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Chemical Identification and Bioactivity of Rat (*Rattus rattus*) Urinary Compounds

Ramasamy Selvaraj and Govindaraju Archunan*

Department of Animal Science, Bharathidasan University, Tiruchirappalli 620 024, India

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Ramasamy Selvaraj and Govindaraju Archunan (2002) Chemical identification and bioactivity of rat (*Rattus rattus*) urinary compounds. *Zoological Studies* **41**(2): 127-135. House rat urine was analyzed to identify the chemical compounds by gas chromatography-linked mass spectrometry (GC-MS). Male rat urine contained ethanol,2-(octylthio) (I), 1,3,5 triazone-2,4-diamine (II), and 1-chlorodecane (III). Similarly, female urine (during estrus) had the 3 compounds hydroperoxide (IV), 1-nitropentane (V), and 4-azidoheptane (VI). The bioactivity of these identified compounds were assayed using a Y-maze apparatus. Odor preference test revealed that the identified compounds show opposite-sex as well as same-sex attraction. Male urinary compounds such as ethanol,2-(octylthio) (I) and 1-chlorodecane (III) exhibited an attraction to the opposite sex, whereas the compound 1,3,5 triazone-2,4-diamine (II) was shown to attract the same sex. However, the compounds hydroperoxide and 4-azidoheptane were found to show maximum response in the attraction of the opposite sex. By contrast, the compound 1-nitropentane attracted both sexes, with a maximum response to the opposite sex. The results indicate that the compounds identified in the male urine are sex specific. The present investigations also show that rat urine contains a mixture of volatile compounds whose bioactivities differ from compound to compound. http://www.sinica.edu.tw/zool/zoolstud/41.2/127.pdf

Key words: Urine, Pheromone, Chemical characterization, Social behavior, Rat.

Rodents are considered one of the major pests of agricultural crops and stored food grains. Major crop losses caused by field rodents have been estimated in various places in India. Rodents may also adversely affect human health and cause great economic losses. Among rodents, rats are the dominant and highly infectious pests which infest human housing, sewers, animal shelters, day care facilities, warehouses, outdoor recreational areas etc. Furthermore, they serve as a reservoir of several important pathogenic vectors of diseases like plague, leptospirosis, ricketsial pox, rat bite fever, marine typhus fever, and so on (Jackson 1987). These detrimental effects are more pronounced particularly in developing countries like India. Thus, efforts have been made to control rats in some of the large metropolitan areas by using chemical rodenticides over the past few years. However, chemical control methods are made less effective by the rodent's bait shyness.

Although no specific method has overcome the poison bait shyness behavior of rodents, attempts have been made to use urine to reduce the shyness behavior. Kumari and Prakash (1980 1988) found that conspecific urine is able to mask the poison aversion and bait shyness behavior in the desert gerbil (Kumari and Prakash 1980) and Indian gerbil (Kumari and Prakash 1988). Since adding rat urine to poison bait successfully eliminates bait shyness behavior, this indicates that rat urine contains volatile compounds that are involved in reducing the poison bait shyness. It is not known whether a single agent or a mixture of compounds present in the urine acts in reducing the bait shyness.

Chemical identities of mammalian urinary cues in the mouse (Andreolini et al. 1987), tiger (Brahmacharry et al. 1992), elephant (Rasmussen et al. 1996), and bovine (Kumar et al. 2000) are avail-

*To whom correspondence and reprint requests should be addressed. E-mail : garchu@bdu.ernet.in

able. Furthermore, chemical characterization and the biological significance of secretions of the preputial gland (Kannan et al. 1998) and clitoral gland (Kannan and Archunan 2001) of laboratory rats and the cheek gland of the lesser bandicoot rat (Kannan and Archunan 1999) have recently been reported. However, chemical compounds of this type in rat urine have not yet been characterized. Therefore, the present study was undertaken to characterize rat urinary compounds, to investigate the bioactivity of the identified compounds, and to analyze the biological significance of the identified compounds with a view to producing a pheromonal trap to contribute to rodent pest management programs.

MATERIALS AND METHODS

Animals

Male and female rats, *Rattus rattus*, were collected from nearby villages and acclimatized to laboratory conditions for 2 wk prior to the experimental study. They were housed in the laboratory and reared on pelleted food (Hindustan Lever Ltd. Bangalore) and water *ad libitum*. Females weighing approximately 162.1 $\pm$ 0.73 g and with regular estrous cycles and males weighing approximately 205.9 $\pm$ 1.81 g and possessing scrotal testes were used in the present study. Male and female rats were housed separately in polypropylene cages (40 x 25 x 15 cm) with 40 x 25 x 2 cm of rice husk lining the bottom as bedding material. The bedding material was changed once in 3 or 4 d to maintain the hygienic condition of the animals. All animals were maintained on a schedule of a 12 L:12 D photoperiod throughout the experimental period.

Determination of estrus stage

During the reproductive life span, the female house rat, *Rattus rattus*, exhibits cyclic changes in the ovary which may lead periodically to ovulation. In the rat, reproduction is possible throughout the year (polyestrus). Vaginal smears obtained from an animal at different times of the cycle show respectively: nucleated epithelial cells during the preovulatory stage (proestrus), cornified epithelial cells during estrus, a mixture of cornified cells with an infiltration of leucocytes during the post ovulatory stage (metestrus), and a sparse smear of leucocytes and few epithelial cells during diestrus. An animal is considered to be in the estrus stage when the smear contains a major proportion of cornified cells (Archunan and Dominic 1991).

Urine collection

Fresh urine was collected from animals by gently massaging the flank region. The collected urine was used for solvent selection, GC-MS analysis, and compound separation by column chromatography.

Solvent selection

A suitable solvent was selected for extracting the appropriate chemical compounds from the urine. The 10 tested solvents, benzene, chloroform, dichloromethane, diethyl ether, ethanol, n-hexane, methanol, pentane, petroleum ether, and water, were mixed with urine individually. After extraction, a preference test was conducted with a Y-maze apparatus for 30 min. The solvent was smeared onto slides and was tested on a responder. The time spent by the responder with the scented and non-scented slides was noted for each solvent. Finally solvents were evaluated according to the time spent with the scented slides. Dichloromethane (DCM) was chosen as the most appropriate solvent in the present investigation, since the house rat responders spent more time with urine mixed with DCM as compared with other solvents. All data were subjected to statistical analysis of the mean, standard error of the mean and Student's *t*- test (Table 1).

Fractionation and GC-MS analysis

Fresh urine was used in the present study. The solvent (DCM) was added to the urine at a 1:1 ratio, which was able to extract the pheromonal compounds. After removing the supernatant, the sample (solvent-compound mixture) was collected in a glass vial and sealed with an airtight screw-type cap. Sample vials were stored at -20 ºC until further analysis.

The sample was fractionated, and chemical compounds were identified by gas-chromatography-linked mass spectrometry (GC-MS) analysis by comparison with standard compounds. The GC-MS analysis was performed on a Shimadzu QP5000 (Japan) instrument under computer control at 70 eV. Chemical ionization was performed by using ammonia as the reagent gas at 95 eV (Kannan et al. 1998). The chemical compounds were identified from the library NIST62. Urine with solvent was subjected to fractionation to separate the compounds. Samples were distilled for 30 min at room tempera-

ture under a vacuum of 226.6644 Pa. The distillate was reduced to 1/5 of its original volume by cooling with liquid nitrogen to condense it. Volatiles from the distilled fraction were subjected to gas chromatography for cross checking and confirmation.

Compound separation (column chromatography)

As per the results obtained by GC-MS analysis, the identified urinary compounds were separated by column chromatography. For this, fresh urine was used with the solvent DCM at a 1:1 ratio. The supernatant was discarded, and the remaining compound mixture was used for separating the compounds on the basis of retention time shown in GC-MS analysis.

Odor preference test

Since urine is constituted of many compounds, it is necessary to determine the bioactivity of each compound through an odor preference test with individuals of the same and opposite sex. Assuming the importance of the compounds, an odor preference test was conducted in a Y-maze apparatus. The Y-maze apparatus (150 x 15 x 15 cm) was made of tin sheeting. The sides were made of glass plates, whereas the top was covered with an iron mesh. This apparatus was able to provide food and water *ad libitum*. The size of the central arm was about 80 cm long and 15 cm wide. The remaining 2 choice arms were each 75 cm long and 15 cm wide.

Scented slides were placed on the right arm and solvent-smeared control slides were placed in the left arm during behavior analysis. At the top of the apparatus, a 0 W bulb was placed and switched on during the experimental period because the rat is a nocturnal animal. Both male and female rats were used as test animals for odor preference tests. In the case of female rats, the tests were conducted during estrus. The test animals were considered to be the responders and were taken from a pool of rats already maintained in the lab. In 3 different sets of the same and opposite sexes, 9 individuals (randomly taken from a pool of 50 rats) were used in each set for the odor preference tests. Fresh samples were used for each trial. The number of visits and the time spent near the urinary sample by the test animals were assessed for 15 min with the identified compounds and the solvent mixture being used as a control. Time devoted to visiting each fraction by tested animals was recorded and subjected to standard error of mean and Student's *t*- test (Zar 1984).

Table 1. Responses of male and female rats towards urine with various solvent candidates (5-min test)

S. No.	Name of solvent	Male to female	Female to male
1.	Benzene + urine	73.4 ± 1.71*	50.8 ± 3.23**
	Benzene	26.2 ± 1.36	24.2 ± 1.46
2.	Chloroform + urine	60.5 ± 1.94*	79.2 ± 3.34*
	Chloroform	23.2 ± 1.30	24.8 ± 1.65
3.	Dichloromethane + urine	140.8 ± 2.91*	135.6 ± 3.31*
	Dichloromethane	35.4 ± 1.21	24.2 ± 1.53
4.	Diethyl ether + urine	91.4 ± 2.15*	104.2 ± 5.93*
	Diethyl ether	29.0 ± 2.41	31.2 ± 1.10
5.	Ethanol + urine	79.0 ± 2.51*	66.6 ± 2.23*
	Ethanol	30.2 ± 1.63	23.4 ± 1.66
6.	n-Hexane + urine	61.4 ± 2.34*	126.6 ± 3.88*
	n-Hexane	23.4 ± 2.30	27.4 ± 1.08
7.	Methanol + urine	80.4 ± 2.12*	62.6 ± 2.79*
	Methanol	28.4 ± 0.96	26.8 ± 1.1
8.	Pentane + urine	75.2 ± 2.23*	86.0 ± 2.87*
	Pentane	23.2 ± 1.42	26.6 ± 0.96
9.	Petroleum ether + urine	78.6 ± 1.84*	51.61 ± 1.21*
	Petroleum	26.8 ± 1.60	22.8 ± 0.76
10.	Water + urine	46.4 ± 2.25*	42.2 ± 2.10**
	Water	26.2 ± 1.63	24.4 ± 1.84

Data are presented as the mean and the standard of error of the mean.
* Level of significance at $p < 0.001$ (Student's *t*- test).
** Level of significance at $p < 0.01$.

The urinary compounds were separated by column chromatography based on the results of the GC-MS study. Equal amounts (1:1) of the solvent, dichloromethane, was added to the urine. After removing the supernatant, the mixture of compounds present in the solvent was separated by column chromatography. Each separated compound was placed on a glass slide and tested against a blank slide (solvent slide) in the Y-maze apparatus with a procedure modified from Ferkin and Seamon (1987). Rat responses to the separate urinary compounds were noted. Three different sets of same- and opposite-sex animals were tested.

RESULTS

Among the 10 solvents tested, dichloromethane (DCM) was found to be the best, since urine mixed with DCM elicited maximum attraction by the responders (Table 1). GC-MS analysis showed that male and female rat urine contained 6 different extractable compounds. The identified compounds present in male urine were ethanol,2-(octylthio) (I), 1,3,5 triazone-2,4-diamine (II), and 1-chlorodecane (III) (Fig. 1). Female rat urine contained hydroperoxide (IV), 1-nitropentane (V), and 4-azidoheptane (VI) (Fig. 2). Behavior analysis showed that the male urinary compounds of ethanol,2-(octylthio) (I) and 1-chlorodecane (III) attracted the opposite sex at a maximum level, whereas the compound 1,3,5,triazone-2,4 diamine (II) attracted other males at a higher level (Table 2). All extractable female urinary compounds were found to show maximum attraction to the opposite sex, with the compound hydroperoxide (IV) exhibiting a maximum effect in opposite-sex attraction among the 3 compounds identified (Table 3).

Individual behavior analysis showed differences in responses among the responders towards male and female urinary compounds. In the behavior test with male urinary compounds, i.e., I vs. II, the male responder spent maximum time oriented towards compound II, whereas female responders were found to spend more time oriented towards compound I (Table 4). When the odor preference test was carried out between compounds I and III, compound I was found to attract the female responder more than did compound II. By contrast, compound III was found to attract male responders at a maximum level. When compound II was tested against compound III, male responders spent more

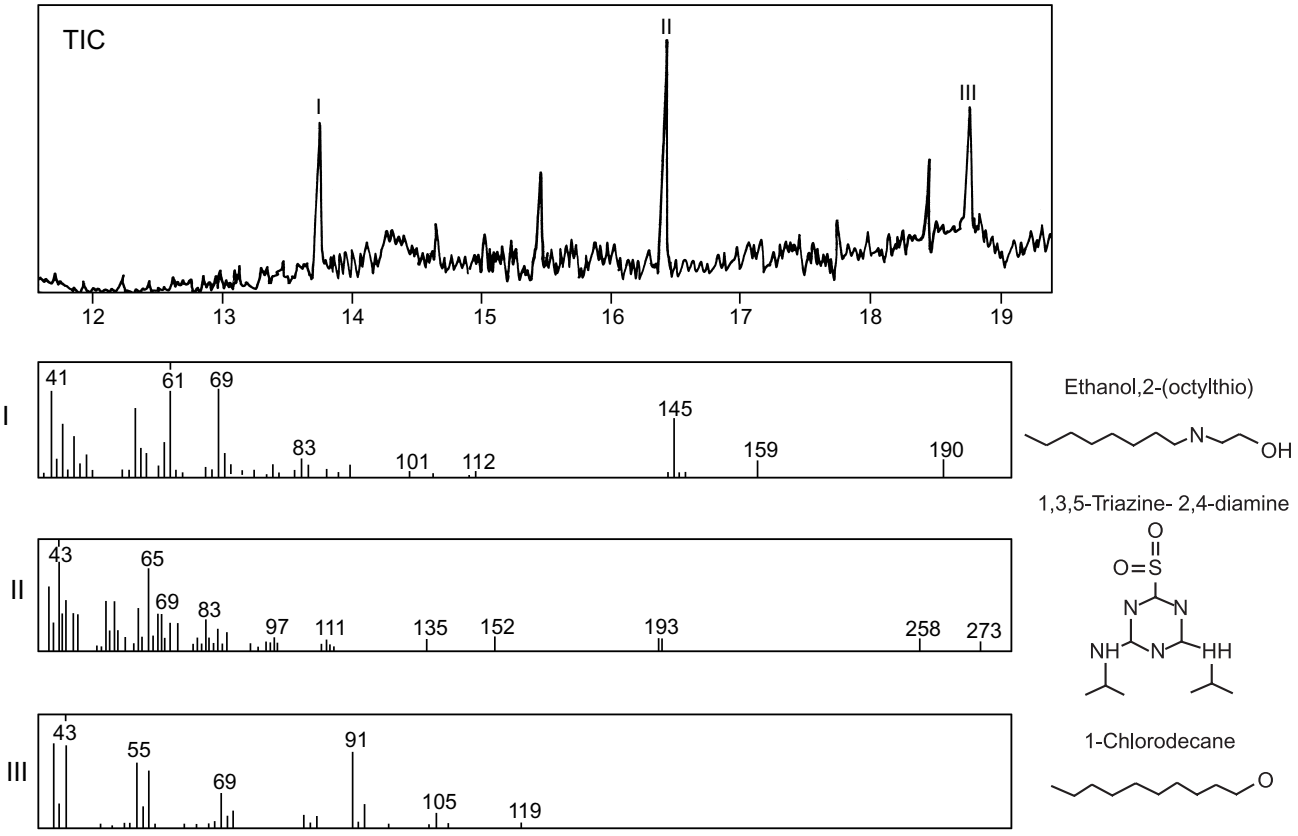

Fig. 1. Gas chromatographic profile, mass spectra, and chemical structures of identified compounds of male urine of *Rattus rattus*.

time oriented towards compound II. However, when the male urinary compounds I, II, and III were tested against urine, male urine was shown to attract both sexes at a maximum level. Yet the responses shown by male responders to compound II and urine were almost the same (Table 4).

In the odor preference test carried out between female urinary compounds, i.e., IV vs. V, IV vs. VI, and V vs. VI, male responders spent more time oriented towards all the compounds

Table 2. Bioassay responses of male and female rats to 3 different fractions of male urinary compounds

Responder	Time (in seconds) spent by an individual oriented towards the fractions of male urine in a 15-min trial with 3 replicates					
	Compound I	Blank slide	Compound II	Blank slide	Compound III	Blank slide
Same sex (*n* = 3/set)	365±3.81	127±1.26	625±3.82*	98±1.25	303±5.0	97±1.26
Opposite sex (*n* = 3/set)	444±1.5*	214±1.26	451±2.75	216±1.0	423±1.8*	178±2.02

Compound I = ethanol,2-(octylthio); Compound II = 1,3,5 triazone-2,4-diamine; Compound III = 1-chlorodecane.
± Data are presented as the mean and the standard of error of the mean.
* Level of significance at $p < 0.005$ compared with the blank (Student's *t*-test).
Level of significance in the same sex:
I vs. II significant at the $p < 0.005$ level; I vs. III significant at the $p < 0.005$ level; I vs. III significant the at $p < 0.005$ level.
Level of significance in the opposite sex:
I vs. II significant at the $p < 0.01$ level; I vs. II significant at the $p < 0.005$ level; II vs. III significant at the $p < 0.005$ level.

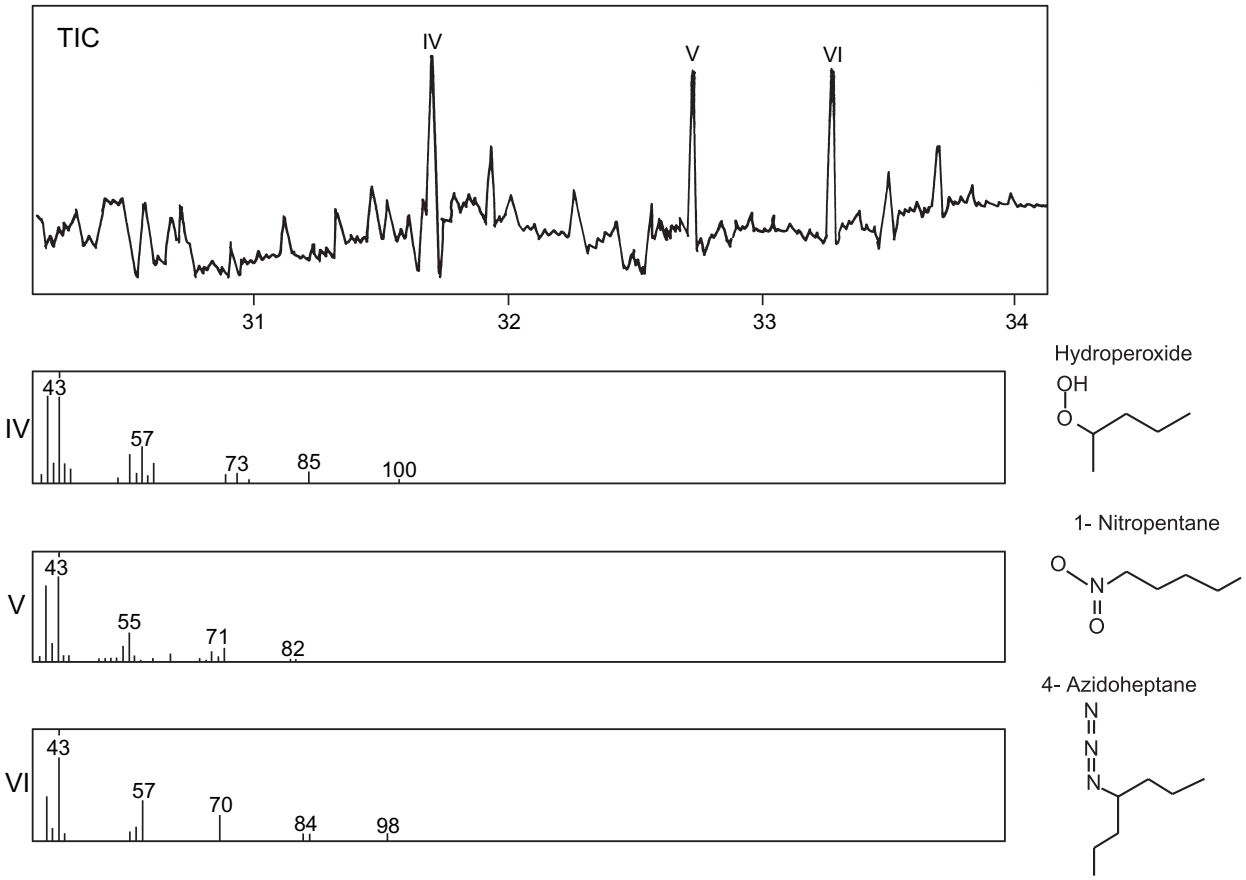

Fig. 2. Gas chromatographic profile, mass spectra, and chemical structures of identified compounds of feamale urine of *Rattus rattus*.

than did females. Among the 3 female urinary compounds, compound IV exhibited a greater response in attracting male responders. However, when female urinary compounds were tested against female urine, urine was found to attract both sexes at a higher level. The time spent by male responders oriented towards compound IV was almost the same as the time responders spent oriented towards female urine (Table 5).

DISCUSSION

The present study reveals that both male and female rat urine contains many volatile substances which mediate both opposite- and same-sex attraction. Three major fractions of chemical compounds were identified in both male and female rat urine. The results are in agreement with a report of male mouse urine which showed 2 active components of 2-(s-butyl) thiazoline and 2,3 dehydro-exo-brevicomin which are involved in primary pheromonal effects (Novotny et al. 1985). Our recent laboratory study indicated that bovine urine contains 7 different compounds during the estrus cycle (Kumar et al. 2000) in which the compounds 1-iodo undecane and di-n-propyl phthalate are exclusively present in the estrus phase and are assumed to attract the opposite sex.

Odor preference tests demonstrated that the

Table 3. Bioassay responses of male and female rats to 3 different fractions of female urinary compounds

Responder	Time (in seconds) spent by an individual oriented towards the fractions of male urine in a 15-min trial with 3 replicates					
	Compound I	Blank slide	Compound II	Blank slide	Compound III	Blank slide
Same sex (*n* = 3/set)	385±3.81	138±2.64	595±3.81	124±0.76	273±1.8	122±1.04
Opposite sex (*n* = 3/set)	565±2.5*	271±1.26	705±2.5*	137±1.8	476±1.32*	216±1.0

Compound IV = hydroperoxide; Compound V = 1-nitropentane; Compound VI = 4-azidoheptane.
± Data are presented as the mean and the standard of error of the mean.
* Level of significance at $p < 0.005$ compared with the blank (Student's *t*-test).
Level of significance in the same sex:
IV vs. II significant at the $p < 0.005$ level; IV vs. VI significant at the $p < 0.005$ level; IV vs. VI significant the at $p < 0.005$ level.
Level of significance in the opposite sex:
IV vs. V significant at the $p < 0.01$ level; IV vs. VI significant at the $p < 0.005$ level; V vs. VI significant at the $p < 0.005$ level.

Table 4. Bioassay responses of male and female rats to male rat urinary compounds

S. No.	Compound	Time (seconds) spent by responder in a 15-min trial with 6 replicates			
		Male		Female	
1.	I vs. II	311±1.31	435±1.31*	515±3.45*	210±1.37
2.	I vs. III	286±1.56	497±0.97*	430±2.32*	316±2.2
3.	II vs. III	584±1.5*	207±0.83	182±1.27	404±1.6*
4.	U vs. I	537±1.5*	269±1.47	310±1.61#	300±2.0
5.	U vs. II	333±2.0*	315±2.48	466±2.36*	171±1.46
6.	U vs. III	369±1.27*	203±1.25	306±1.16	321±2.2♦
7.	U vs. C	414±0.95*	108±1.24	677±1.25	170±1.26

Compound I = ethanol,2-(octylthio); Compound II = 1,3,5 triazone-2,4-diamine; Compound III = 1-chlorodecane; U=urine.
± Data are presented as the mean and the standard error of the mean.
* Significant at the $p < 0.001$ level (Student's *t*- test).
# Significant at the $p < 0.01$ level.
♦ Significant at the $p < 0.05$ level.

compounds ethanol,2-(octylthio) (I) and 1-chlorodecane (III) identified in male urine, and hydroperoxide (IV), 1-nitropentane (V) and 4-azidoheptane (VI) in female (estrus) urine, produced maximum responses in the opposite sex, whereas the compound 1,3,5 triazine-2,4-diamine (II) present in male urine elicited same-sex attraction. Preputial and cheek glands of the house rat (*Rattus rattus*) produce more than 20 volatile compounds (Kannan 1998). Yet, none of the compounds is related to the urinary compounds identified in the present study. Therefore, this possibly suggests that each pheromonal source secretes a unique volatile substance, and probably each compound has its own function in maintaining reproductive and social behaviors. The present study further provides evidence that rat urinary compounds are species specific and are involved in same- as well as opposite-sex attraction. It may also reveal that the female urinary compound, (Z)-7-dodecen-1yl acetate identified in the Asian elephant acts as a sex pheromone (Rasmussen et al. 1997). However, in the present study, all 3 female urinary compounds act as opposite-sex attractants. It is also reported that 1 or more compounds are involved in pheromonal communication (Johnston 1990).

Compounds identified in the present study do not correlate with chemical compounds identified in the preputial gland of the albino rat (Kannan et al. 1998) or the cheek gland of the lesser bandicoot rat (Kannan and Archunan 1999). It appears that chemicals involved in pheromonal communication differ from one source to another. Hydroperoxide, present in female rat urine elicits a maximum response in opposite-sex attraction. Therefore, the biological importance of hydroperoxide should be studied further.

In the behavioral responses of male rats to the male rat urinary compounds, the compound ethanol,2-(octylthio) is more effective than the compounds 1,3,5 triazone-2,4-diamine, and 1-chlorodecane. Further, the response elicited by ethanol,2-(octylthio) in males was almost the same as that of normal urine. The behavior test convincingly demonstrates that the compound ethanol,2 (octylthio) attracts the same sex (Table 4). Similarly in the bioassay responses of male and female rats to female rat urinary compounds, all female rat urinary compounds produced maximum responses in male responders. This clearly indicates that the 3 tested compounds present in female urine are opposite-sex attractants. Furthermore, the compound hydroperoxide produced a maximum response in females which was almost the same as that of normal urine. Our data show that all female urinary compounds may act in opposite-sex attraction.

Rodent control programs have been unsuccessful due to various factors. Since poison bait shyness is a serious problem faced by farmers, there is an urgent need to develop a suitable eco-friendly method to manage rodents. Scent gland secretions (Selvaraj and Archunan 2002) and urine (Soni and Prakash 1987) have been reported to overcome poison bait shyness to some extent in laboratory rats and soft-furred field rats respectively. Developing pheromonal traps would be the best method for rodent pest management programs. The present study is a preliminary attempt to make use of urinary compounds in developing pheromonal traps.

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Table 5. Bioassay responses of male and female rats to female rat urinary compounds

S. No.	Compound	Time (seconds) spent by responder in a 15-min trial with 6 replicates			
		Male		Female	
1.	IV vs. V	443 ± 0.74*	406 ± 1.72	210 ± 1.37	274 ± 1.0*
2.	IV vs. VI	453 ± 1.62*	397 ± 0.97	316 ± 2.2*	244 ± 1.27
3.	V vs. VI	432 ± 2.62	417 ± 3.5	226 ± 3.2	232 ± 2.5
4.	U vs. IV	310 ± 2.0	300 ± 2.2	228 ± 2.8	257 ± 0.8*
5.	U vs. V	360 ± 2.14*	270 ± 1.39	184 ± 1.12*	166 ± 1.48
6.	U vs. VI	385 ± 1.48*	276 ± 1.98	118 ± 1.59	115 ± 0.7
7.	U vs. C	456 ± 0.91*	282 ± 2.5	117 ± 1.26#	101 ± 1.41#

Compound IV = hydroperoxide; Compound V = 1-nitropentane; Compound VI = 4-azidoheptane; U=urine.
± Data are presented as the mean and the standard error of the mean.
* Significant at the $p < 0.001$ level (Student's *t*- test).
# Significant at the $p < 0.01$ level.

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家鼠 (*Rattus rattus*) 尿液成分的鑑定及其生物活性的分析

Ramasamy Selvaraj　Govindaraju Archunan

本研究利用氣相層析質譜儀分析家鼠的尿液，發現雄鼠的尿液中含有 2-(octylthio) ethanol、1,3,5-trizone-2,4-diamine 和1-chlorodecane。雌鼠在發情期時的尿液中則含有Hydroperoxide、1-intropentane 和 4-azidoheptane。利用Y型迷宮來檢測家鼠對氣味的喜好程度，結果顯示這些成分的氣味對異性與同性的家鼠都具有吸引力。雄鼠的 ethanol,2-(octylthio) 和 1-chlorodecane 吸引雌鼠，1,3,5-trizone-2,4-diamine 則吸引雄鼠。雌鼠尿液的 Hydroperoxide 和 4-azidoheptane 顯示出其最大的作用在於吸引異性老鼠，1-nitropentane 則對兩性都有吸引作用，但對吸引異性比較有效。本研究的結果顯示老鼠尿液中這些被鑑定的成分具有性別專一性，且含有揮發性物質，其生物活性亦各不相同。

關鍵詞：尿液，費洛蒙，化學特性，社會行為，鼠。

Department of Animal Science, Bharathidasan University, Tiruchirappalli 62024, India