Behavioral Ecology and Sociobiology (2019) 73:15
https://doi.org/10.1007/s00265-018-2627-y

ORIGINAL ARTICLE

Sex pheromone levels are associated with paternity rate in brown rats

Yao-Hua Zhang[1] · Lei Zhao[1,2] · Xiao Guo[1,2] · Jin-Hua Zhang[1] · Jian-Xu Zhang[1]

Received: 18 April 2018 / Revised: 4 December 2018 / Accepted: 12 December 2018

Abstract

In muroid rodents, urine-borne volatile compounds and major urinary proteins (MUPs) constitute the key male pheromones that shape the sexual attractiveness of males. Here, we aimed to examine whether male pheromone levels were related to sexual attractiveness and reproductive success in the North China subspecies of the brown rat (*Rattus norvegicus humiliatus*). According to the abundance of 2-heptanone (2H), the predominant male pheromone in male urine, male rats were first categorized into a high-2H group and a low-2H group. The levels of the whole volatile profile and non-volatile MUPs were found to be higher in the high-2H group than in the low-2H group. Moreover, the abundances of urinary volatile pheromones or pheromone candidates were positively correlated with the abundance of total MUPs. Two-way choice tests revealed that male urine from the high-2H group was more attractive to females than that from the low-2H group. Microsatellite loci analysis of paternal lineage revealed that the females had single-paternity offspring and that the high-2H group sired more offspring and had higher rates of paternity than did the low-2H group. These results suggest that urine-borne volatile pheromones alone or together with MUP pheromones can predict sexual attractiveness and reproductive success in male rats.

Significance statement

Sexual attractiveness can be quantified using volatile and MUP pheromones and their candidates in deposited urine. 2-Heptanone (a major pheromone) and other urine-borne volatile pheromones or their candidates and total MUPs showed the same difference patterns in males and predicted male sexual attractiveness. The abundances of volatile pheromones or their candidates and total MUPs were associated with reproductive success and paternity rate in males.

Keywords Pheromone · MUP · Sexual attractiveness · Paternity

Introduction

In sexual selection studies, the relationships of male attractive traits with female mate choice, male reproductive success, and paternity are of considerable interest (Andersson and Simmons 2006). Mate choice, or intersexual selection, as one of two components of sexual selection, often refers to a female's choice of a male mate and depends on the attractiveness of the male's phenotypic traits (Andersson and Simmons 2006; Møller 2017). Females typically prefer to mate with more attractive males over less attractive ones, which leads to more attractive male members of a population often (but not always) having greater reproductive success and higher rates of paternity (Kortet and Hedrick 2005; Hosken et al. 2008; Hunt et al. 2009; Møller 2017). To date, several mate choice hypotheses have been proposed to explain the evolution of mate choice, which assumes that the traits associated with reproductive success are heritable (Andersson and Simmons

Yao-Hua Zhang and Lei Zhao contributed equally to this work.

Communicated by A. I. Schulte-Hostedde

✉ Jian-Xu Zhang
zhangjx@ioz.ac.cn

Yao-Hua Zhang
zhangyh@ioz.ac.cn

Lei Zhao
zhaolei@ioz.ac.cn

Xiao Guo
guoxiao@ioz.ac.cn

[1] State Key Laboratory of Integrated Management of Pest Insects and Rodents in Agriculture, Institute of Zoology, Chinese Academy of Sciences, Beichen West Road 1-5, Chaoyang District, Beijing 100101, China

[2] University of Chinese Academy of Sciences, Beijing 100049, China

Published online: 24 January 2019

2006; Taylor et al. 2007; Ingleby et al. 2013). Females often prefer to mate with attractive males because of the direct benefits they obtain or the indirect genetic benefits accrued by their offspring, such as good genes and sexy sons, which are conferred by the favorable alleles inherited from their attractive father (Qvarnstrom and Forsgren 1998; Wedell and Tregenza 1999; Andersson and Simmons 2006; Taylor et al. 2007). Some work revealed that the effect of male sexual attractiveness on female mate choice may be more powerful than that of genetic compatibility/similarity (e.g., inbreeding avoidance); for instance, mating among relatives occurs more often than expected by chance (Tregenza and Wedell 2000; Roberts and Gosling 2003; Szulkin et al. 2013; Zhang and Zhang 2014). The evolution of male attractive traits can be driven by female choice, as exemplified by the auditory and visual signals in crickets, fishes, and birds (Andersson and Simmons 2006). However, the relationships of specific male attractive traits with female mate choice and paternity have seldom been experimentally investigated in muroid rodents, particularly wild ones. The lack of such studies might be due to the greater difficulties involved in deciphering released chemosensory signals as determinants of male attractiveness compared to deciphering auditory and visual ones (Drickamer et al. 2000; Roberts and Gosling 2003; Nelson et al. 2013; Kumar et al. 2014; Zhang and Zhang 2014; Nelson et al. 2015; Fang et al. 2016).

Overall sexual attractiveness is a composite trait, but it is difficult to quantify by measuring multiple characteristics (Scheuber et al. 2003; Taylor et al. 2007; Simmons et al. 2013; Lopes and König 2016). Pheromones that function as intraspecific chemosensory signals play vital roles in socio-sexual interactions in rodents, and they can be used by females to precisely assess the quality of a potential mate of the same species (Roberts and Gosling 2003; Roberts et al. 2010; Wyatt 2014; Zhang and Zhang 2014; Fang et al. 2016). In mice and rats, urine-borne male pheromones include volatile organic compounds (VOCs) and major urinary proteins (MUPs) (Dewsbury 1990; Roberts et al. 2010; Kumar et al. 2014; Zhang and Zhang 2014; Guo et al. 2015; Fang et al. 2016). Deposited urine as a pheromone source remains consistently attractive to females in the physical absence of a male and should indicate the overall sexual attractiveness of the male; therefore, urinary VOCs and MUPs can be used to quantify the sexual attractiveness of males to females (Novotny et al. 1990; Penn and Potts 1998a; Johansson and Jones 2007; Roberts et al. 2010; Kumar et al. 2014). Additionally, numerous studies have demonstrated that some volatile male pheromones or MUP pheromones serve as indicators of immunocompetence and male quality and evoke female olfactory preferences for potential mates in mice and rats (Roberts and Gosling 2003; Roberts et al. 2010; Zhang et al. 2010; Nelson et al. 2013; Kumar et al. 2014; Guo et al. 2015, 2018; Fang et al. 2016; Lee et al. 2017). However, direct evidence regarding the relationships between reproductive success and specific pheromone components, particularly those related to overall sexual attractiveness in rodents, remains lacking.

The brown rat (*Rattus norvegicus*) is a worldwide pest species and the wild predecessor to the laboratory rats used for biological research. Based on genetic linkage analysis performed via methods such as DNA fingerprinting and microsatellite loci analysis, female brown rats have been demonstrated to be promiscuous, to be attracted to the specific attributes of a particular male rat, and to make definitive and consistent mate choices rather than random choices (Shimmin et al. 1995; Berdoy and Drickamer 2007; Zewail-Foote et al. 2009; Winland et al. 2012; Kumar et al. 2014; Costa et al. 2016). Female rats typically prefer one male among multiple potential mates and produce the majority of pups in the litter with one male in a group (Lovell et al. 2007; Zewail-Foote et al. 2009; Winland et al. 2012). Urine-borne pheromones have been shown to be potentially related to male sexual attractiveness to females and male reproductive success in rats (Papes et al. 2010; Kumar et al. 2014; Zhang and Zhang 2014). Several male pheromones, such as urinary volatile ketones (4-heptanone, 2-heptanone and 9-hydroxy-2-nonanone) and two MUP pheromones (OBP3 and MUP13), have been recently identified in rats and exhibit androgen-dependency, sexual dimorphism, and sexual attractiveness to females; therefore, it is feasible to examine the relationships between attractive traits and male paternity in rat species (Kumar et al. 2014; Zhang and Zhang 2014; Guo et al. 2018).

Brown rats have morphologically and genetically differentiated into four subspecies in China. Among these subspecies, *R. n. humiliatus* possesses high levels of pheromone expression in male urine and a small body size, and it is mainly distributed in Beijing and Hebei Province of northern China (Wu 1982; Wang 2003; Musser and Carleton 2005; Guo 2016; Teng et al. 2017). Here, using third- or fourth-generation captive-bred adult *R. n. humiliatus*, we investigated the relationships between the abundances of urinary volatile pheromones or pheromone candidates and MUPs in males and the number of sired pups through two-way chemosensory choice tests and paternity tests conducted via microsatellite loci analysis to determine the paternal lineages. We hypothesized that urine-borne pheromones and pheromone candidates would predict sexual attractiveness and rate of paternity in males of the *R. n. humiliatus* subspecies.

Materials and methods

Animals

The ancestors of captive-bred brown rats (*R. n. humiliatus*) were live-captured in rural areas of Beijing, China and

maintained as an outbred colony of approximately 400 individuals in our laboratory. All the rats used for breeding were maintained as male-female pairs in plastic rat cages (37 × 26 × 17 cm) (purchased from the Suzhou Feng Laboratory Animal Equipment Co Ltd., Suzhou, China) with wood-shaving bedding (Beijing KeAoXieLi Feeds Co., Ltd., Beijing, China). Standard rat chow (Beijing KeAoXieLi Feeds Co., Ltd., Beijing, China) and tap water were provided ad libitum. The housing room had a reversed 14:10-h light: dark photoperiod (lights on at 19:00) and was maintained at 23 ± 2 °C. The rats used for the female chemosensory preference and paternity tests were third- or fourth-generation rats and were caged in groups of three or four same-sex siblings after weaning at 4 weeks of age. All the male rats aged 3 to 6 months were caged individually for 2 weeks prior to urine collection and paternity test.

Twelve sex-naïve females aged 12–16 weeks were caged in groups of four and first used as scent recipients to test the chemosensory responses to male urine. Then, they were subjected to breeding trios for mate choice and paternity tests. All the female rats used had a 4- and 5-day estrous cycle, and only the estrous individuals were used for chemosensory preference tests. The phase of the estrous cycle was determined by vaginal smear cytology (Marcondes et al. 2002).

Urine collection

Twelve male individuals aged 3 to 6 months were used urine donors and then assigned to breeding trios for paternity tests. We used 20 clean metabolic rat cages to collect urine from the rats during the dark phase of the light cycle in the room. The urine from each metabolic cage was fed into a collection tube immersed in an ice box. Urine was collected from each rat for 8 h, and food and water were constantly available. The urine samples were stored at − 20 °C until use. The metabolic cages were cleaned thoroughly with water and 75% alcohol between collections.

Gas chromatography-mass spectrometry analysis

To prepare the samples for gas chromatography-mass spectrometry analysis (GC-MS) assay, we used dichloromethane (purity > 99.5%; DIMA Technology, Inc., Muskegon, MI, USA) and added 5 ppm of 1-tridecanol as an internal standard as a solvent for urine extraction. We mixed 150 μL of urine sample from each male donor with 150 μL of dichloromethane, stirred each subsample thoroughly, and stored the subsamples at 4 °C for 12 h. The bottom phase (the layer with dichloromethane) was used for chemical analysis (Zhang and Zhang 2014).

Chemical analysis was performed on an Agilent Technologies Network 6890N GC system coupled with a 5973 Mass Selective Detector (NIST 2008 version; Agilent Technologies, Inc., Santa Clara, CA, USA). The GC was equipped with an HP5-MS capillary column (30-m long, 0.25-mm inner diameter, 0.25-μm film). The carrier gas was helium (1.0 mL/min). The injection temperature was 280 °C. The oven temperature was initially set at 40 °C, then increased by 5 °C/min to 100 °C, then ramped at 10 °C/min to 280 °C, and held for 5 min. Finally, the temperature was increased to 300 °C and held for 10 min to clean the column. Electron impact ionization was accomplished at 70 eV. The transfer line temperature was 280 °C. The scanning mass ranged from 30 to 450 amu. The samples (4 μL) were injected in splitless mode. Tentative identification was made by comparing the mass spectra of the GC peaks with peaks in the MS library (NIST2002). The presence of 4-heptanone, 2-heptanone, and 9-hydroxy-2-nonanone (all purity > 98%; ACROS Organics, Thermo Fisher Scientific Inc., Waltham, MA, USA) was further confirmed by matching their retention times and mass spectra with authentic analogs.

Grouping of male rats according to the abundance of urinary 2-heptanone

Twenty-four sexually naïve males at 3 to 6 months of age were selected from 10 litters, with two or three males selected per litter. After urine collection and GC-MS analysis, the 12 rats that had higher abundances of urinary 2-heptanone (the most predominant volatile pheromone in male rat urine) were placed in a high-2H group, and the remaining 12 rats, which had lower abundances of urinary 2-heptanone, were placed in a low-2H group (2-heptanone abundance: median [inter quartile range]: high-2H 89.98 [63.45–192.79], low-2H 21.56 [15.33–32.59], $z = 4.157$, $n = 12$, $p < 0.001$) (Zhang et al. 2008; Zhang and Zhang 2014). The three breeding trios that failed to produce offspring were excluded from the analysis of urinary volatile compounds and total MUPs, paternity rate, and body weights between paired males.

Quantification of total MUPs via SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Mini Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). We diluted the urine samples by four-fold and mixed them with 5× sample buffer (250-mM Tris-HCl at pH 6.8, 10% (*w*/*v*) SDS, 0.5% (*w*/*v*) bromophenol blue, 50% (*v*/*v*) glycerol, and 5% (*v*/*v*) *β*-mercaptoethanol). We fractionated 2-μL samples by SDS-PAGE on a 15% gel at a 130-V constant voltage (Armstrong et al. 2005; Guo et al. 2015). Images were captured using a ChemiDoc XRS System (Bio-Rad Laboratories, Hercules, CA, USA), and the band intensities were quantified using the ImageJ program (Public Domain Image Processing Program, NIH, Bethesda, MD, USA). The band intensity of each urine sample was normalized by its ratio with the band

intensity of the same urine sample used as a reference in different gels and then used as the measure of MUP abundance for later pairwise comparisons (Ilayaraja et al. 2014; Lanuza et al. 2014).

Two-way chemosensory preference tests

To minimize observer bias, blinded methods were used when all behavioral data were recorded and/or analyzed. One male urine sample selected from the high-2H group was paired with another non-sibling male from the low-2H group and presented to a female to test female chemosensory preferences as previously described (Zhang and Zhang 2014). Tests were carried out under dim red light during the dark phase of the light cycle. For each test, we left one estrous female subject in its home cage after removing its cage-mates. Two scented glass rods (20-cm long, 4-mm diameter each), each of which had been scented with 2 μL of urine sample from either the high-2H group or the low-2H group, were simultaneously presented to the subject through the bars of the cage. We recorded the investigation time for each urine sample for 3 min after the subject first sniffed or licked the rod tip. For each trial, a urine sample from the high-2H group was randomly paired with a sample from the low-2H group, and each pair of urine samples was used only once. To control for experimenter bias, the experimenter was blind to the nature of the sample, and the positions of the high-2H samples and low-2H samples were alternated each trial. The tests of each female subject were replicated with urine from different pairs of males over four consecutive days. Then, the sum of the four investigation times for each female was used to compare chemosensory preferences between the high-2H group and the low-2H group. Each female subject was tested only once a day.

Two-way mate choice tests

Analogous to the chemosensory preference tests, one male from the high-2H group was randomly paired with a non-sibling male from the low-2H group and presented to individual females in a choice test; each male was subsequently tested for paternity. The trials were carried out in a three-chamber testing apparatus constructed from three plastic rat cages (37 × 26 × 17 cm). Two cages served as choice cages (plastic, 37 × 26 × 17 cm) and were symmetrically connected to the long side of the neutral cage by acrylic tubes (50-cm long, 7-cm inner diameter). The connected tubes had a removable perforated galvanized iron sheet as a door. A sexually naïve, non-sibling female was placed in the neutral cage, whereas the male subjects were fitted with small plastic collars and loosely tethered within the choice cages. The two males, one from the high-2H group and one from the low-2H group, were in separate choice cages and had no direct contact with each other (Fig. 1). We acclimated the animals in the apparatus for 24 h and then removed the doors, allowing the female to freely move throughout the apparatus. We checked the animals twice a day, and the doors were closed once the females gave birth to pups. Pups were counted and subjected to tail biopsy at 3 days of age. Three millimeters of the tail tip from each pup was collected using sterile sharp scissors. After the pups weaned, the adults were anesthetized (pentobarbital sodium 45 mg/kg), and 3 mm of tail tip was collected from each adult. Each tail sample was stored in a 1.5-mL centrifuge tube at − 80 °C until DNA extraction.

The paired males in a breeding trio had similar body weights (with differences less than 5%; mean ± SE, 240.50 ± 18.113 g vs. 239.40 ± 22.096 g, $z = 0.102$, $n = 9$, $p = 0.919$).

DNA extraction and microsatellite analysis of paternity

The phenol-chloroform method was performed to isolate genomic DNA. Four hundred microliters of lysis buffer (20-mM Tris-HCl, 5-mM EDTA, 400-mM NaCl, 1% SDS (m/V), 200-μg/mL Trypsin K, pH = 8.0) was mixed with DNA samples in 1.5-mL centrifuge tubes, which were then bathed in 55-°C water overnight. We removed the impurities of RNAs and proteins by adding phenol:chloroform:isoamyl alcohol (25:24:1, Dingguo Biotechnology Co. Ltd., Beijing, China) to the digestion fluid. DNA was obtained via washing and precipitating the supernatant after centrifugation using isopropanol and ethanol separately. We air-dried the DNA

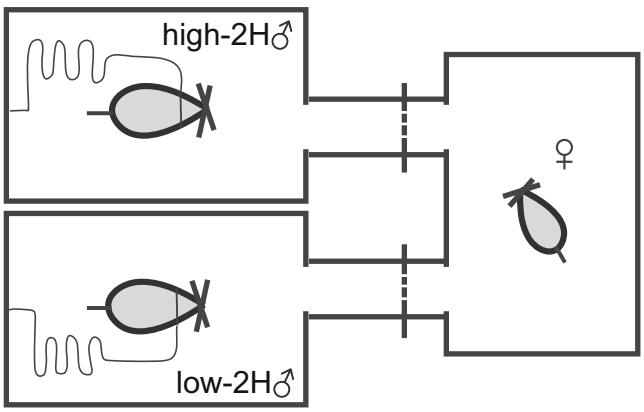

Fig. 1 Two-choice test device. A three-chamber testing apparatus was used in the mating preference and paternity tests. Two rat cages served as choice cages (plastic, 37 × 26 × 17 cm) and were symmetrically connected to the long side of the third, neutral cage (37 × 26 × 17 cm) by acrylic tubes (50-cm long; 7-cm inner diameter). The connected tubes had a removable perforated galvanized iron sheet as a door. Two male rats (high pheromone, dark gray; low pheromone, gray) were fitted with small plastic collars and loosely tethered within the choice cages; they were in separate choice cages and had no direct contact with each other. The female was placed in the neutral cage. The animals were acclimated in the apparatus for 24 h, and then, the doors were removed. The female was free to move throughout the apparatus

for 10 min, dissolved it in sterile water, and stored it at − 20 °C until use.

A NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to determinate the concentration and purity of DNA by measuring OD260/OD280. The concentration of satisfactory DNA was calibrated to 80~90 ng/μL for microsatellite analysis. Nine polymorphic microsatellite loci labeled with FAM, HEX and TAMRA dyes were synthesized from Invitrogen (Thermo Fisher Scientific Inc., Waltham, MA, USA) (Table 1). We genotyped subjects from all groups by PCR amplification (thermal cycler EDC-810, Eastwin Life Sciences Inc., Beijing, China) and capillary electrophoresis performed on an ABI 3130 genetic analyzer (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA, USA). PCR was carried out in a total volume of 20 μL that included 0.8 U of Takara Taq, 0.15 mM of each dNTP, 2-mM $MgCl_2$, 2 μL of PCR Buffer (Takara, Dalian, China), 0.4 μM of each primer (forward primer and reverse primer), and 80–90 ng of genomic DNA. An amplification procedure of denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing for 40 s and extension at 72 °C for 1 min; and a last extension at 72 °C for 10 min was followed.

Amplified products and allele sizes were analyzed and compared by using GeneMarker software (SoftGenetics, State College, PA, USA). In the current work, the relationships between mothers and their pups had already been determined. Based on half of the alleles of a pup being of maternal origin and the other half being of paternal origin, we isolated and identified the candidate fathers of all pups from among the paired males. The Cervus 3.0 program was used to evaluate exclusion probabilities for possible paternity (Marshall et al. 1998; Kalinowski et al. 2007). We excluded an irrelevant candidate male from paternal assignment by setting a minimum of 90% possibility. Power Stats V1.2 software (Promega, Madision, WI, USA) was applied to calculate the combined probability of exclusion through all nine microsatellite loci.

Statistical analysis

The distributions of the raw data were examined using a Kolmogorov–Smirnov test. Parametric tests and non-parametric tests were used for normally and non-normally distributed data, respectively. The abundance of each volatile compound in each urine sample was calculated as the compound's normalized GC peak area, determined by dividing its peak area with the peak area of 1-tridecanol, the internal standard, and multiplying it by 100. We tested for differences in the abundance of each volatile compound and the abundance of MUPs between the high- and low-2H groups using the independent *t* test or Mann-Whitney *U* test. Multiple comparisons were corrected by the false discovery rate (FDR) method (Benjamini and Yekutieli 2001). We also conducted principal component analysis on the abundance of the 11 early-eluting compounds and multivariate analysis of variance (MANOVA) with the principal components (Whittaker et al. 2010; Amo et al. 2012; Zhang et al. 2013). Pearson correlation analysis was used to assess the relationships between the principal components of the abundance of volatile compounds and the abundance of MUPs. The investigation time and litter per dam were examined for differences using Wilcoxon signed-rank test and binomial test, respectively. All statistical analyses were conducted using SPSS 18.0 software (SPSS Inc.,

Table 1 Microsatellite loci selected for paternity determination of individuals of a *R. norvegicus* subspecies (*R. n. humiliatus*) collected in Beijing. Related information (fluorescent dye types, primer sequences for amplification, and products of three microsatellites for capillary electrophoresis) is provided for each locus

Locus	Label	Primer sequences	Multiplex partner
D1Wox31	5′-HEX	5′-CATGCACACCCACTTACACAC-3′ 5′-CCTATTAGAACTTCCCCCTTC-3′	D8Wox7, D12wox1
D8Wox7	5′-FAM	5′-GGTATACAAAGCCTCGTGCA-3′ 5′-TGGGCTAAAGCTTATCCATTTA-3′	D1Wox31, D12wox1
D12wox1	5′-TAMAR	5′-GACATTAAGGGGTCTTCCTAAG-3′ 5′-TATCTTTGCAACGCTGAGG-3′	D1Wox31, D8Wox7
D6wox2	5′-TAMAR	5′-CCAGTCCATACTTATCCATCTG-3′ 5′-CATTTAGATAGGTGATAGAT TCAG-3′	D2Wox27, D19Wox11
D2Wox27	5′-HEX	5′-GATAATTGACATGTCCAGTTCC-3′ 5′-CTGGCTGATGGTAGGATGAG-3′	D6wox2, D19Wox11
D19Wox11	5′-FAM	5′-CTACCCACCCATCTATTCATCC-3′ 5′-GTTTCCAGCACCCATGTCC-3′	D6wox2, D2Wox27
D4Wox7	5′-TAMAR	5′-GATAGCATAAAATCCCTAGAGGTT-3′ 5′-TGCATTTATCTGAAACCATCAC-3′	D3Wox12, D10Wox11
D3Wox12	5′-HEX	5′-TATAGTAAGTTCGAGGCCGG-3′ 5′-AGGGGACCAGTGAGACTCAC-3′	D4Wox7, D10Wox11
D10Wox11	5′-FAM	5′-TCATCTGGTGGGGACATAAC-3′ 5′-GATGAACCAGCACATGGAAG-3′	D4Wox7, D3Wox12

Chicago, IL, USA), and the significance level was set at $p < 0.05$.

Results

The abundance of urinary 2-hepatanone and sexual attractiveness

R. n. humiliatus females preferred the urine of high-2H males to that of low-2H males (median [inter quartile range]: high-2H 6.20 [2.55–8.37], low-2H 4.78 [0.93–5.78], $z = 2.629$, $p = 0.009$, $n = 12$ for each group).

Group differences in the whole volatile profile and total MUPs

We detected and identified 11 early-eluting compounds from voided urine by GC-MS, including seven ketones, three phenols, and one sulfone. As the most abundant one of urinary volatiles judged by GC area values, 2-heptanone were confirmed by statistics to exhibit significant differences between the high-2H group and the low-2H group (2-heptanone, $z = 3.576$, $p < 0.001$, $n = 9$ for each group, Figs. 2 and 3). GC-MS further revealed higher abundances in the high-2H group than in the low-2H group of an additional six ketones that are potential male pheromone components and dimethyl sulfone (4-heptanone, $z = 3.488$, $p < 0.001$; dimethyl sulfone, $t = 4.789$, $p < 0.001$; 6-methyl-5-hepten-2-one, $z = 3.578$, $p < 0.001$; 3-ethyl-2,4-heptanedione, $z = 3.490$, $p < 0.001$; a dialkyl tetrahydro-2H-pyran-2-one, $z = 2.871$, $p = 0.006$; a dialkyl tetrahydro-2H-pyran-2-one, $t = 2.505$, $p = 0.032$; 9-hydroxy-2-nonanone, $z = 3.329$, $p = 0.002$, $n = 9$ for each group for Mann-Whitney U test and $df = 16$ for t test, Figs. 2 and 3). However, those phenols that are non-pheromone compounds showed no significant differences between groups (phenol, $t = 1.500$, $df = 16$, $p = 0.168$; 4-methyl-phenol, $z = 1.810$, $p = 0.086$, $n = 9$ for each group; 4-ethyl-phenol, $z = 1.104$, $p = 0.270$, $n = 9$ for each group; Figs. 2 and 3).

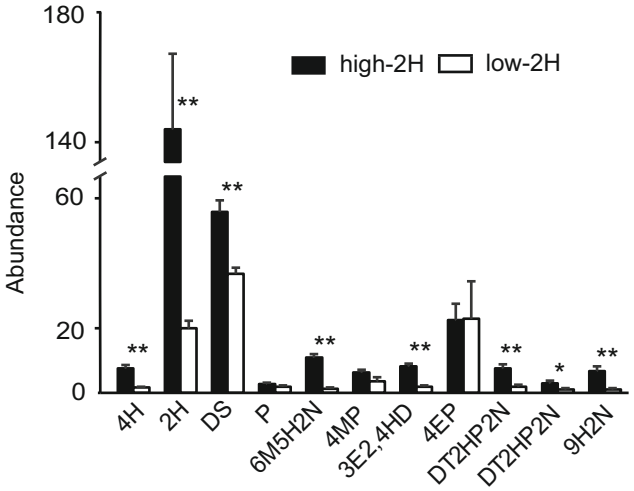

Fig. 3 Pairwise comparisons of the abundances of the 11 major urinary volatiles between the high-2H (black bars) and low-2H (white bars) groups (mean ± SE, $*p < 0.05$, $**p < 0.01$ based on the independent t test or Mann-Whitney U test) (4H = 4-heptanone; 2H = 2-heptanone; DS = dimethyl sulfone; P = phenol; 6M5H2N = 6-methyl-5-hepten-2-one; 4MP = 4-methyl-phenol; 3E2,4HD = 3-ethyl-2,4-heptanedione; 4EP = 4-ethyl-phenol; DT2HP2N = a dialkyl tetrahydro-2H-pyran-2-one; 9H2N = 9-hydroxy-2-honanone)

We extracted the three principal components with eigenvalues over 1 from the 11 early-eluting compounds, which differed significantly between the high-2H and low-2H groups (MANOVA, $F_{3,14} = 23.312$, $p < 0.001$). PC1, accounting for 59.95% of the extracted variation, differed significantly between groups ($F_{1,16} = 39.922$, $p < 0.001$), for which 2-heptanone, 4-heptanone, 6-methyl-5-hepten-2-one, 3-ethyl-2,4-heptanedione, a dialkyl tetrahydro-2H-pyran-2-one, and 9-hydroxy-2-nonanone had PCA loading scores ≥ 0.85. PC2, which accounted for 16.18% of the variation, and PC3, which accounted for 9.97% of the variation, showed no differences between groups (PC2, $F_{1,16} = 0.124$, $p = 0.729$; PC3, $F_{1,16} = 2.010$, $p = 0.175$).

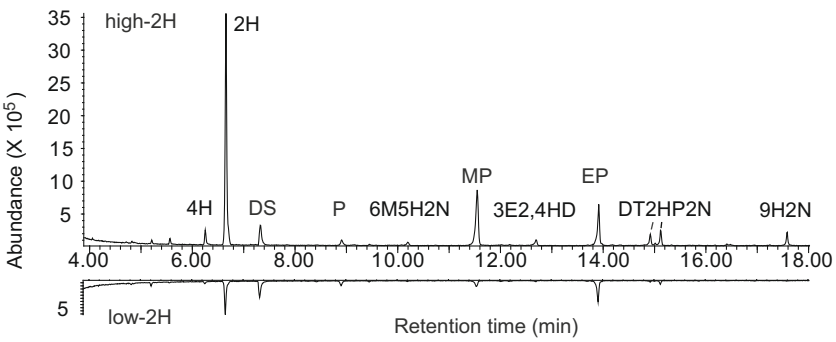

Fig. 2 Representative GC profile of a dichloromethane extract from the urine of males from the high-2H group (**a**) and low-2H group (**b**) in *Rattus norvegicus humiliatus*. GC conditions are described in the methods section (4H = 4-heptanone; 2H = 2-heptanone; DS = dimethyl sulfone; P = phenol; 6M5H2N = 6-methyl-5-hepten-2-one; 4MP = 4-methyl-phenol; 3E2,4HD = 3-ethyl-2,4-heptanedione; 4EP = 4-ethyl-phenol; DT2HP2N = a dialkyl tetrahydro-2H-pyran-2-one; 9H2N = 9-hydroxy-2-honanone)

The total MUPs in the high-2H group were also present at higher abundances than those in the low-2H group ($t = 4.423$, $n = 8$ for high-2H group, $n = 7$ for low-2H group, $df = 13$, $p = 0.001$, Fig. 4). The abundance of MUPs was strongly correlated with PC1 (PC1, $r = 0.831$, $p < 0.001$; PC2, $r = 0.129$, $p = 0.647$; PC3, $r = 0.014$, $p = 0.960$, $n = 15$).

Male pheromones and paternity rates

In the paternity tests, nine females successfully produced 76 pups for parental analysis. Females gave birth to an average of 8.44 pups (ranging from 4 to 10). Remarkably, the parentage testing through microsatellite analysis of genomic DNA revealed that the 69 pups of eight dams were all sired by males of the high-2H group, whereas the seven pups of the remaining dam were sired by a male of the low-2H group. Males of the high-2H group sired more offspring than did males of the low-2H group (binomial test, $n = 9$, $p = 0.039$).

Discussion

Females typically (but not always) prefer to mate with more attractive males over less attractive ones; therefore, the more attractive male members of a population are often believed to have greater reproductive success (Kortet and Hedrick 2005; Hosken et al. 2008). In rats, urine-borne volatile pheromones and MUPs of males have been demonstrated to determine the sexual attractiveness of males to female rats. Therefore, it has been inferred that these compounds might be correlated with reproductive success (Papes et al. 2010; Kumar et al. 2014; Zhang and Zhang 2014). Here, we provided the first experimental evidence that males bearing higher abundances of urinary pheromones were more sexually attractive to females, which then led to siring more offspring by the males, in brown rats.

Urine-borne male pheromones and pheromone candidates have been well documented and can be used to predict sexual attractiveness in rats (Papes et al. 2010; Zhang and Zhang 2011, 2014; Kumar et al. 2014; Takacs et al. 2016; Guo et al. 2018). Our previous work identified seven ketones in urine as male pheromone candidates based on their significantly higher levels in males than in females and their being under androgen control. Among these ketones, 4-heptanone, 2-heptanone, and 9-hydroxy-2-nonanone were synthesized and experimentally demonstrated to act as male pheromones and attract females in SD rats, BN rats, and Lewis rats (Zhang et al. 2008; Zhang and Zhang 2014). In this previous work, the band of approximately 19 kDa (predicted molecular weight) in SDS-PAGE clearly showed both male-biased sexual dimorphism and testosterone dependency, and the MUP pheromones corresponding to this band were consequently regarded as male pheromone candidates and putative male attractants to females. Among these candidates, OBP3 and MUP13 have since been verified to evoke the attraction and sexual arousal of female rats based on research using their recombinant analogs (Calzada-Garcia et al. 1996; Gómez-Baena et al. 2014; Kumar et al. 2014; Guo et al. 2018). Therefore, the abundances of VOCs analyzed by GC-MS and the abundance of total MUPs revealed by SDS-PAGE may be used to roughly calculate the sexual attractiveness of male urine in rats. In addition, we need to improve the procedures to quantify the amount of MUPs using protein assay, such as Western blot and optimizing urine sample loadings in our future work (Ilayaraja et al. 2014; Kumar et al. 2014; Lanuza et al. 2014).

As for *R. n. humiliatus*, the above-mentioned pheromones and pheromone candidates apparently exist in voided urine (Guo 2016). Here, we found that the abundances of both urine-borne volatile pheromones and MUPs exhibited quantitative variations among male individuals in *R. n. humiliatus*. In the high-2H rats and low-2H rats, which were categorized based on the abundance of urinary 2-heptanone, other ketones and total MUPs of pheromones or pheromone candidates expressed the same difference patterns. In particular, the PC1 of urine-borne volatile compounds, which had high factor loadings for the ketones and represented the whole VOC profile, was correlated with the abundance of total MUPs,

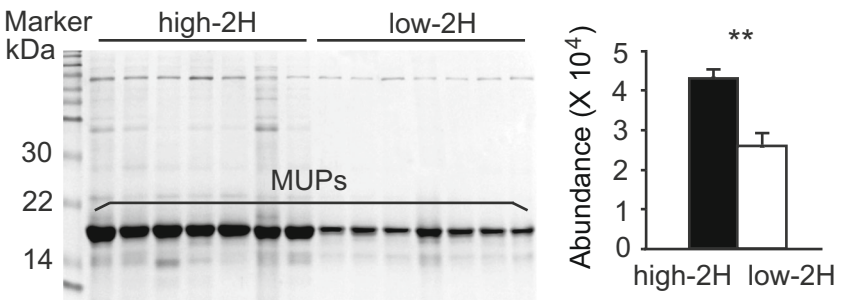

Fig. 4 Pairwise comparisons of the abundances of total major urine proteins (MUPs) between the high-2H and low-2H groups. The predominant bands between the marker of 22 and 14 kDa are MUPs. The abundance of total MUPs was determined by SDS-PAGE. The band intensities were quantified using the ImageJ program, and each band was normalized by the ratio with the same band from the same urine sample in different gels and used for pairwise comparison (mean ± SE, $^{**}p < 0.01$ based on the independent *t* test)

suggesting that the volatile pheromones and the MUP pheromones might work together to enhance sexual attractiveness (Roberts and Gosling 2003; Roberts et al. 2010; Fang et al. 2016). Furthermore, more volatiles require more MUPs to be bound to slow down their release for lasting communicative function (Armstrong et al. 2005; Roberts et al. 2010; Kumar et al. 2014). In this study, two-way chemosensory tests revealed that male urine containing higher abundances of these VOC components and MUPs had higher sexual attractiveness to females than did that with lower abundances in *R. n. humiliatus*.

Furthermore, using two-way mate choice tests and microsatellite analysis, we found that the more attractive male rats sired more offspring and exhibited higher rates of paternity than did the less attractive males, consistent with previous descriptions in many other animals (Kortet and Hedrick 2005; Hosken et al. 2008). Although the brown rat is a promiscuous species and females typically mate with multiple males within an estrous cycle, a single male typically sires the majority of a litter (Shimmin et al. 1995; Zewail-Foote et al. 2009; Winland et al. 2012; Costa et al. 2016). Here, we showed that the male rats possessing higher abundances of volatile pheromones and MUPs sired the vast majority of the pups; however, only single paternity was observed in each of the nine litters: In the two-way mate choice tests, eight litters were sired by males from the high-2H group, and one litter was sired by a male from the low-2H group. This study is the first to demonstrate that the pheromone-related attractiveness of male rats predicts the reproductive success of the bearers in the rat subspecies *R. n. humiliatus*. Our results are consistent with previous reports in house mice (*Mus musculus*) but inconsistent with previous results in other rat subspecies or laboratory rats (Drickamer et al. 2000; Hinson et al. 2006; Winland et al. 2012).

Multiple mate choice hypotheses, such as indicator mechanisms ("good genes" or "handicap mechanisms") and the Fisherian sexy son mechanism, can explain how the attractive traits or ornaments of males are often used by female choosers to assess the quality of potential mates and select males that yield indirect benefits through their offspring (Shimmin et al. 1995; Andersson and Simmons 2006; Zewail-Foote et al. 2009). Indicator mechanisms ("good genes" or "handicap mechanisms") may best illustrate the relationship between attractive traits and male genetic quality (Andersson and Simmons 2006). The immunocompetence handicap hypothesis (ICHH) states that the expression of male sexual signals involves the interaction of the endocrine system and immune function and that only high-quality males can afford to fully display sexual characteristics without experiencing immunosuppression or high parasite loads. Females that choose such males can provide heritable disease resistance to their offspring via the inheritance of favorable alleles from the sire (Folstad and Karter 1992; Penn and Potts 1998b; Lopes and König 2016). It is well-documented that the pheromone production is associated with androgen levels and immunocompetence in animals (Zhang et al. 2010; Guo et al. 2015, 2018). For example, immunocompetence-deficient male mice show reduced abundances of certain urinary volatile pheromones and Darcin (a male MUP pheromone) and consequently lower sexual attractiveness to females (Roberts et al. 2010; Zhang et al. 2010; Lopes and König 2016). In *R. n. humiliatus*, we speculate that if females prefer to mate with males carrying an abundance of pheromones such as 2-heptanone, they might choose higher quality male mates and pass on the genetic benefits, such as disease resistance, to their offspring (Rantala et al. 2002; Berdoy and Drickamer 2007; Lovell et al. 2007; Zewail-Foote et al. 2009; Winland et al. 2012; Kumar et al. 2014).

The evolution of sexual traits in natural populations of brown rats might also be affected by factors other than female mate choice. For example, rats bearing high abundances of sex pheromones might be subject to higher mortality because of increased predation risk (Brooks 2000; Fang et al. 2016; Zhang et al. 2016). In some cases, male-male competition can generate selection on sexually dimorphic traits that opposes that generated from female mate choice (Hunt et al. 2009). Therefore, further research on the influences of intrasexual selection, especially sperm competition, and natural selection on male pheromones is necessary to understand the evolution of male pheromones in natural populations of *R.n. humiliatus*.

Conclusions

VOCs, either alone or together with MUPs, act as male pheromones to attract females for mating in rats. Male brown rats with higher pheromone abundance were more sexually attractive to female mates and had higher rates of paternity compared to male rats with lower pheromone abundance.

Acknowledgments We thank the editors and the two anonymous reviewers for their constructive comments and suggestions, which helped to improve this manuscript.

Author contribution JXZ and YHZ conceived and designed the experiments. YHZ and JHZ performed the behavioral experiments and GC-MS analysis. LZ analyzed the paternity. LZ and XG performed the SDS-PAGE analysis. YHZ, LZ and JXZ wrote the paper. All authors read and approved the final manuscript.

Funding information This work was supported by grants from the Strategic Priority Research Program of the Chinese Academy of Sciences (grant number XDB11010400 to JXZ), and the National Natural Science Foundation of China (grant number 31672306 to YHZ and 31572277 to JXZ).

Data availability statements All data generated or analyzed during this study are included in this published article.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Ethical approval The procedures for animal care and use were performed in strict accordance with the guidelines of the Animal Use Committee of the Institute of Zoology, Chinese Academy of Sciences (IOZ 2015) and were approved by the Animal Use Committee of the Institute of Zoology, Chinese Academy of Sciences. Information regarding the animals' origin and housing conditions are described above. Handling and experimental monitoring were conducted in a manner aimed at reducing stress and maximizing animal welfare.

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

Amo L, Aviles JM, Parejo D, Pena A, Rodriguez J, Tomas G (2012) Sex recognition by odour and variation in the uropygial gland secretion in starlings. J Anim Ecol 81:605–613

Andersson M, Simmons LW (2006) Sexual selection and mate choice. Trends Ecol Evol 21:296–302

Armstrong SD, Robertson DH, Cheetham SA, Hurst JL, Beynon RJ (2005) Structural and functional differences in isoforms of mouse major urinary proteins: a male-specific protein that preferentially binds a male pheromone. Biochem J 391:343–350

Benjamini Y, Yekutieli D (2001) The control of the false discovery rate in multiple testing under dependency. Ann Stat 29:1165–1188

Berdoy M, Drickamer LC (2007) Comparative social organization and life history of *Rattus* and *Mus*. In: Wolff J, Sherman P (eds) Rodent societies: an ecological and evolutionary perspective. University of Chicago Press, Chicago, pp 380–393

Brooks R (2000) Negative genetic correlation between male sexual attractiveness and survival. Nature 406:67–70

Calzada-Garcia JA, Gonzalez MNP, BenitoAndres FJ, Garcia LJM, Cabezas JA, SanchezBernal C (1996) Evaluation of patterns of urinary proteins by SDS-PAGE in rats of different ages. Mech Ageing Dev 87:1–13

Costa F, Richardson JL, Dion K, Mariani C, Pertile AC, Burak MK, Childs JE, Ko AI, Caccone A (2016) Multiple paternity in the Norway rat, *Rattus norvegicus*, from urban slums in Salvador, Brazil. J Hered 107:181–186

Dewsbury DA (1990) Fathers and sons—genetic-factors and social-dominance in deer mice, *peromyscus-maniculatus*. Anim Behav 39:284–289

Drickamer LC, Gowaty PA, Holmes CM (2000) Free female mate choice in house mice affects reproductive success and offspring viability and performance. Anim Behav 59:371–378

Fang Q, Zhang YH, Shi YL, Zhang JH, Zhang JX (2016) Individuality and transgenerational inheritance of social dominance and sex pheromones in isogenic male mice. J Exp Zool B 326:225–236

Folstad I, Karter AJ (1992) Parasites, bright males, and the immunocompetence handicap. Am Nat 139:603–622

Gómez-Baena G, Armstrong SD, Phelan MM, Hurst JL, Beynon RJ (2014) The major urinary protein system in the rat. Biochem Soc Trans 42:886–892

Guo H, Fang Q, Huo Y, Zhang Y, Zhang J (2015) Social dominance-related major urinary proteins and the regulatory mechanism in mice. Integr Zool 10:543–554

Guo HL (2016) Interspecific chemical signals and behavioral interactions between *Rattus tanezumi* and *R. norvegicus*. Master Dissertation, University of Chinese Academy of Sciences, Chinese Academy of Sciences

Guo X, Guo H, Zhao L, Zhang YH, Zhang JX (2018) Two predominant MUPs, OBP3 and MUP13, are male pheromones in rats. Front Zool 15:6

Hinson ER, Hannah MF, Norris DE, Glass GE, Klein SL (2006) Social status does not predict responses to Seoul virus infection or reproductive success among male Norway rats. Brain Behav Immun 20:182–190

Hosken DJ, Taylor ML, Hoyle K, Higgins S, Wedell N (2008) Attractive males have greater success in sperm competition. Curr Biol 18:R553–R554

Hunt J, Breuker CJ, Sadowski JA, Moore AJ (2009) Male-male competition, female mate choice and their interaction: determining total sexual selection. J Evol Biol 22:13–26

Ilayaraja R, Rajkumar R, Rajesh D, Muralidharan AR, Padmanabhan P, Archunan G (2014) Evaluating the binding efficiency of pheromone binding protein with its natural ligand using molecular docking and fluorescence analysis. Sci Rep 4:5201

Ingleby FC, Hunt J, Hosken DJ (2013) Heritability of male attractiveness persists despite evidence for unreliable sexual signals in *Drosophila simulans*. J Evol Biol 26:311–324

Johansson BG, Jones TM (2007) The role of chemical communication in mate choice. Biol Rev 82:265–289

Kalinowski ST, Taper ML, Marshall TC (2007) Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol 16:1099–1106

Kortet R, Hedrick A (2005) The scent of dominance: female field crickets use odour to predict the outcome of male competition. Behav Ecol Sociobiol 59:77–83

Kumar V, Vasudevan A, Soh LJT, Le Min C, Vyas A, Zewail-Foote M, Guarraci FA (2014) Sexual attractiveness in male rats is associated with greater concentration of major urinary proteins. Biol Reprod 91:150

Lanuza E, Martin-Sanchez A, Marco-Manclus P, Cadiz-Moretti B, Fortes-Marco L, Hernandez-Martinez A, McLean L, Beynon RJ, Hurst JL, Martinez-Garcia F (2014) Sex pheromones are not always attractive: changes induced by learning and illness in mice. Anim Behav 97:265–272

Lee W, Khan A, Curley JP (2017) Major urinary protein levels are associated with social status and context in mouse social hierarchies. Proc R Soc B 284:20171570

Lopes PC, König B (2016) Choosing a healthy mate: sexually attractive traits as reliable indicators of current disease status in house mice. Anim Behav 111:119–126

Lovell JL, Diehl A, Joyce E, Cohn J, Lopez J, Guarraci FA (2007) "Some guys have all the luck": mate preference influences paced-mating behavior in female rats. Physiol Behav 90:537–544

Marcondes F, Bianchi F, Tanno A (2002) Determination of the estrous cycle phases of rats: some helpful considerations. Braz J Biol 62:609–614

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. Mol Ecol 7:639–655

Møller AP (2017) Experimental manipulation of size and shape of tail spots and sexual selection in barn swallows. Curr Zool 63:569–572

Musser GG, Carleton MD (2005) Superfamily Muroidea. In: Wilson DE, Reeder DM (eds) Mammal species of the world a taxonomic and geographic reference. Johns Hopkins University Press, Baltimore, pp 894–1531

Nelson AC, Cauceglia JW, Merkley SD, Youngson NA, Oler AJ, Nelson RJ, Cairns BR, Whitelaw E, Potts WK (2013) Reintroducing domesticated wild mice to sociality induces adaptive transgenerational effects on MUP expression. P Natl Acad Sci USA 110:19848–19853

Nelson AC, Cunningham CB, Ruff JS, Potts WK (2015) Protein pheromone expression levels predict and respond to the formation of social dominance networks. J Evol Biol 28:1213–1224

Novotny M, Harvey S, Jemiolo B (1990) Chemistry of male dominance in the house mouse, *Mus domesticus*. Experientia 46:109–113

Papes F, Logan DW, Stowers L (2010) The vomeronasal organ mediates interspecies defensive behaviors through detection of protein pheromone homologs. Cell 141:692–703

Penn D, Potts W (1998a) MHC-disassortative mating preferences reversed by cross-fostering. Proc R Soc Lond B 265:1299–1306

Penn D, Potts WK (1998b) Chemical signals and parasite-mediated sexual selection. Trends Ecol Evol 13:391–396

Qvarnstrom A, Forsgren E (1998) Should females prefer dominant males? Trends Ecol Evol 13:498–501

Rantala MJ, Jokinen I, Kortet R, Vainikka A, Suhonen J (2002) Do pheromones reveal male immunocompetence? Proc R Soc Lond B 269:1681–1685

Roberts SA, Simpson DM, Armstrong SD, Davidson AJ, Robertson DH, McLean L, Beynon RJ, Hurst JL (2010) Darcin: a male pheromone that stimulates female memory and sexual attraction to an individual male's odour. BMC Biol 8:75

Roberts SC, Gosling LM (2003) Genetic similarity and quality interact in mate choice decisions by female mice. Nat Genet 35:103–106

Scheuber H, Jacot A, Brinkhof MWG (2003) Condition dependence of a multicomponent sexual signal in the field cricket *Gryllus campestris*. Anim Behav 65:721–727

Shimmin GA, Sofronidis G, Bowden DK, Temple-Smith PD (1995) DNA fingerprinting to determine paternity in laboratory rat sperm competition experiments. Electrophoresis 16:1627–1632

Simmons LW, Thomas ML, Simmons FW, Zuk M (2013) Female preferences for acoustic and olfactory signals during courtship: male crickets send multiple messages. Behav Ecol 24:1099–1107

Szulkin M, Stopher KV, Pemberton JM, Reid JM (2013) Inbreeding avoidance, tolerance, or preference in animals? Trends Ecol Evol 28:205–211

Takacs S, Gries R, Zhai HM, Gries G (2016) The sex attractant pheromone of male brown rats: identification and field experiment. Angew Chem Int Ed 55:6062–6066

Taylor ML, Wedell N, Hosken DJ (2007) The heritability of attractiveness. Curr Biol 17:R959–R960

Teng HJ, Zhang YH, Shi CM, Mao FB, Cai WS, Lu L, Zhao FQ, Sun ZS, Zhang JX (2017) Population genomics reveals speciation and introgression between brown Norway rats and their sibling species. Mol Biol Evol 34:2214–2228

Tregenza T, Wedell N (2000) Genetic compatibility, mate choice and patterns of parentage: invited review. Mol Ecol 9:1013–1027

Wang YX (2003) A complete checklists of mammal species and subspecies in China. China Forestry Publishing House, Beijing

Wedell N, Tregenza T (1999) Successful fathers sire successful sons. Evolution 53:620–625

Whittaker DJ, Soini HA, Atwell JW, Hollars C, Novotny MV, Ketterson ED (2010) Songbird chemosignals: volatile compounds in preen gland secretions vary among individuals, sexes, and populations. Behav Ecol 21:608–614

Winland C, Bolton JL, Ford B, Jampana S, Tinker J, Frohardt RJ, Guarraci FA, Zewail-Foote M (2012) "Nice guys finish last": influence of mate choice on reproductive success in Long Evans rats. Physiol Behav 105:868–876

Wu DL (1982) Subspecies of the brown rat (*Rattus norvegicus Berkenhout*) in China. Acta Theriol Sin 2:107–112

Wyatt T (2014) Pheromones and animal behavior: chemical signals and signatures. Cambridge University Press, Cambridge

Zewail-Foote M, Diehl A, Benson A, Lee KH, Guarraci FA (2009) Reproductive success and mate choice in Long-Evans rats. Physiol Behav 96:98–103

Zhang JX, Sun L, Zhang YH (2010) *Foxn1* gene knockout suppresses sexual attractiveness and pheromonal components of male urine in inbred mice. Chem Senses 35:47–56

Zhang JX, Sun LX, Zhang JH, Feng ZY (2008) Sex- and gonad-affecting scent compounds and 3 male pheromones in the rat. Chem Senses 33:611–621

Zhang YH, Du YF, Zhang JX (2013) Uropygial gland volatiles facilitate species recognition between two sympatric sibling bird species. Behav Ecol 24:1271–1278

Zhang YH, Liang HC, Guo HL, Zhang JX (2016) Exaggerated male pheromones in rats may increase predation cost. Curr Zool 62:431–437

Zhang YH, Zhang JX (2011) Urine-derived key volatiles may signal genetic relatedness in male rats. Chem Senses 36:125–135

Zhang YH, Zhang JX (2014) A male pheromone-mediated trade-off between female preferences for genetic compatibility and sexual attractiveness in rats. Front Zool 11:73