

# Identification of urinary sex pheromones in female Rajapalayam breed dog and their influence on stud male reproductive behavior

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## ABSTRACT

The dog has been a good companion animal to humans. Companionship, security, hunting, and herding are their best utilities for the owner. The amazing sense of smell of some of the breeds of dogs is taken to advantage to train for accurately tracking and sniffing out criminals. The heralding and tracking efficiencies of the majestic Rajapalayam breed of dog are outstanding but little is documented about the reproductive status of females through volatile compounds to which the males respond appropriately to facilitate sexual attraction and mating of this breed. Therefore, the present study was carried out to investigate the reproductive behaviors and urinary volatiles at various stages of the estrous cycle. The repeated behaviors such as approach, attempted investigation, investigation, pre-mount contact, and mounting were significantly higher in male dogs exposed to females in estrus. By contrast, such reproductive behaviors were totally absent in males exposed to females in post-estrus. Pre-copulatory behaviors and urinary volatiles of female dogs were hypothesized to be the guiding/exhibitor factors. As many as, 30 urinary volatile compounds were identified during the estrous cycle. Among these, 2-methyl-hexadecane, 3-methylene-4-methylpentan-1-ol, (E)-9-methyl-7-undecenoic acid, 4,5-dimethyl-4-hexen-2-one, and 2-octyl-dodecan-1-ol were specific to estrus phase. Behavioral observation clearly revealed that stud males exhibited the highest attraction to females, with repeated attempts of investigation, actual investigation, mounting, and penile erection being significantly high toward estrus urine sample as well as mixture of synthetic estrus-specific volatile compounds. However, pro-estrus-specific 2, 3-dimethyl-1-butanol and postestrus-specific pentadecane did not evoke such sexual behaviors in male dogs. It is concluded that the female dog in estrus phase releases specific volatiles chemicals in the urine, which is instrumental in provoking the manifestations of premating behaviors in male dogs. Thus, the present study provides evidence that non-invasive methods such as assessment of behaviors and estrus-specific urinary volatiles would serve as good for detection of estrus in the Rajapalayam dog. Among the volatiles identified in estrus urine, based on the behavioral assay, urinary (E)-9-methyl-7-undecenoic acid proves to be the most effective male-attracting pheromone in the female dog. Thus, the present study provides evidence to the effect that assessment of male behaviors to estrus-specific urinary volatiles can facilitate detection of estrus in the Rajapalayam dog so as to precisely time the mating.

## 1. INTRODUCTION

The dog has been a good companion animal to humans and it contributes greatly to criminal investigations deriving from the keen

sense of smell. There are several dog breeds registered in India as livestock breeds, including Bhutia Dog, Caravan Hound, Combai, Chippiparai, Himalayan Sheep Dog, Indian Mastiff, Kanni, Mudhol Hound, Rajapalayam, and Rampur Hound [1-3]. The Rajapalayam dog (*Canis lupus familiaris*) is one of the most popular indigenous breeds in the state of Tamil Nadu, India. Furthermore, known as Paleiyakaran and Poligar Hound breeds, it is the companion of royal families and aristocrats in Southern India, particularly in and around the town of Rajapalayam in Virudunagar district [2,4]. In the light of its robust physique and agility, the Rajapalayam breed is utilized for bravery purposes such as hunting, guarding the farms and farm houses, and shepherding the livestock during grazing and migration [2].

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At present, there are only a few pure Rajapalayam breed dogs present in isolated pockets in and around southern Tamil Nadu (<https://www.dogspot.in/rajapalayam/>). The reproductive efficiency of Rajapalayam dog varies from place to place, but it is at the verge of extinction but the underlying reason is not known [5]. Therefore, it is necessary to develop methods to improve breeding for this species. A non-invasive method of detection of estrus of this dog to ensure timed mating will be in order in this context.

Non-invasive fecal steroid metabolite evaluation has already been done in the domestic dog [6,7]. In the past few years, non-intrusive urinary pheromones have become widely used in a variety of disciplines, such as animal science, behavioral ecology, conservation biology, pet, and farm animal management. Pheromones are powerful species-specific chemical signals that organize both social and reproductive behaviors of all organisms including insects, reptiles, birds, and mammals [8].

Primer pheromones play a critical role in both behavioral and reproductive physiology of mammals [8-10]. The female discharges conspecific chemical signals through excretion (i.e., urine/feces) during the heat period (i.e., estrus), which acts as sexual attractants including sexual provocation and mating behavior [11]. According to Ziegler *et al.* [10], the ovulation timing and sexual behavior coordination are considered to be essential factors for successful fertilization [12]. A number of scientists have demonstrated that females release specific odour(s) during estrus through urine [13-16], feces [17], and vaginal fluid [18] which constitute the major avenues for chemical communication in mammals [19] and may be considered an accurate indicators of female receptivity [9,20].

Aspects of reproductive behavior of the dog, *Canis familiaris*, in general, have been studied rather extensively [21,22], although the Rajapalayam breed has been largely ignored. Raja *et al.* [2] investigated the cytogenetic profile of this dog to throw some light on chromosomal abnormalities that would affect growth and fertility. Based on a morphometric analysis, it was indicated that the Rajapalayam dog population has been declining and the purity of the breed is at risk requiring conservation measures [1]. Very specially, there has been no research pertaining to reproductive behaviors and pheromone evaluation in Rajapalayam dog [5]. In pursuance of all these considerations, the present study was conducted to investigate non-invasive monitoring of reproductive status from the behavior and the profiles of volatiles in urine during the three phases of canine female reproductive cycle, namely, pro-estrus, estrus, and post-estrus [15,23]. The evaluation of the stud male behavior in response to the estrus-specific compounds identified in urine to ascertain the chemical information of urinary pheromones to find the potential use of those chemical cues in the management of reproduction in Rajapalayam dogs, *C. lupus familiaris* has been the focused objective of this study.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Twelve Rajapalayam dogs (Rajapalayam breed, *C. lupus familiaris*; eight sexually mature female dogs and four sexually mature male dogs), 2–5 years old, were used [Figure 1]. The animals were maintained indoor in individual cages under a lighting schedule of 12 h L/D cycle. All the investigations (sample collection and behavioral observations) were carried out at Provokennel, a private Rajapalayam breeds breeding center, as well as at a pet store in Rajapalayam (9°27'0N and 77°33'0E) located at an altitude of MSL + 175 m], Virudunagar



**Figure 1:** The Rajapalayam dog. (a) Male and (b) Female.

District, Tamil Nadu, India. The samples were collected with the assistance of a veterinarian while adhering to the standard CPCSEA-IAEC guidelines.

### 2.2. Determination of Estrous Cycle and Observation of Behaviors

A preliminary investigation was carried out for about 1 month to develop familiarity of animal handling and data collection. In general, the domestic dogs pass through, at an average, two estrous cycles per year, the period of which may differ between geographic locations, years, etc. The anestrus dogs (i.e., non-estrous female) were closely monitored for pro-estrous signs of vulvar swelling and vaginal discharge. Each female was allowed to remain with a mature stud male for 10 min twice daily; proceptivity of the female and response of the stud male to the female were observed. When the females indicated to be in pro-estrus, the male was attracted but the female was not at all receptive. Estrus was characterized by the female adopting a rigid stance, with her tail held to the side, and allowed mounting by the male.

Day of “heat” or “estrus” was defined as day 0 of the cycle. Four–to fifteen days before estrus, the females were considered as in pro-estrus, and 5–10 days after estrus, the females were considered as in post-estrus. When the females and stud males were together, the researchers were observed for receptive behavior. A scan-sampling method [24] was used to measure the frequency of receptive behavior and was observed in four stud males over eight estrous cycles of the corresponding female (i.e., pro-estrus, estrus, and post-estrus) [Table 1].

The behavioral observations were conducted in two shifts: Mornings from 7.00 am to 10.00 am and evenings from 15.00 pm to 18.00 pm, for a total of 6 h each day.

### 2.3. Urine Sample Collection

The urine samples were collected during the pro-estrus, estrus, and post-estrus phases in female dogs to analyze the volatile compounds. Urine was collected from the clean floor immediately after urination using a sterile syringe, and transferred to 2 mL vials. Once collected, vials containing urine were labeled and placed in a cold thermos flask until reaching the camp at 10.30 am and 5.30 pm, where the samples were placed in a freezer at –20°C for GC-MS analysis.

**Table 1:** Ethogram (reproductive behaviors) of Rajapalayam Dog's are described.

S. No.	Reproductive behavioral pattern	Description
1.	Approach ♂	Male walks straight toward the female
2.	Attempted investigation ♂	The male touches the tail region of the female with his nose or makes momentary contact with that area
3.	Roaring/Mating call ♂	Roaring/mating call is associated solely with reproductive behavior; the male receives the proceptivity of the bitch and immediately expresses the mating call to convey its availability/readiness for courtship and mating.
4.	Investigation ♂	Sniffs and/or licks the anogenital area of the female dog
5.	Avoid ♀	Withdraws, whirls around, throws off, falls, or sits down
6.	Pre-mount Contact ♂	Attempts mounting, paws on back, chin over back, etc.
7.	Urination ♀	Elimination of urine with the associated sign of heat or estrus
8.	Urination ♂	Elimination of urine with the associated urine marking behavior
9.	Threat ♀	Snarls, barks, snaps, lunges, throws off, falls or sits down, or any combination
10.	Mount ♂	Approaches rear position, and clasps, but no thrusting
11.	Stand ♀	Maintains location and orientation (tail usually deviated) for 5–10 s., or until male dismounts if mount is <5 s
12.	Intromission ♂	Insertion, rapid thrusting, pelvic oscillation, stepping and treading
13.	Standing/Insertion ♀	Maintains location and orientation for verified insertion of more than 10 s., if no lock.
14.	Lock ♂	Scored whenever pair remains engaged after mount is discontinued

#### 2.4. Sample Preparation for GC-MS Analysis

Dichloromethane (DCM) was utilized as the solvent in GC-MS analysis. 5 ml of urine sample was taken from collection at each stage, mixed with 5 ml of DCM, and filtered through a silica gel column with a mesh size of 60–120 for 30 min at room temperature. The filtered extract was condensed to 1/5 of its original volume by cooling with liquid nitrogen [25].

#### 2.5. Identification of Volatile Compounds by GC-MS

The urine sample was fractionated and gas chromatography-linked mass spectrometry was used to identify the volatile compounds (QP-5050, Shimadzu, Japan). The solvent extract (2 µL) was injected into the GC-MS analyzer on a 30 m glass capillary column with a film thickness of 0.25 µm (30 m × 0.2 mm i.d. coated with UCON HB 2000) at the following temperatures: Initial oven temperature was 40°C for 4 min, then escalated to 250°C at 15°C/min for 15 min, and finally kept at 250°C for 10 min. The GC unit had a FID detector that was connected to an integrator. Each component's relative amount was expressed as a percentage of the total ion current. The GC-MS was controlled by a computer at 70 eV, and ammonia was used as the reagent gas at 95 eV. Unknown individual compounds were identified using their mass spectra compared to the library (Wiley and NIST) through probability-based matching.

#### 2.6. Behavioral Assay

The behavioral assay modified from Rajanarayanan and Archunan [25] was adopted. Observation of behavioral aspects of stud male, such as attempted investigation, sniffing, mounting, and penile erection, in reaction to synthetic compounds, was conducted in the Provokennel. Six non-estrous females and four stud males were observed and recorded. The synthetic versions of compounds specific to estrus [2-methyl-hexadecane (I), 3-methylene-4-methylpenta-1-ol (II), (E)-9-methyl-7-undecenoic acid (III), 4,5-dimethyl-4-hexen-2-one (IV) and 2-octyldodecan-1-ol (V)], pro-estrus [2,3-dimethyl-1-butanol] and post-estrus [Pentadecane] were procured from Aldrich Chemical Company, Bangalore. The synthetic estrus-specific compounds were dissolved in various concentrations of DCM, such as 0.5%, 1.0%, 2.0%, and 5.0%. For behavioral assay, synthetic compounds were

soaked in cotton wool and applied manually to the genital region of non-estrous female dogs. It has been found that the estrus-specific compounds at 0.5% concentration are effective in eliciting sniffing and other behaviors, so the same concentration (0.5%) has been utilized throughout the experiment.

A bioassay was conducted in four groups:

The bioassay was carried out in four different groups:

1. Control group: The stud male was exposed to pro-estrus (2, 3-dimethyl-1-butanol, 0.5% concentration)- and post-estrus (Pentadecane, 0.5% concn.)-specific synthetic compounds.
2. Confirmation of estrus phase: The stud male was exposed to neat estrus urine.
3. Exposure to individual estrus-specific synthetic compounds: The stud male was exposed to each estrus-specific synthetic compound [2-methyl-hexadecane (I), 3-methylene-4-methylpenta-1-ol (II), (E)-9-methyl-7-undecenoic acid (III), 4, 5-dimethyl-4-hexen-2-one (IV) and 2-Octyldodecan-1-ol (V)], separately.
4. Exposure to a mixture of estrus-specific synthetic compounds: The stud male was exposed to the mixed estrus-specific synthetic compounds.

The stud males were allowed to sniff, for 15 min in a single exposure, the genital region of the non-estrous female dogs to which the synthetic compound(s) was/were rubbed. The frequency of reproductive behaviors (attempted investigation, sniffing, etc.) displayed by the stud male in reaction to each sample (neat urine and synthetic compounds) was observed and recorded. The genital region was washed thoroughly with water after every assay, and a lag-time of 30 min was allowed between two assays. All experiments were performed in six replicates. The data in respect of stud male behavioral study were compiled using SPSS statistical software (11<sup>th</sup> version), and subjected to analysis of DMRT test across pro-estrus, estrus and post-estrus phases.

### 3. RESULTS

#### 3.1. Reproductive Behavior and Mating

During pro-estrus phase, the female's vulva was swollen and there was vaginal mucous secretion but it showed unwillingness by way of aggressive/submissive behaviors and did not permit mounting of male



when the latter exhibited courtship behaviors such as roaring mating calls, sniffing of the female's face and flank, licking of the vulva, whirling around, approach, and attempted investigation. The stud male made mating call(s) frequently during pro-estrus and estrus phases followed by sniffing and licking the vulva. The females exhibited frequent urination and showed teasing behavior and were attracted toward the stud male. During the receptive periods, the female readily allowed the stud male to mount, standing still with her tail folded to one side and presenting her hindquarters to him. After the coitus, the stud male dismounted by lifting one hind leg over the female's back and placing both front feet to one side. The enlarged bulbous glandis of the stud male did not allow withdrawal of the penis and thus the male and female remained disengaged for about 12–50 min. After the final disengagement, both the stud male and estrus female commonly self-licked their genitals.

Thus, 14 different reproductive behaviors were observed, nine in males and five in females [Tables 1 and 2]. Based on the Duncan Multiple Range Test, stud males' reproductive behaviors (except attempted investigation, avoid, and threat) exhibited during estrus were significantly higher ( $P < 0.05$ ) compared to pro-estrus and post-estrus [Table 2].

### 3.2. GC-MS Urinary Profiles

In the gas chromatography analysis, the major volatile compounds were identified between 5 and 45 min of retention time. Table 3 and Figure 2 show the GC-MS profiles of urinary compounds found in three different reproductive phases (i.e., pro-estrus, estrus, and diestrus) of female dogs. A urine sample showed 14–24 detectable peaks at each stage, and nearly 30 detectable peaks were noted between the three phases. Among 30 volatile compounds, 21 compounds were identified in the pro-estrus urine sample, 24 in the estrus, and 14 in the diestrus. The peak numbers corresponding to the list of the compounds are presented in Table 3. The chemical constituents identified in the urine

**Table 2:** The reproductive behavioral patterns (in frequency) in Rajapalayam dogs during various stages of the estrous cycle analyzed by DMRT test.

S. No.	Types of male and/or female behavior	Estrous cycle phase		
		Pro-estrus	Estrus	Post-estrus
1.	Approach ♂	4.26±0.28 <sup>b</sup>	7.66±0.51 <sup>a</sup>	1.16±0.28 <sup>c</sup>
2.	Attempted Investigation ♂	11.3±0.82 <sup>a</sup>	5.16±0.35 <sup>b</sup>	0.83±0.43 <sup>c</sup>
3.	Mating call ♂	4.60±0.83 <sup>ab</sup>	5.80±1.32 <sup>a</sup>	0.00±0.00
4.	Investigation ♂	3.83±0.95 <sup>b</sup>	6.66±0.61 <sup>a</sup>	1.33±0.19 <sup>c</sup>
5.	Avoid ♀	6.26±1.47 <sup>a</sup>	2.87±0.81 <sup>b</sup>	0.00±0.00
6.	Pre-mount Contact ♂	0.83±0.68 <sup>b</sup>	4.66±0.56 <sup>a</sup>	0.50±0.31 <sup>c</sup>
7.	Urination ♀	2.66±0.30 <sup>b</sup>	4.16±0.28 <sup>a</sup>	1.50±0.20 <sup>bc</sup>
8.	Urination ♂	3.50±0.39 <sup>b</sup>	5.20±0.33 <sup>a</sup>	1.66±0.18 <sup>c</sup>
9.	Threat ♀	2.83±0.43 <sup>b</sup>	1.16±0.28 <sup>c</sup>	3.50±0.38 <sup>a</sup>
10.	Mount ♂	4.16±0.25 <sup>b</sup>	10.16±1.09 <sup>a</sup>	0.00±0.00
11.	Stand/Mount ♀	0.83±0.28 <sup>b</sup>	5.50±0.39 <sup>a</sup>	0.00±0.00
12.	Behavior of Intromission: ♂	0.00±0.00	3.16±0.26 <sup>a</sup>	0.00±0.00
13.	Stand/Insertion ♀	0.00±0.00	2.40±0.23 <sup>a</sup>	0.00±0.00
14.	Lock ♂	0.00±0.00	2.66±0.30 <sup>a</sup>	0.00±0.00

Values are expressed as Mean±SE. Means in the same vertical column that are not marked with the same superscript (alphabets) letters are significantly different at  $\alpha=0.5$  level (Duncan's test).

sample were alkanes, alcohols, ketones, carboxylic acid, and ester. Among the different compounds, alkanes were invariably present in the urine as compared to all other compounds. Comparison of the compounds across the phases of estrous cycle revealed that certain compounds are specific to a particular phase. For instance, among the 30 volatiles, six compounds – 2-octanone, 2-methylundecane, propionic acid, 2-methyl-dodecane, nonanone, and dodecane were present in the pro-estrus and estrus urine. Further, five compounds – 2-methyl-hexadecane (I), 3-methylene-4-methylpenta-1-ol (II), (E)-9-methyl-7-undecenoic acid (III), 4,5-dimethyl-4-hexen-2-one (IV), and 2-octyl-dodecan-1-ol (V) – were specific to estrus, and not present in pro-estrus and post-estrus urine samples. As many as, 21 volatiles were identified as major compounds of the pro-estrus urine of which five – 2,3-dimethylbutan-1-ol, 1-hydroxy-1-phenylpropan-2-one, pentanoic acid, 3-hydroxy-2-hexanone, and tetradecanoic acid – showed to be specific for pro-estrus urine. The compounds 2-pentanone, 1,3-propanediol, methyl propyl sulfide, benzoic acid, heptadecane, octadecane, nonadecane, docosane, hexacosane, and tricosane were found in all phases of the estrous cycle.

### 3.3. Behavioral Responses of Stud Males to the Urinary Volatiles

The Duncan's Multiple Range Test revealed that the stud males exhibited significant differences in reproductive behaviors in response to the specific but synthetic urinary volatiles of pro-estrus, estrus, and post-estrus phases and neat estrus urine. The reproductive behaviors (i.e., attempted investigation, investigation, mounting, and penile erection) of stud males were substantially higher when exposed to the estrus urine followed by mixture of five estrus-specific synthetic compounds and the lone compound, that is, (E)-9-methyl-7-undecenoic acid (III). There was no significant influence of frequency of reproductive behaviors of stud males to individual estrus-specific compounds I, II, IV, and V [Table 4]. Behaviors such as attempted investigation, investigation, and penile erection of stud males were greatly ( $P < 0.05$ ) influenced by neat estrus urine which evoked equal response as the mixture of five estrus-specific compounds and (E)-9-methyl-7-undecenoic acid (III). In contrast, the mounting behavior of stud males was greatly ( $P < 0.05$ ) influenced by mixture of five estrus-specific compounds followed by neat estrus urine and (E)-9-methyl-7-undecenoic acid (III). Nonetheless, among the estrus-specific compounds, (E)-9-methyl-7-undecenoic acid (III) was found to invoke the highest response of stimulation of pre-copulatory behaviors in the stud male.

## 4. DISCUSSION

The olfactory stimuli are an essential component that regulates reproductive physiology and sexual activity in most of the animals [26]. The results herein show that the exhibition of male and female reproductive behaviors was significantly more during estrus phase than during pro-estrus and post-estrus phases. The attempted investigation and mounting frequencies of the male dogs are really remarkable. In support of this findings, it has been reported that male domestic dogs show higher rate of mounting behavior in response to estrus urine than non-estrus urine [27,28], and buffaloes showed frequent mounting behavior in response to estrus urine as opposed to non-estrus urine [25]. Further, the frequency of attempted investigation was significantly high during pro-estrus; thus, this behavior could be considered as a sign of early estrus in the Rajapalayam dog. It has been reported that estrus urine emits an crucial chemical signal that provokes the manifestation of pre-mating behavior in male dogs [14,15,27].

**Table 3:** Volatile compounds identified in the urine of the Rajapalayam dog during three phases of its estrous cycle.

S. No.	RT	Compound name	Molecular formula	Molecular weight	Group	Estrous cycle		
						Proestrus	Estrus	Postestrus
1.	11.34	2-pentanone	C <sub>5</sub> H <sub>10</sub> O	86	Ketone	+	+	+
2.	11.69	2,3-dimethylbutan-1-ol	C <sub>6</sub> H <sub>14</sub> O	102	Alcohol	+	-	
3.	13.87	2-ethyl-1-hexanol	C <sub>8</sub> H <sub>18</sub> O	130	Alcohol	-	+	+
4.	15.97	1,3-propanediol	C <sub>3</sub> H <sub>8</sub> O <sub>2</sub>	76	Alcohol	+	+	+
5.	16.62	2-octanone	C <sub>8</sub> H <sub>16</sub> O	128	Ketone	+	+	-
6.	17.94	2-methyl-undecane	C <sub>12</sub> H <sub>26</sub>	170	Alkane	+	+	-
7.	18.41	Propionic acid	C <sub>3</sub> H <sub>6</sub> O <sub>2</sub>	74	Carboxylic acid	+	+	-
8.	18.82	1-hydroxy-1-phenylpropan-2-one	C <sub>9</sub> H <sub>10</sub> O <sub>2</sub>	150	Ketone	+	-	-
9.	19.20	2-methyl-hexadecane (I)	C <sub>17</sub> H <sub>36</sub>	240	Alkane	-	+	-
10.	20.02	2-methyl-dodecane	C <sub>13</sub> H <sub>28</sub>	184	Alkane	+	+	-
11.	20.63	Nonanone	C <sub>9</sub> H <sub>18</sub> O	142	Ketone	+	+	-
12.	21.00	Dodecane	C <sub>12</sub> H <sub>26</sub>	170	Alkane	+	+	-
13.	22.50	Pentadecane	C <sub>15</sub> H <sub>32</sub>	212	Alkane	-	-	+
14.	24.54	4-methylphenol	C <sub>7</sub> H <sub>8</sub> O	108	Phenol	-	+	+
15.	24.95	Pentanoic acid	C <sub>5</sub> H <sub>10</sub> O <sub>2</sub>	102	Carboxylic acid	+	-	-
16.	25.54	Methyl propyl sulfide	C <sub>4</sub> H <sub>10</sub> S	90	Sulfur	+	+	+
17.	26.28	3-hydroxy-2-hexanone	C <sub>6</sub> H <sub>12</sub> O <sub>2</sub>	122	Ketone	+	-	-
18.	26.50	3-ethyl-4-methylpentan-1-ol (II)	C <sub>8</sub> H <sub>18</sub> O	130	Alcohol	-	+	-
19.	28.25	(E)-9-methyl-7-decenoic acid (III)	C <sub>11</sub> H <sub>20</sub> O <sub>2</sub>	184	Carboxylic acid	-	+	-
20.	29.52	Tetradecanoic acid	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	228	Carboxylic acid	+	-	-
21.	30.37	Benzoic acid	C <sub>7</sub> H <sub>6</sub> O <sub>2</sub>	122	Carboxylic acid	+	+	+
22.	32.31	Heptadecane	C <sub>17</sub> H <sub>36</sub>	240	Alkane	+	+	+
23.	34.04	Octadecane	C <sub>18</sub> H <sub>38</sub>	254	Alkane	+	+	+
24.	35.81	Nonadecane	C <sub>19</sub> H <sub>40</sub>	268	Alkane	+	+	+
25.	36.42	4,5-dimethyl-4-hexen-3-one (IV)	C <sub>8</sub> H <sub>14</sub> O	126	Ketone	-	+	-
26.	37.95	Docosane	C <sub>22</sub> H <sub>46</sub>	310	Alkane	+	+	+
27.	39.52	3-methyloctadecane	C <sub>19</sub> H <sub>40</sub>	268	Alkane	-	+	+
28.	40.32	Hexacosane	C <sub>26</sub> H <sub>54</sub>	366	Alkane	+	+	+
29.	41.76	2-octyldodecan-1-ol (V)	C <sub>20</sub> H <sub>42</sub> O	298	Alcohol	-	+	-
30.	42.72	Tricosane	C <sub>27</sub> H <sub>56</sub>	378	Alkane	+	+	+

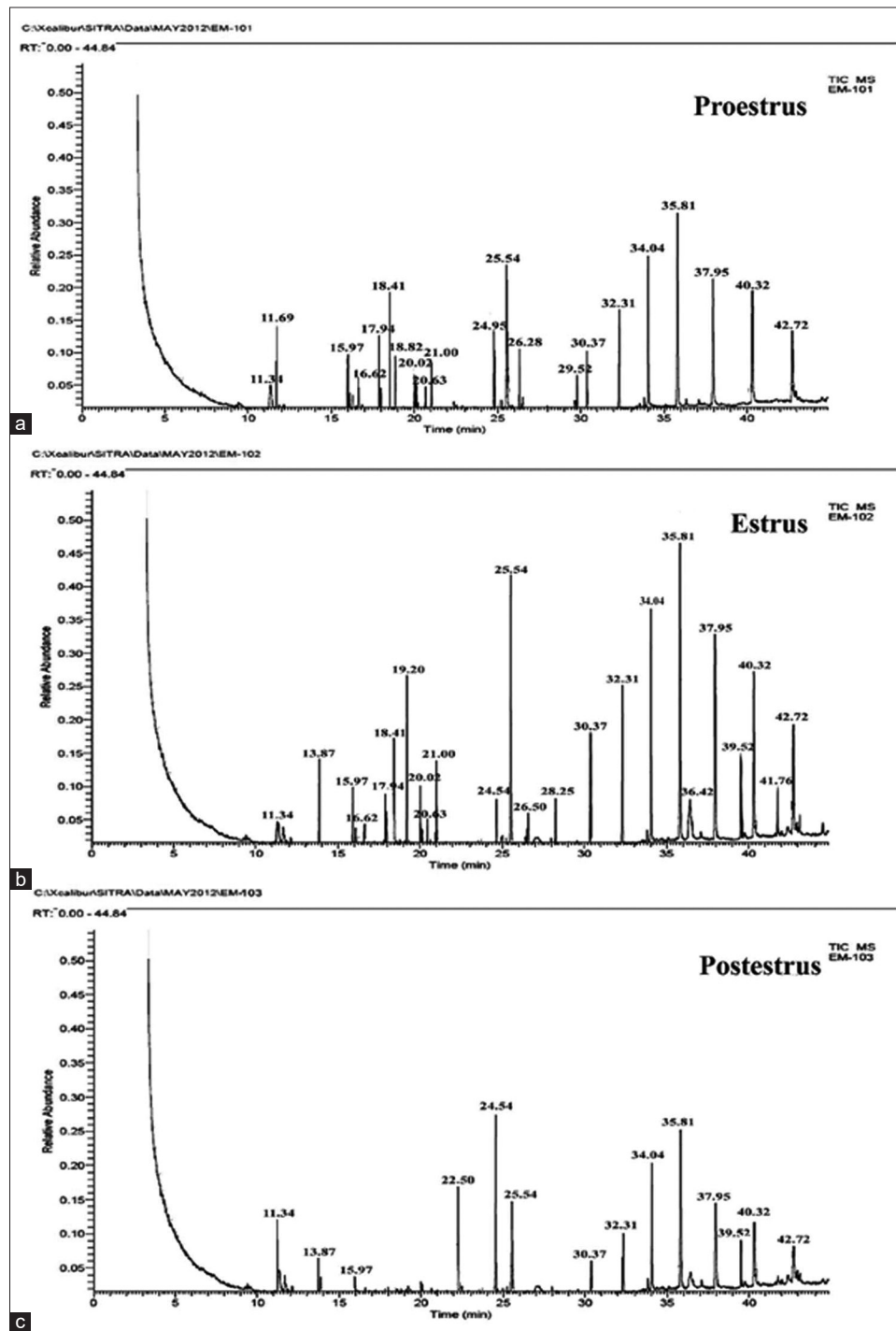
RT: Retention time, +: Present, -: Absent

Hoogland [27] found that the attracted stud male exhibits several copulatory behaviors such as self-licking of genitals, dust-bathing, and making mating calls. The findings suggest that the estrus females emit conspecific chemical signals through their urine, which may have an influence on male's pre-copulatory behavior.

Pheromones are important molecules playing roles in animal communication, particularly breeding, territoriality, and conspecific recognition. In dog, urine is one of the major sources of pheromones, involving scent or territorial marking and conveys various signals such as sex attraction and aggression. [28,29]. In the present study, the 30 volatile compounds, identified across the three phases of estrous cycle, were qualitatively different between the phases. One of the striking features of the identified volatile profiles in the present study is the presence of many alkane compounds as compared to other compounds such as carboxylic acid, alcohol, phenol, aldehyde, ester, and ketones. For instance, certain alkanes are present in female urine of two or even three phases, indicating that these alkanes probably are common metabolic end products in mammals [30]. It has been reported that

the alkane compounds play a significant role in sexual attraction in several mammalian species. For example, the 1-iodoundecane, found in estrus bovine urine, is reported to play a key role in bull attraction [31]. It has been found that the alkane 1, 5-dimethyl-6-8-dioxodicyclo (3,2,1) octane act as a pheromone during musth in elephants [32]. In insects, five alkanes (hexacosane, heptacosane, octacosane, nonacosane, and tritriacontane) are responsible for bitrophic herbivore-plant interactions and also acts as an attractant for oviposition and feeding [33]. In the present study, the alkane compound [i.e., 2-methyl-hexadecane (I)] is identified in the estrus female urine as involved in stimulating the pre-copulatory behaviors in the stud male.

The compounds 2-pentanone, 1,3-propanediol, methyl propyl sulfide, benzoic acid, heptadecane, octadecane, nonadecane, docosane, hexacosane, and tricosane are present throughout the estrous cycle which may be considered as pheromone candidates since they are known to associate with communication in the dogs. Benzoic acid has been reported in the urine of African wild dog [34]. The phase-



**Figure 2:** GC-Profiles of volatile compounds in female urine during pro-estrus (a), estrus (b), and post-estrus (c). For peak number, please refer to Table 3.

dependence of expression of the compounds methyl propyl sulfide, heptadecane, octadecane, nonadecane, docosane, hexacosane, and tricosane (peak area and height) in that they are only slightly expressed during pro-estrus, reach the peak during estrus, and then decrease during post-estrus phase makes an interesting observation. This is different from the situation in the mouse, as reported by Andereolini *et al.* [35] that urinary volatiles across estrous cycle differed only quantitatively and not qualitatively and that there is no estrus-specific compound in the urine of female mouse. Dzieciol *et al.* [15] reported that the amount (peak heights) of methylketone compounds (e.g., 2-octanone,

2-pentanone, and 3-hexanone) increased during estrus phase and simultaneously the amounts (peak heights) of sulfide compounds (e.g., 1-methylotio propane; 1-methylotio butane, 1-methylotio pentane, and dimethyl trisulfide) decreased during the period of estrus and abruptly increased in diestrus of Beagle breed dog. In the present study, the peak areas and heights of the compounds methyl propyl sulfide, heptadecane, octadecane, nonadecane, docosane, hexacosane, and tricosane had higher in the GC profile during estrus than pro-estrus and post-estrus phases. The result suggests that the volatile compounds are maximally produced during estrus despite their initiation at pro-

**Table 4:** The stud male reproductive behavior (frequency number: mean±SD) in responding to neat estrus urine and identified compounds.

Group	Exposed to	Male reproductive behaviours			
		Attempted investigation	Investigation	Mounting	Penile erection
A (Control)	2,3-dimethylbutan-1-ol	1.06±0.16 <sup>f</sup>	1.66±0.21 <sup>e</sup>	0.00±0.00*	0.00±0.00*
	Pentadecane	0.00±0.00*	0.00±0.00*	0.00±0.00*	0.00±0.00*
B	Neat estrus urine	6.86±0.30 <sup>a</sup>	8.33±0.36 <sup>a</sup>	3.83±0.28 <sup>ab</sup>	3.88±0.26 <sup>a</sup>
C	2-methyl-hexadecane (I)	3.73±0.37 <sup>d</sup>	3.33±0.92 <sup>d</sup>	1.50±0.43 <sup>d</sup>	1.00±0.60 <sup>d</sup>
	3-methyl-4-methylpentan-1-ol (II)	4.73±0.85 <sup>c</sup>	4.83±0.65 <sup>c</sup>	2.33±0.33 <sup>c</sup>	1.83±0.43 <sup>c</sup>
	(E)-9-methyl-7-decenoic acid (III)	5.40±0.42 <sup>b</sup>	5.66±0.56 <sup>b</sup>	3.16±0.31 <sup>b</sup>	2.33±0.36 <sup>b</sup>
	4,5-dimethyl-4-hexen-3-one (IV)	2.60±0.22 <sup>c</sup>	3.16±0.95 <sup>d</sup>	1.16±0.52 <sup>d</sup>	1.16±0.55 <sup>cd</sup>
	2-octyl-dodecan-1-ol (V)	4.46±0.70 <sup>c</sup>	4.00±0.81 <sup>c</sup>	1.66±0.45 <sup>d</sup>	1.50±0.50 <sup>c</sup>
D	Mixture of 5 estrus-specific compounds	6.46±0.65 <sup>a</sup>	8.16±0.50 <sup>a</sup>	4.00±0.35 <sup>a</sup>	3.50±0.29 <sup>a</sup>

Values are expressed as Mean±SE. Mean in the same vertical column that is not marked with the same superscript (alphabets) letters is significantly different at  $\alpha=0.5$  level (Duncan's test). \*No Response. Means followed by the same superscript letters (a-f) within vertical columns are not significantly different ( $P<0.05$ ); comparison by DMRT (Duncan Multiple Range Test).

estrus. Hence, these volatile compounds may be considered as possible candidates appearing during early estrus.

The five unique estrus-specific urinary volatiles identified in the present study are in molecular weight range 130 and 298 Da and carbon atoms  $C_8-C_{20}$ . Pheromones are usually in the molecular weight range 50–350 Da and 5–20 carbon atoms and must be volatile to reach the receiver [36]. It is reported that several volatile compounds appear in the urine of mammalian species in estrus; for example, methyl propyl sulfide, methyl butyl sulfide, and acetone in feral dog [37]; dimethyl sulfone, 1,3-propanediol, benzoic acid, 1-methyl-2,4-imidazolidinedione, and squalene in wild dog [34]; methyl paraben in domestic dog [38-40]; 1-chlorooctane, 4-methyl phenol, and 9-octadecenoic acid in buffalo [25]; 2-methyl-3-butyn-2-ol, 3,7-dimethylnonane, 3-phenyl-2-propen-1-ol, and 2-hydroxy-benzoic acid in blackbuck [9]; and 1-octadecanol in goat [41]. All these estrus-specific urinary pheromones possess 12 carbon atoms and the molecular weight is around 300 Da. Hence, the unique compounds observed in estrus urine of Rajapalayam dog possess physical properties necessary for consideration of putative urinary chemosignals.

Detection of putative pheromones as well as confirmation of specific odors with reference to estrous cycle has been addressed using stud males. The bioassay revealed that males exhibit repeated behaviors, that is, attempted investigation, sniffing, mounting, and penile erection, in response to estrus urine, mixture of synthetic estrus-specific compounds and the most promiscuous estrus-specific compound, (E)-9-methyl-7-undecenoic acid (III), as compared to the other estrus-specific Compounds I, II, IV, and V. The outcome of the study clearly shows that (E)-9-methyl-7-undecenoic acid is the most efficient sex attractant compared to the other compounds present during the estrus phase. Dzięcioł *et al.* [40] observed signs of sexual arousal, copious blood flow in the penile artery, and extended time of sniffing only with natural estrus pheromones. The sex pheromone present in estrus vaginal fluid can influence cardiac function as well as change the extent of blood flow in penile vessels in stud dogs [26]. The flehmen behavior of bulls was higher when exposed to a mixture of estrus-specific synthetic compounds than to the individual compounds and control sample [42]. Similarly, in the present study, the mixture of 2-methyl-hexadecane (I), 3-ethyl-4-methylpentan-1-ol (II), (E)-9-methyl-7-decenoic acid (III), 4,5-dimethyl-4-hexen-3-one (IV), and 2-octyl-dodecan-1-ol (V) induced high incidence of pre-copulatory behavior in stud males. Behavioral investigations demonstrated that individual estrus-specific compounds primarily influence the pre-

copulatory behaviors in the male whereas mixture of estrus-specific compounds influence the frequency of pre-copulatory behaviors in male dogs. Thus, it is possible that more than one chemical signal emanating from urine may act in concert to initiate the pre-copulatory behaviors and lead to successful coitus.

This is the first report of identification of estrus-specific volatiles in urine of female South Indian breed Rajapalayam dog. The findings of this study suggest that the high incidence of reproductive behavior exhibited by male dog is in view of the receptive females releasing conspecific chemical signals through urine, which would influence the pre-copulatory behavior in male dogs. The behavioral analysis showed that stud males were attracted toward the females and performed repeated pre-mating behavior in response to estrus urine as well as a mixture of the synthetic estrus-specific compounds. Based on the behavioral studies and the role of estrus-specific compounds, it is concluded that the volatiles identified in estrus urine are sex-attractant pheromones which induce sexual arousal and copulatory behavior of stud male. Further, the specific volatile compounds appearing in estrus urine would serve as non-invasive indicator of estrus in the Rajapalayam dog so as to time the controlled mating.

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## 6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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## 8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

## 9. ETHICAL APPROVALS

The Provokennel, a privately owned dog breeding center approved by Tamil Nadu Government, provided permission to collect the urine samples and ethological observation. This study does not involve experiments on animals, but the care and use of animals adheres to CPCSEA-IAEC guidelines.

## 10. DATA AVAILABILITY

The data used to support the findings of this study are included within the article.

## 11. PUBLISHER'S NOTE

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