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# Identification of Heat Period through the Pheromonal Compounds in the Urine of Malaimadu, *Bos indicus*

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**ABSTRACT: Early oestrus and pregnancy detection are the major challenging issues in farm animals. Also some other problems like irregular or prolonging of the oestrus cycle, anoestrus, fighting behavior among young ones, mother-young bond, unmotivated males, and poor farm animal management including stress are considered to be threatening factor. The present investigation aimed to address the detection of heat period in the malaimadu, *Bos indicus* by studying the urinary pheromonal compounds during the different cycles of oestrus by analytical and molecular validation and electrophoretic profiling. We found significant results by identifying peculiar pheromonal compounds released by the experimental animal in the oestrus urine.**

**Keywords:** Detection; diagnosing; oestrus cycle; pheromonal compounds and GC-MS analysis.

**INTRODUCTION:** Malaimadu is locally called as 'hill cattle' and it is native to Madurai, Virudhunagar, Tiruneveli, Theni, Dindigul and Karur Districts of Tamil Nadu State, India. There are about 20,000 in number in different parts of Tamil Nadu. This breed is known for supplying bullocks for ploughing and for draught purpose but nowadays is utilized mainly for penning and manuring farmers field. They are short sturdy and body colour varies from place to place based on availability of vegetation. The decline of population of these breeds from few lakhs to 20,000 is due to various factors such as, difficulty in identifying the oestrus period of the animal for the reproduction, the denial of grazing permits by forest officials, the unavailability of labour to look after the herds and tractorization, which reduces the demand for plough bullocks for draught animals.[1]

Cattle reproduction is vital for successful management of farm animals. In the reproductive cycle, oestrus has a central role in cattle reproduction. The period of the oestrus cycle of cattle is calculated from one oestrus (heat or phase of sexual receptivity) to the next oestrus. The average oestrus periods are 21 days in bovine and buffalo and 17 days in ewes.[2] The oestrus cycle is subdivided into four phases based on the dominant hormone or ovarian structure and reproductive behavior during each phase. The stages of the bovine cycle are pre-oestrus, oestrus, metoestrus, and dioestrus. The days are calculated as follows: day 0 is considered to be oestrus, day 1–5 are metoestrus, day 6–17 are dioestrus, and day 18–20 are pre-oestrus.[3]
In ungulates the period of oestrus is recognized by the bulls through the routine investigation of urine along the anogenital areas of females. The involvement of chemosignals in the female cow's reproductive process is well documented in urine during the oestrus and that is perceived by the bull. The dispersion of oestrus-specific compounds in the cow's body fluids has been demonstrated previously in swabs from the fluids of vaginal secretion, urine, milk, and blood.[4]

The pheromone signals are known to have a potential role in farm animal's communication, reproduction and development. Communication of the timing of the physiological event of ovulation and coordination of sexual behavior are important for successful mating and fertilization in farm animals. On the other hand, success rate of artificial insemination in cattle mainly depends on the time of oestrus when it is artificially inseminated.[5] Hence the present study is made to identify the peculiar pheromonal compounds released by the experimental animal during the oestrus cycle and thereby identifying the oestrus period to allow the females for mating or artificial insemination to have a successful reproduction.

## MATERIALS AND METHODS:

**Sample Collection:** The stages of oestrus cycle in the experimental animal malaimadu, *Bos indicus* were carefully determined for 2-3 consecutive cycles and affirmed by rectal palpation of ovarian structure.

**Cold extraction:** The solvent Di-Chloro-Methane (DCM) was added to the urine at 3:1 ratio and stored in ice cold contain in 24hours or 48 hours. After removing the supernatant, the sample (solvent - com-

pound mixture) was collected in a glass vial and sealed with an airtight screw type cap (Figure 1).

**Figure 1: Cold extraction using Di-Chloro-Methane.**

**GC-MS analysis:** The sample was fractionated and chemical compounds were identified by gas chromatography – linked mass spectrometry (Shimadzu GC-17 A with QP5050) with the following specifications. A polar 30 m DB-5 column (0.25 mm i.d. and 0.25um film thickness) and helium as carrier gas were used (Agilent techniques, USA). Injector temperature was 250$^{o}$C; interface heating was 300$^{0}$C; ion source heating: 200$^{\circ}$C, EI mode; scan range was 40-600 amu. For compound identifications NIST library spectra as well as reference MS – spectra were used.

**Biochemical estimation:** The urine sample collected from periods of oestrus were analysed for protein (Bradford[6]), carbohydrate (Dubois[7]) and lipid (Folch[8]).

**Analysis of Fatty acid:** The urinary lipid content was extracted by using chloroform and methanol (2:1) and lipid was estimated by the method of Folch[8]. The extracted lipid was analysed for fatty acid using gas chromatography.

**Electrophoretic analysis:** For the separation of pheromone carrier protein, the extracted urine samples from oestrus cycle was loaded in a well and a marker was also added separately in a well. Initially an electric current of 50 mV was applied till the dye enters the separating gel. Subsequently the electric field was increased to 100 mV till the tracking dye reached the bottom of the gel. After electrophoresis, the gel was removed from the glass plate and the resolved peptides were revealed by Coomassie Brilliant Blue staining solution Laemmli[9].

## RESULTS AND DISCUSSION:

**Results:** GC-MS analysis inferred that the following compounds were founds during the pro-oestrus stage of experimental animal namely brophenyl-5-hydropyrimidine, Furancarboxaldehyde, bromophenyl, Di-butyl methoxy-1,4-dihydrobenzaldehyde, Pyridinamine, dibromo, Tetrahydrotrimethoxy methylbenzocycloheptene, Benzene,1-methoxy cyanophenylethenyl, Benzo naphtha thiophene, Cinnamic acid, p-(trimethylsiloxy), methyl ester, chloromethyl, Methaqualone, Oxazolo xanthine, 2,3-dihydro-2-hydroxymethyl, dibromo Silane, 9-anthracenyltrimethyl, Thiophene. By contrast, compounds such as acetophenone, Dibutoxyhexamethyl bi-tetrasiloxane, phenyl pyridin-4-ylmethylidene, thioxoimidazolidin-4-one, isopropoxy-hexamethyl tri-tetrasiloxane were only found in the metoestrus urine sample of experimental animal. Compounds such as pentadecadien-1-ol, acetoxyethyl diazahomoadamantan-9-one, propanediamine, aminopropyl -N-methyl-, undecen-1-al, methyl-1H-Imidazole ethanamine, Dimethyl aminopropyl aminobenzothiazole, Dimethyl-amino-4'-methoxyacetophenone, Pentadecanone, trimethyl-2-Undecanone, methoxy-6-methy dihydro-4H-furo pyran, hepten-2-amine, Cycloheptadecen-1-one, cyanotetramethyl piperidin-4-ylidene acetic acid, ethyl ester, Cyclohexadecanone, Hexadecanal, 3-Methylaminopropyl aziridine, n-Hexadecanoic acid, Oxirane and pentadecanoic acid were found to be present in oestrus phase (Table 1, 2 & 3).

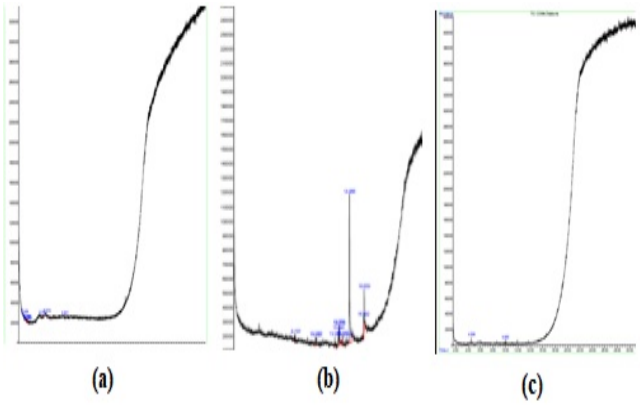


**Figure 2: GC-MS analysis of urine sample (a) Pro-oestrus (b) Oestrus (c) metoestrus.**

***MALDI-TOF analysis:*** From the previous work in the urine sample, the band formed between 20-40KDa were excised and then subjected to trypsinolysis for peptide mass finger printing. The mass spectrum of 20-40 KDa proteins were obtained by MALDI-TOF and the mono isotopic member of mass spectra were scored and analysed with MASCOT search.

From the search it was found that the sequence was with the *Rattus norvegicus* 60S ribosomal protein L37a and covers protein sequence coverage of 16%. The obtained sequence was compared with BLAST sequences of *Bos taurus* and observed a score of 38.

**Table 1: Profile of volatile compounds identified from cow urine in prooestrus by GC-MS.**

| S.No. | Compound Names | RT |
|---|---|---|
| 1 | 2-(4-Broophenyl)-5-hydroxypyrimidine | 51 |
| 2 | 2-Furancarboxaldehyde, 5-(4-bromophenyl)- | 37 |
| 3 | 3,5-Di-t-butyl-4-methoxy-1,4-dihydrobenzaldehyde | 45 |
| 4 | 4-Pyridinamine, 3,5-dibromo- | 65 |
| 5 | 6,7,8,9-Tetrahydro-1,2,3-trimethoxy-9-methyl-5H-benzocycloheptene | 204 |
| 6 | Benzene, 1-methoxy-3-(2-cyano-2-phenylethenyl) | 15 |
| 7 | Benzo[b]naphtho[2,3-d]thiophene, 9,10-dihydro-7-methyl- | 117 |
| 8 | Cinnamic acid, p-(trimethylsiloxy)-, methyl ester | 45 |
| 9 | Mercury, chloromethyl- | 49 |
| 10 | Methaqualone | 28 |
| 11 | Oxazolo[3,2-E]xanthine, 2,3-dihydro-2-hydroxymethyl-5,7-dimethyl- | 83 |
| 12 | Phenol, 2,4-dibromo- | 63 |
| 13 | Phenol, 2,6-dibromo- | 63 |
| 14 | Silane, 9-anthracenyltrimethyl- | 29 |
| 15 | Thiophene, 2-(methylselenyl)-5-(propylthio)- | 43 |

**Table 2: Profile of volatile compounds identified from cow urine in metoestrus by GC-MS.**

| S.No. | Compound Names | RT |
|---|---|---|
| 1 | Acetophenone | 51 |
| 2 | 3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane | 43 |
| 3 | 3-Phenyl-5-(pyridin-4-ylmethylidene)-2-thioxoimidazolidin-4-one | 41 |
| 4 | 3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane | 39 |

**Table 3: Profile of volatile compounds identified from cow urine in oestrus by GC-MS.**

| S.No. | Compound Names | RT |
|---|---|---|
| 1 | (Z)6,(Z)9-Pentadecadien-1-ol | 41 |
| 2 | 1-(2-Acetoxyethyl)-3,6-diazahomoadamantan-9-one | 58 |
| 3 | 1,3-Propanediamine, N-(3-aminopropyl)-N-methyl- | 15 |
| 4 | 10-Undecen-1-al, 2-methyl- | 27 |
| 5 | 1H-Imidazole-4-ethanamine, N,N,2-trimethyl- | 28 |
| 6 | 2-[3-Dimethylaminopropylamino]benzothiazole | 42 |
| 7 | 2-Dimethylamino-4'-methoxyacetophenone | 42 |
| 8 | 2-Pentadecanone, 6,10,14-trimethyl- | 29 |
| 9 | 2-Undecanone, 6,10-dimethyl- | 29 |
| 10 | 4-Methoxy-6-methyl-6,7-dihydro-4H-furo[3,2-c]pyran | 58 |
| 11 | 5-Hepten-2-amine, N,6-dimethyl- | 41 |
| 12 | 9-Cycloheptadecen-1-one, (Z)- | 14 |
| 13 | Cyano(2,2,6,6-tetramethylpiperidin-4-ylidene)acetic acid, ethyl ester | 42 |
| 14 | Cyclohexadecanone | 27 |
| 15 | Hexadecanal, 2-methyl- | 43 |
| 16 | N,N'-Diisopropyl-N''-3-[2-thiophospha-toethyl]aminopropylguanidine | 44 |
| 17 | N-[3-Methylaminopropyl]aziridine | 42 |
| 18 | N-acetyl, N-ethyl-2,3-methylenedioxyphenethylamine | 43 |
| 19 | N-Acetyl-4-methoxy-N-.alpha. dimethyl-benzenepropanamine | 43 |
| 20 | n-Hexadecanoic acid | 27 |
| 21 | Oxirane, 2-methyl-2-(1-methylethyl)- | 29 |
| 22 | Pentadecanoic acid | 27 |

From the biochemical estimation of urine samples, it was found that the amount of protein, lipid and carbohydrate was maximum during the oestrus period when compared with prooestrus and metoestrus periods (Table 4, 5 & 6). Similarly the number of fatty acids also found to be maximum during the oestrus period when compared with prooestrus and metoestrus periods (Table 7).

**Table 4: Protein estimation of cow urine samples during oestrus cycle.**

| S.No. | Stages | Protein (µg in µl) |
|---|---|---|
| 1 | Prooestrus | 34.5 ± 1.22 |
| 2 | Metoestrus | 39.90 ± 0.42 |
| 3 | Oestrus | 46.50 ± 0.75 |

**Table 5: Carbohydrate estimation of cow urine samples during oestrus cycle.**

| S.No. | Stages | Carbohydrate (µg in µl) |
|---|---|---|
| 1 | Prooestrus | 1.63 ± 0.15 |
| 2 | Metoestrus | 1.76 ± 0.15 |
| 3 | Oestrus | 2.13 ± 0.15 |

**Table 6: Lipid estimation of cow urine samples during oestrus cycle.**

| S.No. | Stages | Lipid (µg in µl) |
|---|---|---|
| 1 | Prooestrus | 0.32 ± 0.03 |
| 2 | Metoestrus | 0.39 ± 0.01 |
| 3 | Oestrus | 0.55 ± 0.04 |

In the electrophoretic analysis of urine samples of experimental animal using SDS-PAGE analysis had revealed the presence of low molecular weight proteins (14.4 – 21.1 K Da) in Lane 1 when compared with molecular markers loaded in Lane 2.

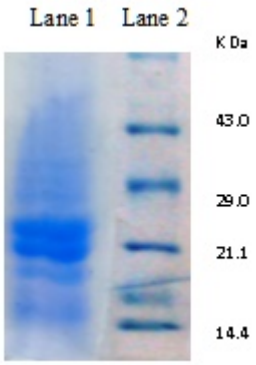


**Figure 3: Electrophoretic analysis (12%) of urine sample across the oestrus period of experimental animal.** ***[L1-oestrus; L2- Molecular weight marker]***

**Table 7: Occurrence of fatty acids in urine sample of Malaimadu (*Bos indicus*) during the different phase of oestrus cycle**

| S.No | Name of the fatty acids | Pro-oestrus | Metoestrus | Oestrus |
|---|---|---|---|---|
| 1 | Acetic acid | + | - | + |
| 2 | Arachidonic acid | + | - | + |
| 3 | Caproic acid | - | - | + |
| 4 | Formic acid | + | + | + |
| 5 | Gadoleic acid | - | - | + |
| 6 | Gallic acid | + | + | - |
| 7 | Malonic acid | - | + | - |
| 8 | Myristic acid | - | - | + |
| 9 | Oleic acid | + | - | + |
| 10 | Palmitic acid | + | + | + |
| 11 | Pelargonic acid | - | - | + |
| 12 | Phthalic acid | + | + | + |
| 13 | Stearic acid | + | + | + |
| 14 | Succinic acid | - | + | - |
| 15 | Valeric acid | - | + | - |

**Discussion:** In farm animals, the comprehensive use of artificial insemination (AI) has made reproduction more successful. Efficiency of AI partly depends on the precise detection of female receptivity, which is made by breeders on visual cues or with the help of Flehman behavior of males. In the present investigation different volatile compounds have been identified in different phases of oestrus cycle. The compounds such as Undecen-1- al, 2-methyl, pentadecanone, undecanone, cycloheptadecen-1, cycloheptadecen-1 and hexadecanal were founds to be reported in earlier studies. The fact that 1-hexadecanol is a natural ligand of aphrodisin and could be a mammalian pheromone, which is further supported by other studies of Zhang *et al*[10]; Hagemeyer[11]). Interestingly form the review it was found that 1-hexadecanol has been identified as a natural ligand of aphrodisin (Briand*et al*.[12]). Aphrodisin is a lipocalycin protein found in vaginal discharge of golden hamsters (*Mesocricetus auratus*) that induces mating behaviour in males. It is not known whether aphrodisin itself has pheromonal properties or whether it carries smaller, volatile pheromones (Tirindelli*et al*.[13]).

Earlier reports indicated that lipids play a crucial role in the sexual attraction in rat and vary considerably on basis of physiological status Kannan *et al*.[14] Ramesh kumar *et al*.[15] revealed that there is a significant variation in the excretion of lipid content across various reproductive phases that lipids have got specific role in olfactory communication depending upon the physiological state of the individuals and responders. These reviews support the presence of different number of free fatty acid found during the different cycles of oestrus in the experimental animal.

The presence of 19 k Da proteins as found in the present study has similar to the studies from the pheromonal sources of mammals such as mouse, rat, hamster, pig, horse and human (Robertson *et al*.[16], Rajkumar *et al*.[17], Singer *et al*.[18], Marchese *et al*.[19], D'Innocenzo *et al*.[20], Zeng *et al*.[21]). For instance, Major Urinary Proteins (MUPs, 19 k Da) have been identified in the urine of mice (Robertson[16] *et al*.), globulin (18 k Da) 2µ has been demonstrated in the urine (Rajkumar *et al*.[17], 2009) and preputial gland of rat (Ponmanickam and Archunan[22]; Ponmanickam[23] *et al*.), aphrodisin (17 k Da) in hamster vaginal mucous (Singer *et al*. [18]) and salivary lipocalin (20 k Da) in the boar salivary gland (Marchese *et al*.[19]). These proteins carrying hydrophobic ligands are involved in pheromone communication.

**CONCLUSION:** The present investigation revealed the presence of low molecular pheromone carrying protein and peculiar compounds during the oestrus period when compared with other cycles of the experimental animal. Hence in the future, efforts will be made to find the compounds in the urine of these animals by designing pregnancy strips and thereby allowing the animal for mating at a correct oestrus period to have a high rate of conception.

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