Study of chemical communication based on urine in tree shrews *Tupaia belangeri* (Mammalia: Scandentia: Tupaiidae)

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Abstract

Chemical communication plays a key role in mammalian reproductive and social behaviour. The chemical constituents of urine are the main signal resource that can encode sex, quality and social status. In order to investigate the role of urine in the reproductive biology of *Tupaia belangeri*, the volatile components of urine were analysed by gas chromatography-mass spectrometry, and the behaviour of the tree shrew in response to urinary odour was investigated in a Y-maze test. The results show that hydrocarbons were the major components of urine in wild, acclimated and laboratory-breeding animals. The concentrations of the chemical components in urine from individuals in the wild population were higher than those in the acclimated and breeding animals. *Tupaia belangeri* showed significant differences in reactions of individuals and urinary odour. Males and females had different components in their urine. The stay duration of male tree shrews to the urinary odour of females in oestrus or lactating was significantly longer than that to the odour of pregnant females. The chemical components were different at different reproductive stages. Taken together, these results suggest that the odour of urine can encode female reproductive status and gender. *Tupaia belangeri* relies on these odours to recognise sex and choose a mate. Chemical communication based on signals in urine plays an important role in the reproduction of *T. belangeri*.

Keywords: *Tupaia belangeri*, chemical communication, urine, sex, behaviour

Introduction

Chemical communication refers to the use of mammalian signals from saliva, urine, faeces, vaginal secretions, semen or glandular secretions from the skin. Some chemical signals are received by the olfactory organs, and the signal receiver stimulates the corresponding changes in behaviour and physiology after a comprehensive analysis by the nervous system (Gray et al. 1984; Osada et al. 2008). The chemical composition of mammalian urine is various, and includes small organic molecules containing volatile and semi-volatile substances (alkanes, alcohols, acids and esters), peptides and proteins. Some components with biological activity can be used as pheromones to transfer important information about sex, body mass, species and the sexual partner. These urine odours play important roles in animal gender identification or selection, identification of species, and mammalian reproduction (Hurst et al. 2001).

Mutual recognition and social behaviour among individuals of the same species are the basis for the maintenance of life in mammals. Urine of Brandt’s vole *Lasiopodomys brandti* contains complex individual odour information, particularly sex information, which can be used for sex identification (Ferkin & Johnston 1995). *Mus musculus* and *Mus musculus domesticus* are the two subspecies of the house mouse that can be identified by differences in the urinary components of the males (Mucignat-Caretta et al. 2010). Males and females of the same species can be recognized by the odour of the urine. For example, E-β-farnesene, E,E-α-farnesene and 1-hexadecanol are found in higher concentrations in male urine than that of females. Squalene, 2-heptanone, and 4-ethyl...
phenol are found in higher concentrations in females than in males (Zhang et al. 2008b). (s)-2-Sec-buty1-4,5-dihydrothiazole is a special sex recognition compound found in male M. musculus that is used for species competition and to mark individual territory. Other study showed that 2,5-dimethylpyrazine is a female-specific substance in mouse urine (Zhang et al. 2007). Therefore, urine plays an important role in animal sex and species identification.

The chemical composition of urine and its function have been reported in many animals, such as Ailuropoda melanoleuca (Liu et al. 2012), Arvicola amphibius (Nazarova & Proskurnyak 2013), Mus musculus (Zhang et al. 2007), Panthera leo (Andersen & Vulpius 1999) and Lynx rufus (Mattina et al. 1991). Ailuropoda melanoleuca rely on urine to distinguish distant relatives and avoid inbreeding or excessive inbreeding (Liu et al. 2012). Female A. amphibius rely on a urinary protein in males to select a mate (Nazarova & Proskurnyak 2013). A urinary protein in M. musculus acts as a cue for gender identification and encodes age information (Kwak et al. 2013). Levels of pheromone-producing compounds in the urine of immunocompromised M. musculus (such as inbreeding coefficient offspring) are lower, reducing attractiveness to females, whereas female mice rely on pheromones (such as E-β-farnesene, E,β-farnesene, 1-hexadecanol, R,R-3,4-dehydro-exo-brevicomin and (s)-2-sec-buty1-4,5-dihydrothiazole) to assess the immune competence of male mice (Zhang et al. 2007). Male mice have large amounts of odourant molecules in their urine, such as 6-hydroxy-6-methyl-3-heptanone, stimulating female mice to enter oestrus (Novotny et al. 1999). The chemical composition of urine is remarkably different between lactating and non-lactating female Microtus oeconomus, and the male can identify the differences and choose in favour of the lactating female (Zhang & Zhang 2011). The urine of female house mice in oestrus contains a specific signal substance called 1-iodo-2-methylundecanec that stimulates the male to mature (Achiraman & Archunan 2006). Thus, differences in the composition or content of urine can affect mate choice in mammals, and urine plays an important role in animal reproduction.

The tree shrew, Tupaia belangeri (Mammalia: Scandentia: Tupaiaidae), is a squirrel-like lower primate and a unique Oriental species that is being used as a promising animal model in biomedical research (Wagner 1841; Wu et al. 2013). Tree shrews often perch in coniferous and broad-leaved forests, where they mainly eat fruit, insects and other animal food. The tree shrew is a monogamous animal, and spring and summer are the peak breeding periods. Pregnancy is generally 41–45 days, with a litter size of only 1–6, usually 2–4. Young tree shrews of 3–4 months of age up to maturity have been captured in the wild (Zheng et al. 2014). In the laboratory, frequent sniffing, licking of genitals, and sniffing of the female’s genitals occurs when female shrews are in oestrus, which ultimately leads to mating, but the success rate of mating is low (Zou et al. 1991). In the present study, we analysed chemical communication signals in the urine to increase reproductive success in tree shrews. (1) What are the volatile components of urine from three sources (wild, laboratory-acclimated, and laboratory-breeding shrews) during the non-breeding period? What are the differences in the urine? (2) Can a tree shrew in non-breeding condition be identified by urine odour? What is the difference in volatile components between male and female urine? (3) Can male tree shrews identify different breeding females according to their urine odour? We were also interested in understanding the reproductive biology characteristics of tree shrews through an analysis of their urine chemical communication system to improve the artificial reproduction rate.

**Materials and methods**

**Animals and experimental design**

All animals were healthy adults and housed individually in wire cages (40 × 40 × 40 cm³), and water with vitamins was provided ad libitum. The cage environment was maintained at 12 h L: 12 h D (lights on at 08:00), 25 ± 1°C, and 65–92% relative humidity. All animal procedures were approved by the Animal Care and Use Committee of School of Life Sciences, Yunnan Normal University (Permit No. 13-0901-011).

**Experiment 1: study of volatile components in urine from three sources**

Urine samples were collected from 14 wild animals (n = 7 females and 7 males), which were captured (25°25′–26°22′N, 102°13′–102°57′E, at 1679 m altitude) at the boscage of Luquan County, Yunnan Province, China. Twelve laboratory-acclimated tree shrews (n = 6 females and 6 males) were used, which were captured (25°25′–26°22′N, 102°13′–102°57′E, at 1679 m altitude) at the boscage of Luquan County, Yunnan Province, China, and maintained at the School of Life Sciences, Yunnan Normal University, Kunming for 4 weeks. Finally, 12 laboratory-breeding animals from the Laboratory of Medical and Biological Chinese Academy of
Medical Sciences Institute (n = 6 females and 6 males) were used, which were in non-breeding condition.

**Experiment 2: identification of body and urine odours from non-breeding animals**

Twelve laboratory non-breeding animals (n = 6 females and 6 males) were used. All animals were in non-breeding condition. The tree shrews were used as a stimulus to determine the preferred sex by body odour using females, males and controls. Then, urine was used to determine the preferred sex using females, males and controls. Non-reproductive shrew urine was first used as the stimulus to determine the response to same-sex urine odour by males and females. Before the Y-type maze behavioural test, six non-breeding tree shrews (n = 3 females and 3 males) were used as the subjects placed in the box to test the left and right preference, which the stimulate source was the empty container, it found that tree shrews have no preferences (P > 0.05). The identification of body and urine odours from non-breeding animals was carried out.

**Experiment 3: identification of female urine odour at different reproductive stages**

Six males were used. Females were in oestrus (n = 6), pregnant (n = 7) or lactating (n = 9). Oestrus criteria: female genital pore regularly open, decreased food intake, restless, activity increased significantly, prefers to do “backflip” movement (Zheng et al. 2014). Before the Y-type maze behavioural test, six non-breeding tree shrews (n = 3 females and 3 males) were used as the subjects placed in the box to test the left and right preference, which the stimulate source was the empty container, it found that tree shrews have no preferences (P > 0.05). The identification of female urine odour at different reproductive stages was carried out. The urine of female subjects was collected at oestrus and during pregnancy and lactation.

**Urine sample collection and extraction**

We placed odour donors individually in a clean plastic mouse cage (40 × 40 × 40 cm) with a wire grid floor (0.5 × 0.5 cm) 1 cm above the bottom. We collected urine samples from 8:00 to 11:00 AM of the dark phase during 4 consecutive days. Once an animal urinated, its urine was absorbed by a glass capillary, transferred to a vial, and stored on ice. The duration of urine collection for each animal was 20 min. Potentially contaminated urine deposited next to faeces was not collected. The samples were held in 12-mL sample bottles (HM-1266A, Ningbo Hamai, Ningbo Zhejiang, China), and 5–10 mL of urine was collected. Five millilitres of each sample was added to a 20-mL headspace bottle, capped and placed in a 55°C water bath for 40 min. Fiber solid-phase microextraction adsorption was performed for 20 min. The sample was subjected to HS-SPME (headspace solid phase microextraction) extraction fibre at 250°C without shunt desorption for 10 min, cooled to room temperature and extracted with methanol, ether and hexane, washed with deionised water and used for the gas chromatography-mass spectroscopy (GC-MS) analysis.

**GC-MS analysis**

Analytical GC-MS was performed on an Agilent Technologies Network 6890N GC system (Palo Alto, CA, USA) coupled with the 5973 Mass Selective Detector and the NIST 2002 library. Xcalibur (Windows XP) was used for data acquisition and processing. The GC was equipped with a 30-m glass capillary column (internal diameter 0.25 mm × 0.25 µm film) coated with HP5MS. Helium was used as the carrier gas, at a flow rate of 1.0 mL/min. The temperature of the injector was set to 230°C. Oven temperature was programmed as follows: 50°C was the initial temperature, which was increased by 4°C/min up to 240°C and then held for 5 min. Electron impact ionisation was used at 70 eV. The transfer line temperature was 280°C. The scanning mass ranged from 30 to 350 amu. The amount of urine sample injected was 1 µL in split mode (10:1). Tentative identification was made by comparing the mass spectra of the GC peaks with those in the MS library (Wiesler et al. 1984).

**Y-maze test**

The method described by Zhang et al. (2008a) served as the reference. The Y-maze apparatus had a conductive grid floor and consisted of three identical arm boxes (40 × 10 × 20 cm³) made of dark, opaque Plexiglas that were symmetrically opposed 120° to each other. Arms 1 and 3 were stimulus source boxes and arm 2 was an adaptation box. The behavioural observation room was equipped with a 15-W incandescent lamp and a real-time monitoring infrared camera. The formation rate was about 1 m/s in arms 1 and 3. The animals adapted for 15–20 min in arm 2, the front door of the box was opened and performance was recorded for 10 min.
Statistical analysis

Data were analysed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Prior to all statistical analyses, data were examined for assumptions of normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively. Differences in behaviour of tree shrews in the Y-maze test were detected using the paired t-test. Results are presented as mean ± standard error (SE), and $P < 0.05$ was considered significant.

Results

Volatile components of urine from the three sources

The chromatograms of urine components in wild, lab-acclimated, and laboratory-reproducing groups are shown in Figures 1–3. The GC-MS results reveal that many of the compounds in the urine samples had similar retention times, and were mainly aldehydes, ketones, acids, aromatic hydrocarbons, phenols and alkanes. A total of 92 compounds were detected in the urine of wild tree shrews (Table I), including eight kinds of alcohols, four aldehydes, 16 alkanes, two amides, three ethers, 15 esters, seven ketones, two phenols, six acids, three aromatic hydrocarbons, one alkene and one sulphur compound dimethyl sulphoxide (DMSO). The compounds with the highest concentrations were decanoic acid, ethyl ester 2-propenoic acid and 2-methyl-2-ethyl-2-[[2-methyl-1-oxo-2-propenyl]oxy]methyl]-1,3-propanediyl ester. Sixty-five compounds were detected in the urine of the laboratory-acclimated tree shrews, including five kinds of alcohols, eight alkanes, three ethers, eight esters, seven ketones, two phenols, two acids, two
Table I. Comparison of compounds identified in urine of *Tupaia belangeri* in the wild, laboratory-acclimated and laboratory-breeding.

| RT (min) | Compound | Wild | Acclimation | Breeding |
|----------|----------|------|-------------|----------|
| 2.35     | Thirane, methyl- | +    |             |          |
| 2.47     | Ethanedioic acid, diethyl ester | +    |             |          |
| 2.51     | Oxalic acid | +    |             |          |
| 2.58     | 2-Butanone | +    |             |          |
| 2.66     | 1,3-Butanediol, (S)- | +    |             |          |
| 2.79     | 2-Butanol | +    |             |          |
| 2.79     | Pentane, 3-methyl- | +    |             |          |
| 2.96     | Diborane(6), α-mercaptopro- | +    |             |          |
| 2.96     | Ethyl oxamate | +    |             | +        |
| 2.99     | Formic acid, 1-methylpropyl ester | + | + |          |
| 3.01     | Acetic acid, oxo- | +    |             |          |
| 3.13     | 1-Propanamine, 3-ethoxy- | +    |             |          |
| 3.21     | Methanamine, 1,1-bis(2,2-dimethylpropoxy)-N,N-dimethyl- | +    |             |          |
| 3.26     | Butane, 2-isothiocyanato- | +    |             |          |
| 3.46     | Dimethyl ether | +    |             |          |
| 3.81     | Methane, nitroso- | +    |             |          |
| 3.85     | 1-Butanol, 2-methyl-, (S)- | +    |             |          |
| 3.89     | Lactic acid | +    |             |          |
| 4.2      | sec-Butyl fluoroformate | +    |             |          |
| 4.76     | Cyclobutene, 2-propenylidene- | +    |             |          |
| 4.88     | Methoxyacetic acid, butyl ester | +    |             |          |
| 5.39     | Undecane | +    | +           |          |
| 6.25     | 1-Hexanol | +    | +           |          |
| 6.31     | Decane, 1-chloro- | +    | +           | +        |
| 6.36     | Hexane, 1-chloro- | +    |             |          |
| 6.69     | p-Xylene | +    | +           |          |
| 6.71     | 2,4-Dithiapentane | +    |             |          |
| 8.89     | 1-Hepten-3-one | +    |             |          |
| 8.91     | 1-Octen-3-one | +    |             |          |
| 9.03     | 1-Heptyln-3-ol | +    |             |          |
| 9.04     | 3-Hexanol, 2,2-dimethyl- | +    |             | +        |
| 9.67     | 2,7-Octadiene-1,6-diol, 2,6-dimethyl- | +    | +          | +        |
| 10.09    | Phenol | +    |             |          |
| 10.42    | 1-Hexanol, 2-ethyl- | +    | +           | +        |
| 10.52    | Octadecanoic acid, phenyl ester | +    |             |          |
| 11.62    | Cyclopropane, pentyl- | +    |             |          |
| 11.63    | 1-Octanol | +    |             |          |
| 11.66    | Cyclopropane, pentyl- | +    |             |          |
| 11.96    | Pyrazine, tetramethyl- | +    |             |          |
| 12.42    | 1,6-Octadien-3-ol, 3,7-dimethyl- | +    | +           |          |
| 12.47    | Nonanal | +    | +           |          |
| 12.85    | Dodecane | +    | +           |          |
| 13.02    | Octanoic acid, ethyl ester | +    | +           |          |
| 13.28    | Cyclohexanecarboxylic acid, ethyl ester | +    |             |          |
| 13.36    | 1-Octanol, 2-methyl- | +    |             |          |
| 14.07    | 2,3,5-Trimethyl-6-ethylpyrazine | +    |             |          |
| 14.14    | Thiophene, 2-pentyl- | +    |             |          |
| 15.05    | 4-Nonene, 5-butyl- | +    |             |          |
| 15.07    | Octanoic acid | +    |             |          |
| 15.2     | Benzoic acid | +    |             |          |
| 15.25    | Methyl salicylate | +    |             |          |
| 15.37    | Estragole | +    |             |          |
| 15.57    | Ethyl 3-cyclohexene carboxylate | +    |             |          |
| 15.67    | Acetic acid, 2-phenylethyl ester | +    | +           |          |
| 16.12    | Butanoic acid, 2-methyl-, hexyl ester | +    |             |          |
| 16.42    | 1-Cyclohexene-1-carboxylic acid | +    |             |          |
| 16.78    | Tridecane | +    | +           | +        |
| 16.95    | 2-Nonen-1-ol, (E)- | +    |             |          |
| 17.23    | Dodecane, 2,7,10-trimethyl- | +    | +           |          |

(Continued)
| RT (min) | Compound                                                                 | Wild | Acclimation | Breeding |
|---------|---------------------------------------------------------------------------|------|-------------|----------|
| 17.55   | Nonanoic acid                                                             | +    |             |          |
| 18.72   | Dodecane, 2,6,11-trimethyl-                                              | +    |             |          |
| 19.51   | Propanoic acid, 2-methyl-, 2,2-di-methyl-1-(2-hydroxy-1-methyl)propyl ester | +    |             |          |
| 19.67   | Dodecane, 2,6,10-trimethyl-                                              | +    |             |          |
| 19.78   | Eugenol                                                                  | +    | +           |          |
| 19.84   | Cyclooctadecane                                                           | +    |             |          |
| 19.89   | Ethoxyl trans-4-decenoate                                                | +    |             |          |
| 20.06   | Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester          | +    |             |          |
| 20.13   | Decanoic acid, ethyl ester                                               | +    | +           |          |
| 20.39   | Tetradecane                                                              | +    | +           |          |
| 20.61   | n-Decanoic acid                                                           | +    |             |          |
| 21.44   | 2-Dodecyl-1-y(+)succinic anhydride                                         | +    |             |          |
| 21.49   | Octanoic acid, 3-methylbutyl ester                                        | +    |             |          |
| 21.72   | Methyl tetrahydroxionol                                                  | +    |             |          |
| 21.96   | (E)-α-Famesene                                                           | +    |             | +        |
| 22.01   | Heptadecane, 2,6,10,14-tetramethyl-                                       | +    |             | +        |
| 22.44   | 2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethyl)                       | +    |             | +        |
| 22.45   | 2,3-Cyclododecenopyridine                                                | +    |             | +        |
| 22.62   | Heptadecane                                                              | +    |             | +        |
| 22.63   | 2-Piperidinone, N-[4-bromo-n-butyl]-                                      | +    |             | +        |
| 23.26   | Butylated Hyroxytoluene                                                  | +    |             | +        |
| 23.38   | Octadecane                                                               | +    |             |          |
| 23.46   | Phenol, 2,4-bis(1,1-dimethyl)                                             | +    |             | +        |
| 23.67   | Hexadecane                                                               | +    |             | +        |
| 23.75   | Eicosane                                                                 | +    |             |          |
| 25.48   | Propanoic acid, 2-methyl-, 1-(1,1-dimethyl)-1,3-propanediyl ester         | +    |             | +        |
| 25.64   | Cedrol                                                                   | +    |             |          |
| 26.28   | Phenol, 2,6-bis(1,1-dimethyl)-4-(1-methyl)                                | +    |             | +        |
| 26.37   | Pentadecane, 2,6,10-trimethyl-                                           | +    |             | +        |
| 26.67   | Decanoic acid, decyl ester                                               | +    |             |          |
| 26.93   | 1,13-Tetradecaadiene                                                     | +    |             |          |
| 27.21   | Benzene, (1-ethyl-1-methyl)propyl-                                        | +    |             |          |
| 27.45   | 3,5,5-Trimethylhexyl acetate                                             | +    |             |          |
| 27.52   | Heptadecane                                                              | +    |             | +        |
| 27.63   | Nonadecane                                                               | +    |             | +        |
| 27.64   | Phenol, 2,4-di-t-butyl-6-nitro-                                           | +    |             | +        |
| 27.65   | Heptadecane, 2,6,10-trimethyl-                                           | +    |             | +        |
| 27.69   | Decanoic acid, decyl ester                                               | +    |             |          |
| 27.75   | 1,13-Tetradecaadiene                                                     | +    |             | +        |
| 27.82   | Decane, 5,6-bis(2,2-dimethylpropilidene)-, (E,Z)-                         | +    |             | +        |
| 28.7    | Diphenyl sulphoxide                                                      | +    |             | +        |
| 28.93   | 2,3-Cyclodecenopyridine                                                  | +    |             | +        |
| 29.29   | Phenol, 2-(1,1-dimethyl)phenyl-                                           | +    |             | +        |
| 29.61   | Hexadecane, 2,6,10,14-tetramethyl-                                       | +    |             | +        |
| 30.34   | Cyclooctadecane                                                          | +    |             | +        |
| 30.35   | 8-Cyclohexadecene-1-one                                                  | +    |             | +        |
| 30.69   | 2-Pentadecano, 6,10,14-trimethyl-                                        | +    |             | +        |
| 30.78   | 2-Pentadecano, 6,10,14-39trimethyl-                                      | +    |             | +        |
| 30.98   | 1-Octadecanol, methyl ether                                               | +    |             | +        |
| 30.99   | 1-Eicosanol                                                              | +    |             | +        |
| 31      | Hexadecene-1-ol, trans-9-                                                | +    |             | +        |
| 31.02   | 1-Hexadecanol                                                            | +    |             |          |
| 31.38   | 1,2-Benzenedicarboxylic acid, (2-methylpropyl) ester                     | +    |             | +        |
| 31.49   | Cyclohexadecane                                                          | +    |             |          |
| 33.25   | Dibutyl phthalate                                                        | +    |             | +        |
| 33.44   | Cyclopentadecane, 4-methyl-                                              | +    |             |          |
| 33.72   | Nonadecane, 2-methyl-                                                    | +    |             |          |
| 33.78   | 9-Octadecenal                                                           | +    |             |          |
aromatic hydrocarbons, two kinds of alkenes and one pyrazine. Ethyl ether and n-hexane were the two compounds with the highest concentrations. Sixty-four chemicals were identified in the urine of laboratory-breeding animals, including six kinds of alcohols, 11 alkanes, two ethers, eleven esters, nine ketones, six phenols, two alkenes and one aromatic hydrocarbon. The two most concentrated compounds were α-farnesene and butylated hydroxytoluene. Among the three sources of animals, hexadecane, 2,6,10,14-tetramethyl-; hexadecanoic acid ethyl ester; hexadecen-1-ol, trans-9-nonadecane; nonanal; n-pentadecycloclohexane were unique chemical constituents in the urine of wild tree shrews. It found that 4-Methyl-cyclopentadecanone was unique to the urine from laboratory-acclimated animals, whereas 1,13-tetradecadiene was unique to the laboratory-breeding animals.

**Identification of non-breeding animals by body and urine odours**

Using male and females as stimuli, females selected females significantly more frequently than other males ($t = -2.13$, $P < 0.05$, Table II). Females and males did not choose the control ($P < 0.01$, Table II). Using male or female urine odour as the stimulus, tree shrews chose the urine odour of the opposite sex (female: $t = -2.13$, $P < 0.05$; male: $t = 1.92$, $P < 0.05$, Table III). Using male or female urine and the control as stimuli, tree shrews did not choose the control (Table III). The differences in volatile substances between male and female urine are shown in Table IV. Thiirane, methyl- and pyrazine were unique volatile substances in female urine, whereas α-farnesene, 9-octadecenal, cyclopropane andpentyl- were unique volatile substances in male urine. When male and female tree shrews were exposed to their own urine and that of the opposite sex as the odour source, females and males showed a significant preference for their own urine (female: $t = 2.29$, $P < 0.01$; male: $t = 2.36$, $P < 0.01$, Table V).

**Identification of female urine odour at different reproductive stages**

Male tree shrews’ stay duration to urinary odour of oestrous and lactating females was significantly

| Table I. (Continued). |
|-----------------------|
| RT (min) | Compound | Wild | Acclimation | Breeding |
|----------|-----------|------|-------------|----------|
| 33.79    | 13-Octadecenal, (Z)- | +    |             |          |
| 34.05    | Octadecanal | +    |             |          |
| 34.1     | Pentadecanal- | +    |             |          |
| 34.28    | 2-Propenoic acid, 2-methyl-2-[[(2-methyl-1-oxo-2-propenyl)oxy]methyl]-1,3-propanediyl ester | +    |             |          |
| 34.37    | 9-Cycloheptadecen-1-one, (Z)- | +    | +          |          |
| 34.41    | Tricosane, 2-methyl- | +    |             |          |
| 34.5     | 8,11,14-Eicosatrienoic acid, (Z,Z,Z)- | +    |             |          |
| 34.63    | Behenic alcohol | +    |             |          |
| 35.89    | n-Pentadecyclohexane | +    | +          |          |
| 35.91    | Cyclohexane, octyl- | +    |             |          |

Note: +: Presence detected; RT: Retention Time.

| Table II. Preference of Tupaia belangeri for conspecific individuals in Y-maze test. |
|---------------------------------|
| Sex of individual | Animal stimulus (s) | Female | Male | Control | $P$ value |
|-------------------|---------------------|--------|------|---------|-----------|
| Female            | 235.70 ± 12.78      | 212.80 ± 11.16 | 85.50 ± 9.65 | < 0.01 |
| Female            | 239.60 ± 30.54      | 180.10 ± 15.54 | 70.20 ± 4.12 | < 0.01 |
| Female            | 162.90 ± 14.43      | 102.00 ± 17.08 | 79.50 ± 4.12 | < 0.01 |
| Male              | 170.50 ± 3.59       | 69.40 ± 2.78  | 70.20 ± 4.12 | < 0.01 |
| Male              | 172.10 ± 16.22      | 102.00 ± 17.08 | 70.20 ± 4.12 | < 0.01 |

Data are means ± standard error (SE); ns: non-significant ($P > 0.05$); $P < 0.05$ indicates significant difference; $P < 0.01$ represents highly significantly different between two groups.
longer than their stay duration to pregnant females (Table VI). Sixty volatile components were detected in female urine during oestrus, including 19 alcohols, eight ketones, six aldehydes and three alkenes. During pregnancy, there were 76 volatile components in female urine, including 23 alcohols, seven ketones, three aldehydes, three phenols and four heterocyclics. There were 69 volatile

Table III. Preference of Tupaia belangeri to different urine odours in Y-maze test.

| Sex of individual | Odour stimulus (s) | Female | Male | Control | P value |
|-------------------|-------------------|--------|------|---------|---------|
| Female            |                   | 319.40 ± 7.02 | 102.70 ± 4.84 | < 0.01 |
| Female            |                   | 316.80 ± 4.18  | 112.70 ± 3.02  | < 0.01 |
| Female            |                   | 141.30 ± 15.21 | 226.40 ± 25.36 | < 0.05 |
| Male              |                   | 298.20 ± 7.54  | 101.80 ± 7.75  | < 0.01 |
| Male              |                   | 265.80 ± 15.60 | 73.80 ± 4.01   | < 0.01 |
| Male              |                   | 214.40 ± 20.70 | 159.30 ± 13.91 | < 0.05 |

Data are means ± standard error (SE); ns: non-significant (P > 0.05); P < 0.05 indicates significant difference; P < 0.01 represents highly significantly different between two groups.

Table IV. Sex difference of volatility compounds in urine of non-breeding Tupaia belangeri.

| RT (min) | Compound                                             | Female | Male |
|---------|------------------------------------------------------|--------|------|
| 4.76    | Cyclobutene, 2-propenylidene-                        | +      | +    |
| 5.39    | Undecane                                             | +      | +    |
| 6.25    | 1-Hexanol                                            | +      | +    |
| 6.36    | Hexane, 1-chloro-                                    | +      | +    |
| 9.67    | 2,7-Octadiene-1,6-diol, 2,6-dimethyl-                 | +      | +    |
| 10.09   | Phenol                                               | +      | +    |
| 10.42   | 1-Hexanol, 2-ethyl-                                  | +      | +    |
| 11.62   | Cyclopropane, pentyl-                                | +      | +    |
| 12.47   | Nonanal                                              | +      | +    |
| 12.85   | Dodecane                                             | +      | +    |
| 14.17   | Pyrazine                                             | +      | +    |
| 16.78   | Tridecane                                            | +      | +    |
| 19.78   | Eugenol                                              | +      | +    |
| 20.13   | Decanoic acid, ethyl ester                           | +      | +    |
| 22      | 5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-           | +      | +    |
| 22.34   | á-Farnesene                                          | +      | +    |
| 22.44   | 2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)- | +      | +    |
| 22.9    | Pentadecane                                          | +      | +    |
| 23.26   | Bulylated Hydroxyltoluene                           | +      | +    |
| 23.26   | Bulylated Hydroxyltoluene                           | +      | +    |
| 23.96   | Phenol, 2,4-bis(1,1-dimethylethyl)-                  | +      | +    |
| 25.24   | Hexadecane                                           | +      | +    |
| 26.37   | Pentadecane, 2,6,10-trimethyl-                       | +      | +    |
| 26.93   | 1,13-Tetradecadiene                                  | +      | +    |
| 27.52   | Heptadecane                                          | +      | +    |
| 29.84   | Hexadecane, 2,6,10,14-tetramethyl-                    | +      | +    |
| 30.35   | 8-Cyclohexadecan-1-one                              | +      | +    |
| 31      | Hexadecen-1-ol, trans-9-                             | +      | +    |
| 31.02   | 1-Hexadecanol                                        | +      | +    |
| 31.38   | 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester | +      | +    |
| 32.41   | Cyclohexadecanone                                    | +      | +    |
| 33.25   | Dibutyl phthalate                                    | +      | +    |
| 33.44   | Cyclopentadecanone                                   | +      | +    |
| 33.44   | Cyclopentadecanone, 4-methyl-                        | +      | +    |
| 33.78   | 9-Octadecenal                                        | +      | +    |
| 34.05   | Octadecanal                                          | +      | +    |
| 34.37   | 9-Cycloheptadecan-1-one, (Z)-                        | +      | +    |

Note: +: Presence detected; RT: Retention Time.
components in lactating female urine, including 22 alcohols, 11 ketones, two aldehydes, three phenols and four heterocyclics. Hexadecane was a unique constituent of urine from females in oestrus, whereas 2,3-hexanedione, 2-buten-1-ol and 3-methyl- were unique during pregnancy. Cyclohexanone and 2-methyl-5-(1-methylethenyl)- were unique chemical constituents in urine from lactating females (Table VII).

**Discussion**

**Volatile components of urine from the three sources**

The urine from the wild, laboratory-acclimated, and reproducing tree shrews had large amounts of ketones. The urine of other mammals, such as *Odocoileus virginianus* (Miller et al. 1998) and *Antechinus stuartii* (Toftegaards et al. 1999) also has ketone compounds. In the present study, the chemical components of the wild populations were at higher concentrations than those in the acclimated and breeding animals. In addition, the types of urine chemicals were similar between the acclimated and breeding animals, probably because indoor tree shrews are fed a single diet and food resources are richer in the field. Moreover, the molecular weights of the tree shrew urine chemicals were < 300, which are small and volatile, and can be used as signals to rapidly transmit to other individuals within a certain range. We speculate that the differences in chemical composition and content of individual urine may help convey information about the individual tree shrew. One study reported that trans-5-hepten-2-one, trans-4-hepten-2-one, n-pentyl acetate and cis-2-penten-1-yl acetate are volatile components in the urine of tree shrews, which are compounds that can delay oestrus in female mice (Novotny et al. 1999). Decanoic acid and cyclopropane can expel the same individual in mongoose (Woodley & Baum 2003).

**Identification of non-breeding animals using to body and urine odours**

Body odour is vital to the maintenance and survival of animals. Many mammals rely on individual odours to recognise the opposite sex, social status and other crucial information, thereby improving the fitness of the individuals. *Lingnania chungin* use body odour to distinguish males from females (Zhang et al. 2008b). *Tscherskia triton* prefers the odour of the opposite sex in the Y-maze odour-selection experiment (Zhang et al. 2008b). *Tscherkia triton* prefers the odour of the opposite sex in the Y-maze odour-selection experiment (Zhang et al. 2008b). In the present study, using the tree shrews as stimulus, females selected females significantly more frequently than they selected males, and males selected females significantly more frequently than they selected males. However, tree shrews did not choose the control when it was offered as a stimulus, suggesting that the tree shrew can use body odour molecules for sex recognition.

Both non-volatile and volatile substances in urine can encode sex information. Major urinary proteins in wild mice differ in males and females, and mice can use the odour to recognise sex (Hayakawa et al.

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**Table V. Preference of *Tupaia belangeri* to urine odour from different period in Y-maze.**

| Sex of individual | Odour stimulus (s) | P value |
|------------------|-------------------|---------|
|                  | Oestrum           | Pregnancy | Lactation |
| Female           | 173.70 ± 13.35     | 129.00 ± 6.83 | < 0.01 |
| Male             | 166.50 ± 9.89      | 110.00 ± 9.97 | < 0.01 |

Data are means ± standard error (SE); *P* < 0.01 indicates very significant difference between two groups.

**Table VI. Preference of male *Tupaia belangeri* to female urine odour from different period in Y-maze.**

| Sex of individual | Odour stimulus (s) | P value |
|------------------|-------------------|---------|
|                  | Oestrum           | Pregnancy | Lactation |
| Male             | 140.10 ± 10.92     | 58.50 ± 7.62 | < 0.01 |
| Male             | 145.80 ± 8.73      | 152.40 ± 10.60 | ns |
| Male             | 95.60 ± 11.12      | 152.40 ± 10.60 | < 0.01 |

Data are means ± standard error (SE); ns: non-significant (*P* > 0.05); *P* < 0.01 indicates very significant difference between two groups.
Table VII. Compounds identified in urine of the *Tupaia belangeri* in estrus, pregnancy and lactation.

| RT (min) | Tentatively identified compound | Oestrum | Pregnancy | Lactation |
|----------|---------------------------------|---------|-----------|-----------|
| 2.67     | Acetone                          | +       |           |           |
| 3.45     | Ethyl Acetate                    | +       | +         | +         |
| 3.92     | Butanal, 3-methyl-               | +       |           |           |
| 4.17     | 2-Butanone, 3-methyl-             | +       |           |           |
| 4.25     | Isopropyl alcohol                | +       |           |           |
| 5.29     | 2-Pentanone                      | +       | +         | +         |
| 5.31     | 2,3-Butanedione                   | +       | +         | +         |
| 5.66     | 3-Buten-2-one, 3-methyl-          | +       | +         | +         |
| 7.52     | 3-Cyclohexene-1-methanol, α,4-trimethyl- | + | + | + |
| 7.80     | 3-Hexanone                       | +       |           |           |
| 8.10     | Disulphide, dimethyl              | +       |           |           |
| 8.45     | Hexanal                          | +       | +         |           |
| 9.87     | 4-Heptanone                      | +       | +         | +         |
| 10.09    | 2,3-Hexanediode                  | +       |           |           |
| 10.11    | (R)-(−)-2-Pentanol                | +       |           |           |
| 10.22    | o-Xylene                         | +       |           |           |
| 10.26    | 1H-Pyrole, 1-methyl-              | +       | +         | +         |
| 10.87    | 1-Butanol                        | +       | +         | +         |
| 11.77    | 2-Heptanone                      | +       | +         | +         |
| 11.84    | Pyridine                         | +       | +         | +         |
| 12.37    | 2-Heptanone, 3-methyl-            | +       | +         | +         |
| 12.77    | 1-Butanol, 2-methyl-              | +       | +         | +         |
| 14.05    | 3-Octanol                        | +       |           |           |
| 14.11    | 1-Pentanol                       | +       | +         | +         |
| 14.89    | 2,4-Dithiapentane                | +       |           |           |
| 14.95    | 2-Butanone, 3-hydroxy-            | +       | +         | +         |
| 14.96    | 2-Butanone                       | +       | +         | +         |
| 15.03    | Cyclohexanone                     | +       |           |           |
| 15.08    | Octanal                          | +       | +         | +         |
| 16.11    | Pyrazine, 2,5-dimethyl-           | +       | +         | +         |
| 16.16    | 2,5-Octanediode                  | +       | +         | +         |
| 16.18    | 2-Buten-1-ol, 3-methyl-           | +       |           |           |
| 16.29    | Pyrazine, 2,6-dimethyl-           | +       | +         | +         |
| 16.52    | 5-Hepten-2-one, 6-methyl-         | +       | +         | +         |
| 17.94    | 3-Hexen-1-ol, (Z)-               | +       |           |           |
| 17.98    | 2-Nonanone                       | +       | +         | +         |
| 18.1     | Nonanal                          | +       | +         |           |
| 18.23    | 3-Octanone                       | +       | +         | +         |
| 19.49    | cis-Linalooloxide                 | +       | +         | +         |
| 19.50    | a-Methyl-a-[4-methyl-3-pentenyl]oxiranemethanol | + | | |
| 19.73    | 1-Octen-3-ol                     | +       | +         | +         |
| 19.81    | Thiophene, 2-pentyl-              | +       | +         | +         |
| 19.87    | 1-Heptanol                       | +       | +         | +         |
| 20.07    | 6-Hepten-1-ol, 2-methyl-          | +       | +         | +         |
| 20.24    | 2-Furanmethanol, 5-ethylnitrohydro-α,α,5-trimethyl-, cis- | + | + | |
| 20.77    | 1-Hexanol                        | +       | +         | +         |
| 20.77    | 1-Hexanol, 2-ethyl-               | +       | +         | +         |
| 20.92    | Pentadecane                      | +       |           |           |
| 21.44    | Benzaldehyde                     | +       | +         | +         |
| 21.52    | 2-Nonanone                       | +       |           |           |
| 22.18    | 1,6-Octadien-3-ol, 3,7-dimethyl-  | +       | +         | +         |
| 22.52    | 1-Octanol                        | +       | +         | +         |
| 23.40    | Hexadecane                       | +       |           |           |
| 23.43    | 1-Octanol, 2-methyl-              | +       | +         | +         |
| 23.78    | Benzaldehyde, 2-methyl-           | +       |           |           |
| 23.88    | 5-Octen-1-ol, (Z)-               | +       |           |           |
| 24.34    | 3,6-Dipropyl-2,5-dimethylpyrazine | +       | +         | +         |
| 24.92    | 1-Nonanol                        | +       | +         | +         |
| 25.13    | Propanol, 2-(4-ethoxyphenyl)-2-methyl- | + | + | |
| 25.30    | 2-Methyl-2-butyl-1,3-benzodioxole | +       | +         | +         |

(Continued)
Chemical information in the form of non-volatile and volatile small-molecule complexes can be perceived in urine in *Microtus mandarinus* (Zhang et al. 2010). Other volatile substances used for recognising sex, such as contents of E-β-farnesene, E,E-α-farnesene and 1-hexadecanol, were found at higher concentrations in male urine than in female, whereas concentrations of squalene, 2-heptanone and 4-ethyl phenol were higher in female urine than in male urine, and the animals use these substances to identify sex. In the present study, sex differences were detected in tree shrew urine components. Thiirane, methyl- and pyrazine were the unique constituents in female urine, which is similar to results found for *Mustela furo* (Zhang et al. 2005). The unique constituents in male urine were α-farnesene, 9-octadecenal, cyclopropane and pentyl- which were combined with the behavioural results.

### Identification of urine odour from females at different reproductive stages

The urine of mammals contains information about reproductive state. For example, significant differences in the chemical composition of urine from lactating and non-lactating female *Microtus oeconomus* were reported from a behavioural study in which males identified differences in females of different reproductive states, in favour of lactating females (Zhang et al. 2010). In the present study, male tree shrews identified females in oestrus.

#### Table VII. (Continued).

| RT (min) | Tentatively identified compound | Oestrum | Pregnancy | Lactation |
|---------|--------------------------------|---------|-----------|-----------|
| 25.31   | 4-(3,4-Methylenedioxyphenyl)-2-butanone | +       |           |           |
| 25.46   | 3-Nonen-1-ol, (Z)- |         | +         | +         |
| 25.46   | Bicyclo[4.1.0]heptane, 3-methyl- | +       |           |           |
| 25.75   | 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)- | +       |           | +         |
| 26.35   | 1,3,6,10-Dodecatetraene, 3,7,11-trimethyl-, (Z,E)- | +       |           |           |
| 26.61   | Naphthalene | +       | +         |           |
| 26.86   | α-Farnesene | +       | +         | +         |
| 27.23   | Cyclohexanone, 2-methyl-5-(1-methylethenyl)- | +       |           |           |
| 27.30   | 6-Octen-1-ol, 3,7-dimethyl- | +       |           | +         |
| 27.30   | 6-Octen-1-ol, 7-methyl-3-methylene- | +       |           |           |
| 27.96   | (Z)-4-Decen-1-ol | +       | +         |           |
| 27.96   | Z-4-Dodecenol | +       | +         | +         |
| 28.05   | 2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)- | +       |           |           |
| 28.29   | Benzenemethanol, α-methyl- | +       | +         |           |
| 29.18   | 5,9-Undecadien-2-one, 6,10-dimethyl- | +       | +         |           |
| 29.23   | Phenol, 2-methoxy- | +       | +         | +         |
| 29.32   | Spiro[2.4]heptane, | +       | +         | +         |
| 29.36   | 1,5-dimethyl-6-methylene- | +       | +         |           |
| 29.66   | Propanoic acid, 2-methyl-, 1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester | +       | +         | +         |
| 30.36   | Phenylethyl Alcohol | +       | +         |           |
| 30.93   | Benzene, 1,4-dimethyl-2,5-bis(1-methylethyl)- | +       | +         |           |
| 31.02   | 3,4-Dihydroxybenzyl alcohol,tris(trimethylsilyl)- | +       | +         |           |
| 31.53   | Cyclodecane | +       | +         |           |
| 31.53   | Cyclooctane | +       | +         |           |
| 32.21   | Phenol | +       | +         |           |
| 33.70   | Phenol, 4-methyl- | +       | +         |           |
| 34.49   | Cedrol | +       | +         |           |
| 34.50   | Epicedrol | +       | +         |           |
| 35.49   | Benzoic acid, 2-ethylhexyl ester | +       |           |           |
| 37.49   | Benzenamine, N-[4-(1-methylethyl)benzyldene]-4-(1-pyrolidylsulphonyl)- | +       | +         |           |
| 37.92   | Phenol, 2,4-bis(1,1-dimethylethyl)- | +       | +         |           |
| 38.71   | 2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl- | +       | +         |           |
| 39.13   | Cyclohexadecanone | +       |           |           |
| 39.14   | Cyclopentadecanone | +       | +         |           |
| 40.15   | Indole | +       | +         |           |
| 41.77   | Dibutyl phthalate | +       |           |           |
| 41.78   | 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester | +       | +         | +         |
| 41.78   | Phthalic acid, butyl isohexyl ester | +       | +         | +         |
| 41.78   | 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester | +       | +         | +         |

Note: +: Presence detected; RT: Retention Time.
pregnant, or lactating based on urine odours, suggesting that the urine encodes reproductive information about female tree shrews.

Studies have shown that some of the volatile chemicals in the urine of lactating females differ from those in urine from non-lactating females. It showed that 2-iodide octane is unique to urine of lactating root voles, whereas 2-iodide hexane and 2,6,10-trimethyl-14-alkyl were unique in non-lactating urine, observed from differences in male individual behaviour towards the urine odors from lactating and non-lactating females, thus affecting the male mate choice (Zhang et al. 2010). In the present study, hexadecane was unique to female tree shrews in oestrus, and 2,3-hexanediol; 2-buten-1-ol, and 3-methyl- were unique constituents in the urine of pregnant tree shrews. Cyclohexanone and 2-methyl-5-(1-methylethenyl)- were unique to the urine of lactating females, suggesting that the urine composition of tree shrews changes during different physiological states, so the pheromones change also, thus affecting the choice made by males.

In conclusion, urine odour encodes female reproductive status and gender information, and tree shrews rely on these cues to choose a mate and recognise sex. Chemical communication based on urine plays an important role in the reproduction of T. belangeri.

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