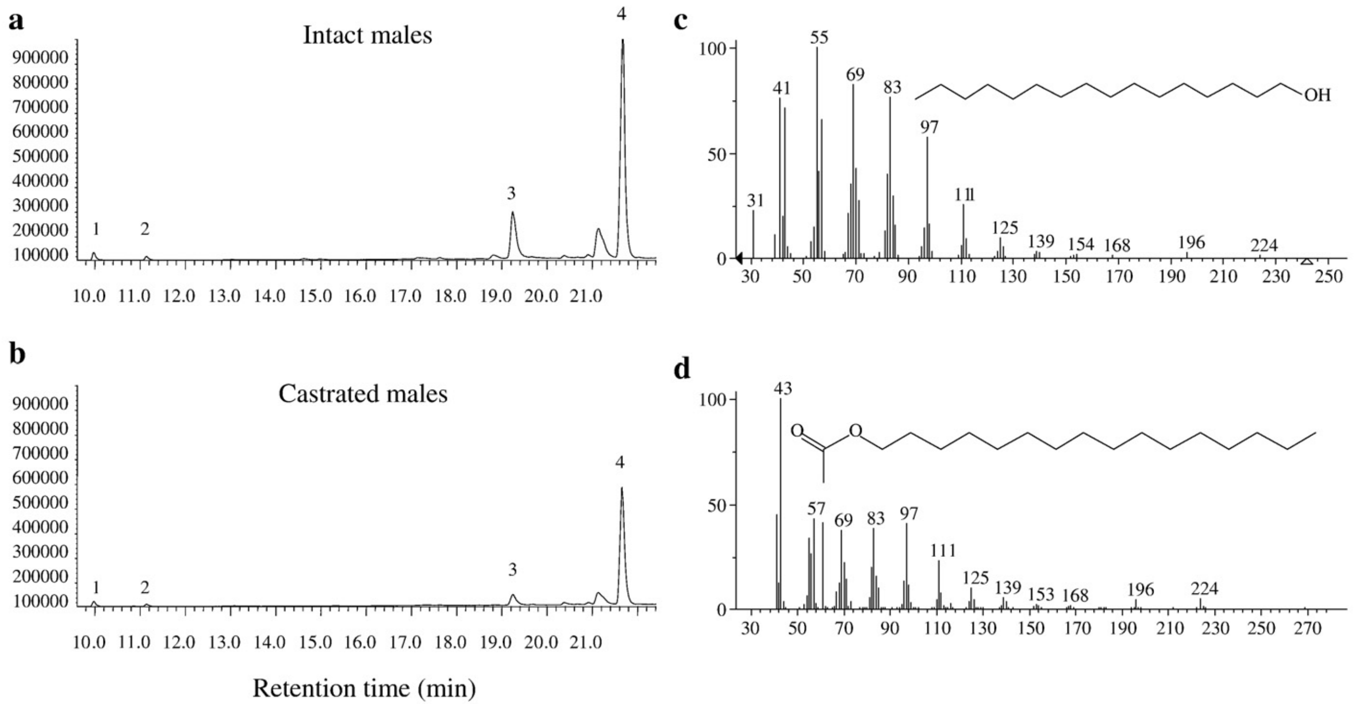

Fig. 1. GC–MS results of preputial gland secretion of the house mouse. Partial gas chromatograms of intact male (a) and castrated males (b), GC peaks 1, 2, 3 and 4 correspond, respectively, to *E*-β-farnesene, *E*, *E*-α-farnesene, hexadecanol (16C:OH) and hexadecyl acetate (16C:Ac). c and d are the spectra and structures of hexadecanol and hexadecyl acetate, respectively.

(Z=2.510, P<0.05) over castrated male urine (Fig. 3a). They investigated water scented with either 0.012 ppm hexadecanol (Z=2.432, P<0.05) or 4 ppm hexadecyl acetate (t=2.511, P<0.05) more than plain water (Fig. 3a). However, VNOx females showed no difference in their investigation on castrated male urine and castrated male urine spiked with 400 ppm preputial gland secretion, and a mixture of hexadecanol (1 ppm) and hexadecyl acetate (30 ppm). The same result held for plain water and water scented by either 0.012 ppm hexadecanol or 4 ppm hexadecyl acetate (Fig. 3b).

3.4. Attractiveness of lower doses of preputial gland secretion, hexadecanol and hexadecyl acetate to males

Contrary to females, males spent more time investigating castrated male urine spiked with lower concentrations of preputial gland secretion (4 ppm) (t=2.546, P<0.05), preputial gland secretion (16 ppm) (t=3.329, P<0.01), a mixture of hexadecanol (0.3 ppm) and hexadecyl acetate (7 ppm) (Z=2.803, P<0.01), hexadecanol (1 ppm) (t=2.665, P<0.05) or hexadecyl acetate (30 ppm) (Z=1.883, P=0.06) as compared with castrated male urine (Fig. 2d, e). This was also true for males investigating water scented with 0.006 ppm hexadecanol (Z=1.961, P=0.05, marginal significance) or 2 ppm hexadecyl acetate (Z=2.353, P<0.05) vs. plain water (Fig. 2f). In contrast, adding 400 ppm preputial gland secretion to castrated male urine did not change the attractiveness of castrated male urine to males (Fig. 2d). This was also true for the addition of hexadecanol (1 ppm) and hexadecyl acetate (30 ppm) to castrated male urine (Fig. 2e). Neither did males show a differential response to water and water spiked with a higher concentration of hexadecanol (0.012 ppm) or hexadecyl acetate (4 ppm) (Fig. 2f).

In addition, males showed an overt preference for a low dose of mixture of hexadecanol (2 ng) and hexadecyl acetate (48 ng) over a high dose of mixture of hexadecanol (0.8 μg) and hexadecyl acetate (19.2 μg) (31.85±9.993 vs. 10.64±2.708 s) (Z=2.040, n=12, P<0.05). However, females' preference was reversed between them (2.197±0.318 vs. 3.817±0.850 s, Z=2.398, n=10, P<0.05).

4. Discussion

Our data suggest that castration did not eliminate hexadecanol and hexadecyl acetate in the preputial gland secretion, but significantly reduced their amounts. In addition to our previous finding that these two compounds are richer in males than in females, their testis-dependency further suggests their association with maleness. Meanwhile, castrated male urine contained less preputial gland secretion, hexadecanol and hexadecyl acetate discharged by the residual preputial glands than intact male urine did. This result is consistent with previous findings that castration does not eliminate other sex-common male pheromone compounds such as farnesenes, dehydrobevicomin and 6-hydroxy-6-methyl-3-heptanone, although it does eliminate male-specific *S*-2-*sec*-butyl-dihydrothiazole [7,17,20,21,23]. Considering individual variation in preputial gland secretion components [40] and residual preputial glands of castrated males, adding 400 ppm preputial gland secretion, 1 ppm hexadecanol and 30 ppm hexadecyl acetate into castrated male urine restored the concentrations approximating their physiological levels in intact males. Hexadecanol and hexadecyl acetate showed attractiveness to females just as preputial gland secretion did at their natural levels, suggesting male pheromones. Adding single hexadecanol or hexadecyl acetate into castrated male urine required a higher concentration to attract sexually naïve females to castrated male urine. 20 ppm for hexadecanol and 2000 ppm for hexadecyl acetate were the respective minimum concentrations in castrated male urine to show sexual attractiveness in our pilot experiments (data not shown). An unusually high dosage of a single pheromone analog to induce the response of sexually naïve female mice has also been exemplified with farnesenes [11]. Therefore, pheromonal activity of the hexadecanol and hexadecyl acetate mixture (at the physiological levels) and an unusually high level of either of the two compounds implied that they acted synergistically as male pheromones in nature. In addition, a much lower concentration of hexadecanol (0.012 ppm) or hexadecyl acetate (4 ppm) dissolved in water than in castrated male urine elicited the preferential response of females and activated c-Fos expression in both main and accessory olfactory bulbs, medial amygdala and hypothalamus of female

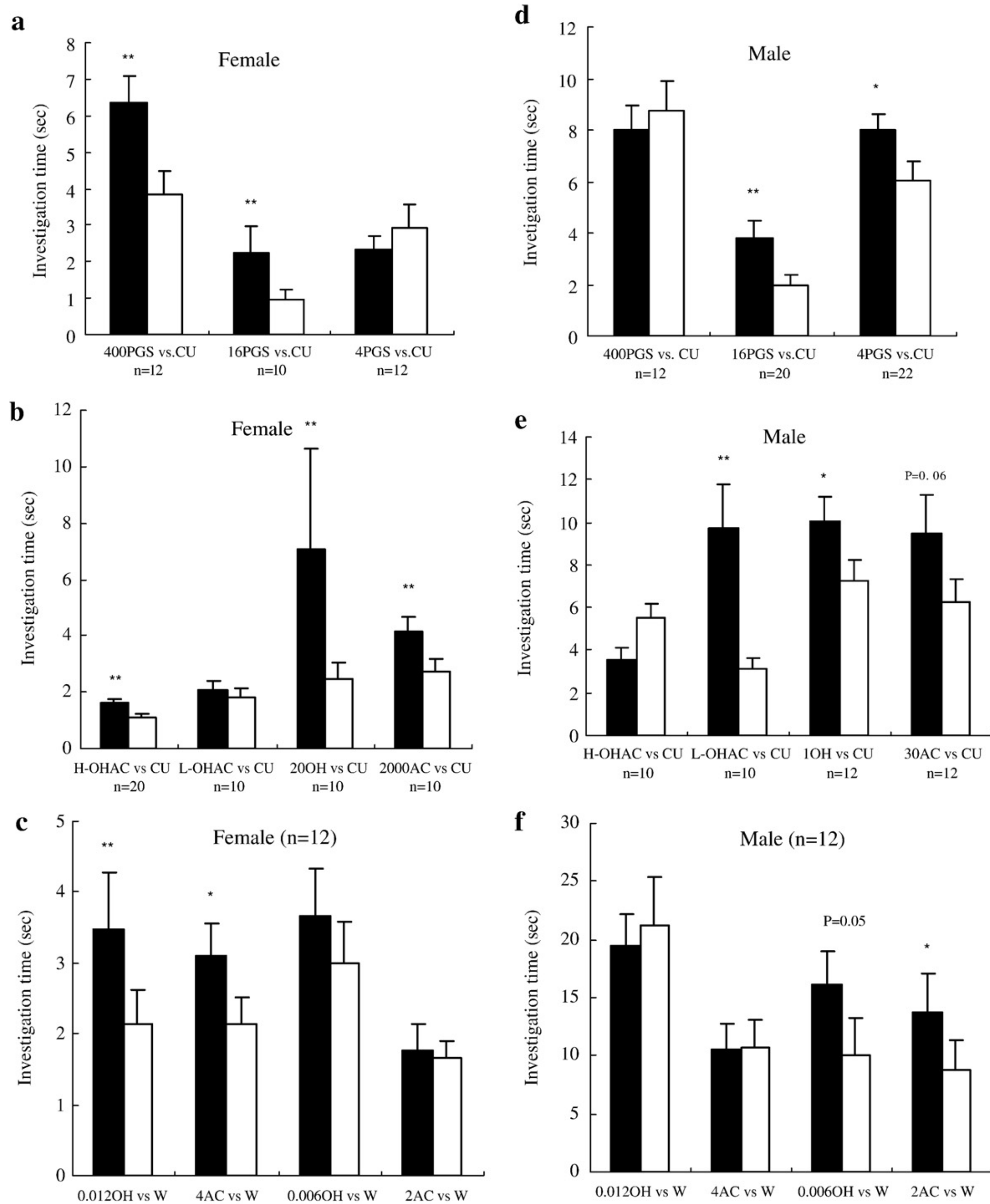

Fig. 2. Responses (mean ± SE, s) of mice to castrated male urine (CU) and the castrate urine added with preputial gland secretion (PGS) (a: female recipient; d: male recipient), or hexadecanol (OH) and/or hexadecyl acetate (AC) (b: female; e: male), and to plain water (W) and the water scented by hexadecanol and hexadecyl acetate and (c: female recipient; f: male recipient). Figures before the PGS, OH and AC refer to the added concentration (ppm). H- and L-OHAC refer, respectively, to 1 ppm hexadecanol plus 30 ppm hexadecyl acetate, and 0.3 ppm hexadecanol plus 7 ppm hexadecyl acetate. *$P<0.05$, **$P<0.01$ using Wilcoxon test or paired *t* test.

mice (X. Ye. and M. Luo, unpublished data). Such low effective concentrations of the compounds added in water relative to those added in castrated male urine might be ascribed to the residual preputial gland secretion and existence of binding molecules/proteins that link to either hexadecanol or hexadecyl acetate or both, and restrain the molecules from escaping in the urine of castrated males. Thus, castrated male urine might need more of hexadecanol and hexadecyl acetate to be scented than water to sharpen the contrasts against the blank counterpart and differentiate the responses of females.

In spite of the overlapping functionality of the main olfactory and vomeronasal systems in detecting pheromonal molecules, surgical lesion of the VNO abolishes olfactory sex preference of mice [2,27,28,39]. However, such VNO-dependent perception of pheromones is only present in rodents but not in other mammals [6]. For instance, 2-methylbut-2-enal, the rabbit mammary pheromone, which guides nipple search in rabbit pups, and androstenone, the boar pheromone, which mediates sexual behavior in estrous sows [5,8,30], are not affected by lesioning the VNO. Our results showed

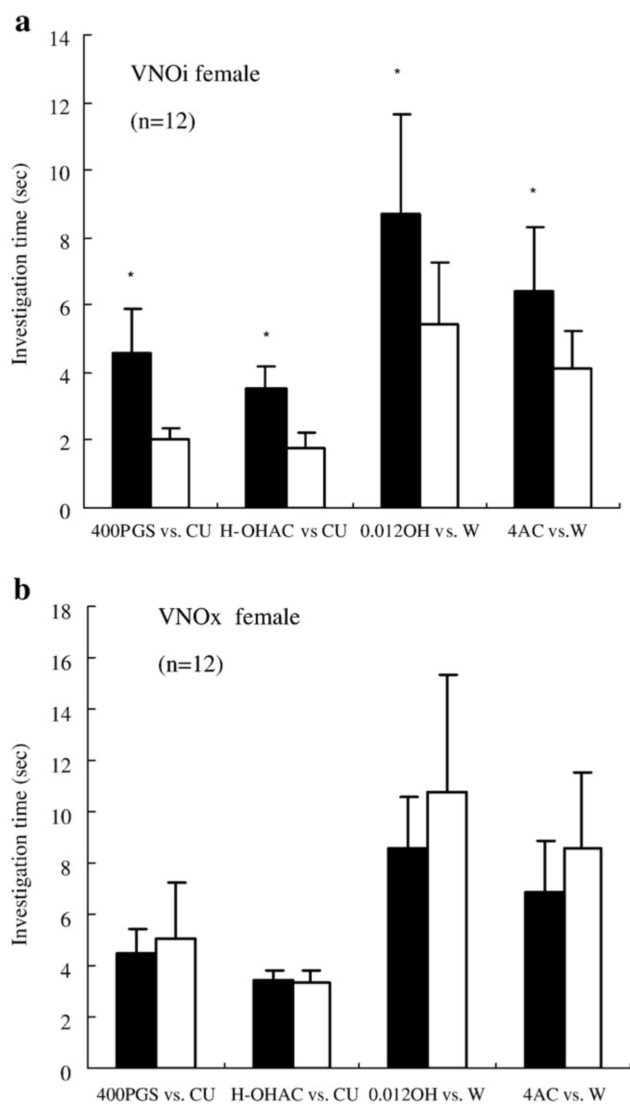

Fig. 3. Responses (mean±SE, s) of sham-operated (VNOi) (a) and vomeronasal organ-removed (VNOx) (b) female mice to the castrate urine (CU) added with 400 ppm preputial gland secretion (400 PGS), or 1 ppm hexadecanol plus 30 ppm hexadecyl acetate (H-OHAC) and CU, or the water scented by 0.012 ppm hexadecanol (0.012OH) or 4 ppm hexadecyl acetate (4AC) and plain water (W). *$P<0.05$, using Wilcoxon test or paired *t* test.

that the effects of hexadecanol and hexadecyl acetate on the sexual preference of females were indeed VNO-dependent. This accorded with the VNO-dependent preference of females for male urine over female urine. The VNO-dependent behavioral effects corroborated the identity of hexadecanol and hexadecyl acetate as two male pheromone molecules in this species.

Our data also revealed that adding hexadecanol and hexadecyl acetate to castrate urine or water at lower concentrations enhanced the attractiveness only to males, whereas at higher concentrations only to females. Owing to both the lower contents of hexadecyl acetate in male and female urine and hexadecanol in female urine, and the coelution of nontargeted urinary constituents, we could not conduct a direct measurement of them by using the dichloromethane extraction and GC–MS analysis. Instead, we used the hexadecanol of male urine with the highest GC peak as a marker and its proportional relationship with others for calculation. Thus, we could only roughly estimate their amounts in voided urine. Together with the fact that castrated males still had larger residual preputial glands and more preputial gland secretion in urine than females, the concentration of male preputial gland secretion added in the castrated male urine should be less than 16 ppm accordingly. Hence 4–16 ppm of male preputial gland secretion used here to scent castrated male urine and simulate female urine should be reasonable. However, the effective concentrations of hexadecanol (0.3 ppm) and hexadecyl acetate (7 ppm) added in castrated male urine to attract males seemed to be slightly higher than those (0.03–0.12 ppm hexadecanol and 0.64–2.56 ppm hexadecyl acetate) present in the 4–16 ppm preputial gland secretion of males. Such effective concentrations of these two compounds might compensate for the lack of other effective components in the preputial gland secretion such as *E*-β-farnesene and *E*, *E*-α-farnesene. Furthermore, because pheromone-binding proteins slow down the release of urinary pheromones, the amounts of urinary pheromones perceived by mouse recipients must be lower than the contents in voided urine measured by solvent extraction and GC–MS. Thus the effective concentrations perceived by mouse recipients should be lower than those present in voided urine [24]. In agreement with male preference for female urine over male urine, it has been shown that male preference for the low dosages of hexadecanol and hexadecyl acetate dissolved in water over plain water also relied on the VNO (authors' unpublished data). The attractiveness of hexadecanol and hexadecyl acetate to males suggests that these two compounds were of female pheromones at the physiological levels in females. On the other hand, unlike *E*-α-farnesene and *E*, *E*-β-farnesene, these two pheromone compounds are unrelated to dominance–subordination relationship in males [7,18,20,40], suggesting that sex pheromone molecules of mice diverge in other functions of chemical communication.

In conclusion, the dosage-dependent reversal of sexual attractiveness of hexadecanol and hexadecyl acetate suggests that they played a dual role in olfactory sex recognition in mice. This system is comparable to the pheromones of some ant species with higher concentrations inducing alarm responses (avoidance) and lower concentrations attracting conspecific individuals [32]. In brandt's voles (*Lasiopodomys brandtii*), we also found that farnesyl acetate from preputial glands is a dosage-dependent male pheromone with lower concentrations attracting females and higher concentrations repelling females [43]. Such dosage-dependent dual role pheromones provide a new insight into how animals use sex-common scent components to signal sex [3]. It has been demonstrated that volatile farnesenes of preputial glands repel male mice, but attract females [11,12]. Our study, however, uncovered a more complex, dosage-dependent opposing dual role of semivolatile pheromones, hexadecanol and hexadecyl acetate, for sex attractiveness or recognition. This also raises the question of how the neural receptors function in response to dosage-dependent pheromones. We expect that our study will stimulate future research in the study of pheromone communication.

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