

Volatile Profiles of Male and Female Urine Samples of Asiatic Elephant, *Elephas maximus* in Captivity

Balaji Sundaramahalingam*, Solomon Stepenraj

Department of Biotechnology, Ayya Nadar Janaki Ammal College (Autonomous), India

Received August 22, 2019; Revised September 19, 2019; Accepted September 26, 2019

Copyright©2019 by authors, all rights reserved. Authors agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

Abstract Asian elephant, *Elephas maximus* is placed in Schedule I and Part I of Indian Wildlife Protection Act (1972) conferring it the highest level of protection. In higher mammals like elephants, reproductive success exclusively depends on odour produced from female during oestrus phase and response made by the male before mating. The odours released by the females qualitatively vary with the reproductive state namely during pre-oestrous, oestrous and post-oestrous periods. The present study aimed to identify and compare the volatile profiles of urine samples of male and female Asiatic elephants in captivity. From our assay it was found that peculiar pheromonal carrying compounds namely p-Cresol, 9-Octadecenal, 9-Hexadecenal, 13-Octadecenal and 6-Octadecenoic acid were present only in female urine sample when compared with the male urine sample. In SDS-PAGE analysis, it was confirmed that the protein with low molecular weight protein below 20 kilo daltons has been found and it depicts that these are the compounds which act as pheromone carrying lipoproteins.

Keywords Asian Elephant, Indian Wildlife Protection Act, Reproductive Success, P-Cresol and Lipoproteins

1. Introduction

India holds the largest number of wild Asian elephants, estimated at about 26,000 to 28,000 or nearly 60% of the population of the species. Historically, the significance of the elephant in Indian culture and mythology, as well as its economic and military role in sub continental armies, has also contributed to a remarkable level of tolerance and support of people towards its survival and conservation. However, the resource needs of a growing human population (over 1.2 billion people: Census 2011) of a country experiencing strong economic growth, growing and dispersing elephant populations at regional scales,

shrinkage and fragmentation of elephant habitat, and increasing human-elephant conflicts emphasize the urgent need for appropriate long-term policies to manage and conserve the species (Sukumar, 2011).

Given its long history of about 4500 years in taming the elephant (Sukumar, 2011), India also presently manages 3400-3600 elephants in captivity (Bist, 2002). Captive elephants have been used for a variety of purposes in India including warfare, logging, cultural and religious ceremonies, recreation in zoos, and circuses and more recently for wildlife tourism and protection of Sanctuaries and National Parks. However, with declining work due to the ban on timber logging in the country and the use of modern machinery, the traditional interest among private owners and state forest departments in managing captive elephants is diminishing. In contrast, demand for elephants in temples, which once received its animals from the state forest departments, continues to increase with their stock getting depleted due to old age deaths and absence of recruitment from breeding.

Elephant reproduction is a very important topic that is very necessary for elephant conservation. Understanding about anatomy, physiology and pathology can improve the conservation strategy for long term conservation, because female only have three chances per year to conceive. Within each cycle, the fertile period can be considered to be from 2 days before or until shortly after the ovulation. Therefore, identification of this brief period is most critical to ensure that males breed females at the proper time. Hence the present study is designed to unearth pheromonal compounds released during the estrus period.

2. Materials and Method

2.1. Life Cycle of Elephants

Elephants are considered to be adults at about 18 years of age and live to approximately 70 years, much the same

as humans. The elephant estrous cycle is 14 to 16 weeks long, with a 4 to 6 weeks follicular phase and an 8 to 12 weeks luteal phase (Plotka et al. 1988).

Elephants show two luteinizing hormone (LH) surges during their estrous cycles: the first LH surge occurs approximately 3 weeks before ovulation to cause non-ovulatory follicles to form accessory corpora lutea, and the second LH surge occurs around ovulation (Brown, 2000). Z7-12: Acetyl compound is first detectable in female Asian elephant urine after the luteal phase, and it increases in concentration linearly with the progression of the follicular phase through ovulation (Rasmussen, 2000).

The experimental animal selected for present investigation is Asian elephant, *Elephas maximus*. In this investigation the urine samples from male and female elephants were collected from Kerala. Female urine samples were collected from Thekkady (Attapallam), kumily District, Kerala (Figure 1). The male elephant urine samples were collected from Kodanad elephant training centre (Ernakulam district), Kerala (Figure 2).



Figure 1. Female elephant



Figure 2. Male elephant

2.2. Experimental Design & Sample Collection

The present study was designed to identify and compare the pheromonal compounds in the adult male and female urine sample of *Elephas maximus* during the oestrus stage. The urine samples were collected from the animals immediately after excretion in a sterile water cane during the morning. The samples were stored and maintained at -20° to -24° C for further analysis.

2.3. Extraction of Compounds from Urine by Cold Extraction

The solvent methanol was added to the urine at 3:1 ratio and stored in ice cold container for 24 to 48 hours. After removing the supernatant, the sample (solvent - compound mixture) was collected in a glass tube and sealed with an airtight screw type cap.

2.4. GC-MS Analysis

The sample was fractionated and chemical compounds were identified by gas chromatography – linked mass spectrometry (Shimadzu GC-17 A with QP5050). For compound identifications NIST library spectra as well as reference MS – spectra were used.

2.5. Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

After sephadex G-200 column chromatography, the pooled concentrated fractions showing the highest specific activity were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed according to Laemmli (1970) using 12.5 % gel and stacking gel (5%). For the separation, the urine sample obtained from the estrus stage was loaded on the gel. The samples were boiled in equal volume of loading buffer and loaded onto wells of the gel. For determination of molecular weight, 5 μ l of protein standard (marker) was applied on the gel. 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.

3. Results and Discussion

The present investigation aimed to find the pheromonal compound in the urine sample of the experiment animal, Asian elephant using GC-MS, and biochemical analysis. The amount of protein in the urine sample of experimental animal was estimated using Bradford method and it was found to be maximum in female urine elephant sample

(24.3 $\mu\text{g}/\mu\text{l}$) when compared with the male urine sample (15.7 $\mu\text{g}/\mu\text{l}$). The amount of carbohydrate was found to be 6.8 $\mu\text{g}/\mu\text{l}$ when compared with male urine sample 5.4 $\mu\text{g}/\mu\text{l}$. The amount of lipid in the sample was estimated using the Folch method. From the result it was found that the amount of lipid was in different stages during the pre-oestrus (4.46 $\mu\text{g}/\mu\text{l}$) and oestrus, (11.8 $\mu\text{g}/\mu\text{l}$) post-oestrus (14.5 $\mu\text{g}/\mu\text{l}$).

In the present investigation, urinary volatile compounds have been identified and compared between the male and female urine sample using GC-MS analysis. In male urine sample seven compounds were identified namely Diethyl Phthalate, Cyclododecanemethanol, Bromoacetic acid,

octadecyl ester, 1H-Indene, 2-butyl-5-hexyloctahydro, 6,9,12-Octadecatrien-1-ol, 6,9,12-Octadecatrien-1-ol,2-chloro, Phthalic acid and 2-ethoxyethyl octyl ester.

While in female elephants the compounds such as, p-Cresol, 4-methylphenol, Cyclononane, Diethylmalonic acid, monochlorid, hexadecyl ester, Palmitoyl chloride, 9-Octadecenal, cis-9-Hexadecenal, 13-Octadecenal, Oleic Acid, cis-9-Hexadecenoic acid, 10-Methyl-E-11-tridece-1-ol acetate, 6-Octadecenoic acid, Cyclohexane, Cycloicosane, Cyclopentadecanone and 9-Octadecenal were identified.

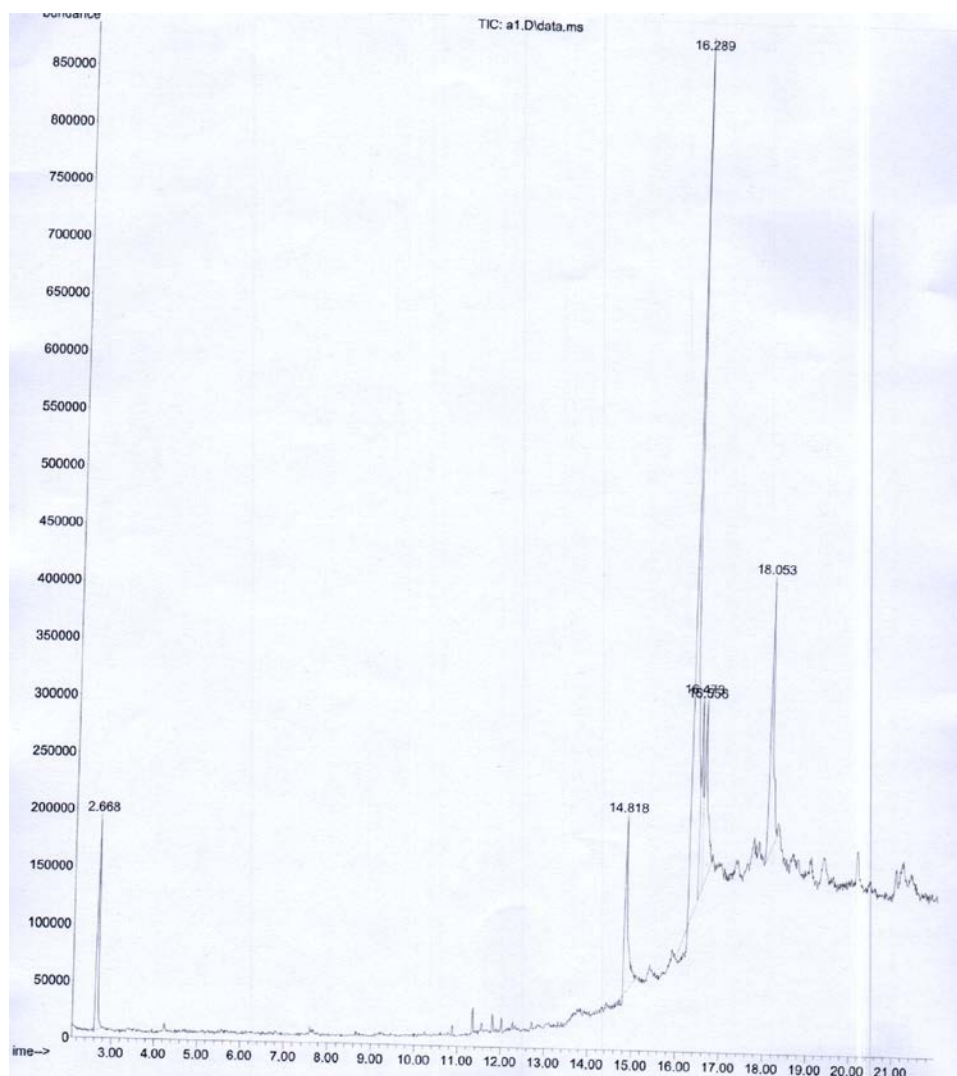


Figure 3. GC-MS analysis of Male urine sample analysis

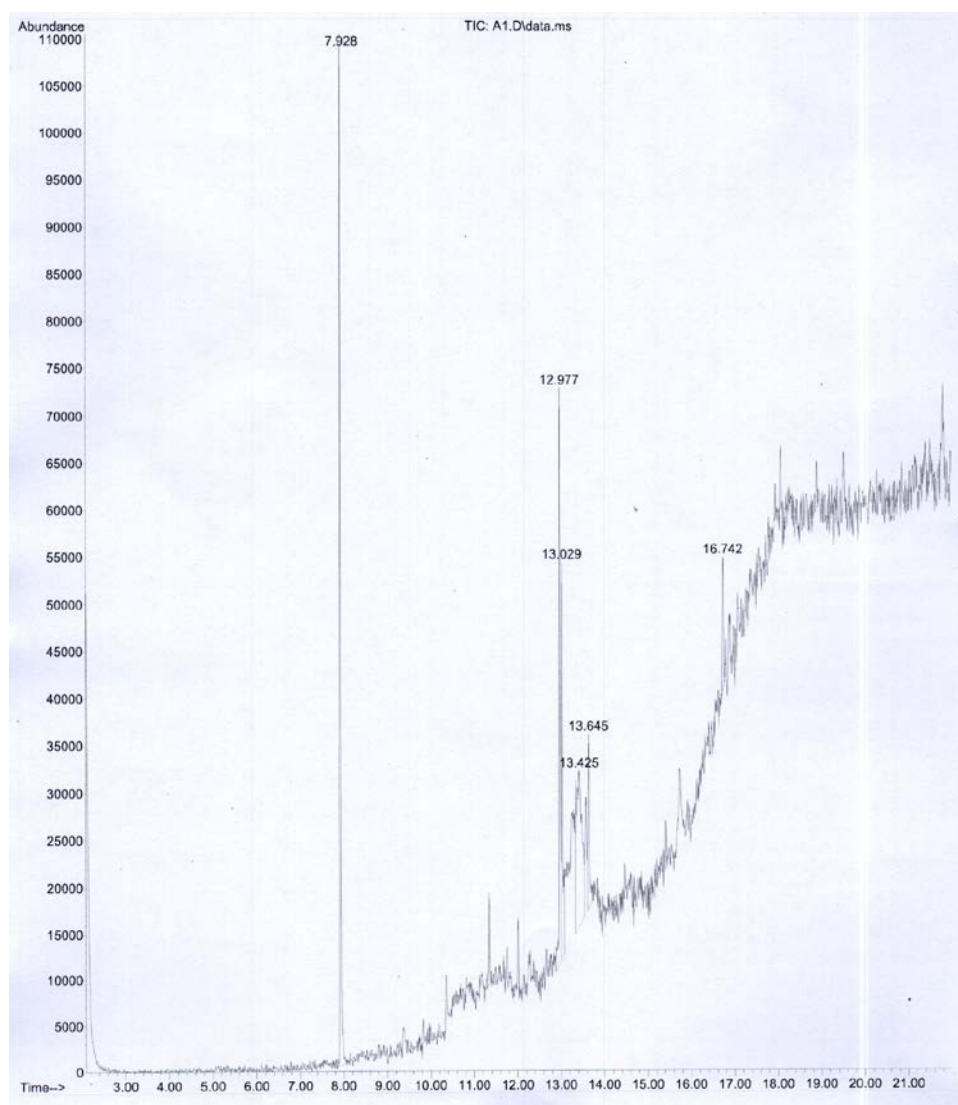


Figure 4. GC-MS analysis of female urine sample

The above results were supported by different authors while studying the oestrous periods in different animals. Interestingly, the compound 4- methyl phenol was reported by Rajagopal and Divya (2012) in the Rajapalayam dog urine sample. The 4- methyl phenol compound was found to be identified during both the estrus and diestrus period of dog (*Canis familiaris*). They suggested that the compound 4- methyl phenol may be used as a chemical signal of estrus. Similarly Rajanarayanan and Archunan (2012) also identified the sex pheromones carrying protein urine sample of female buffaloes and their influence on bull reproductive behaviour. They studied the different phases of estrus cycle and reported the presence of 1-chlorooctane, 4-methyl phenol, and 9-octadecenoic acid occurred only during estrus. The 4-methyl phenol (p-cresol), a unique volatile compound, can be considered as a common compound of estrus-specific. The reports by the above authors convincingly conclude that 4-methyl phenol acts as a sex attractant compound and appear as sex pheromone compounds which initiate the bull's reproductive

behaviour. In the present study 9-octadecenoic acid was also found to be present only in the urine sample of female elephant.

In the present investigation p-Cresol was one of the compounds identified in the female urine sample. Mozuraitis et al. (2012) analyzed the estrus urine samples in mares and found 150 urinary volatile compounds. Among the 150 compounds m- and p-cresols occurred significantly in greater amounts in estrus when compared to nonestrus stages female urine sample. The m-and p-cresol has the function of ovulation marker in horse. A great increase in amounts of p-cresol in urine samples from all mares of different breeds during the most active stallion acceptance periods provides a good signal to stallion that a mare is in estrus; thus p-cresol might be considered as a sex pheromone component. It is further reported that p-cresol is able to influence the penile erection in mares (Buda et. al., 2012)

In the present investigation the protein profiles of the urine sample were compared between the male and female

urine samples of the experimental animal. The electrophoretic profiles revealed high as well as low molecular proteins in the female elephant urine sample (10-20 kDa).

The presence of low molecular proteins in the present study has also been reported in different mammals such as mouse (Robertson et al. 1993), rat (Rajkumar et al., 2009), hamster (Singer et al., 1986), pig (Marchese et al., 1988), horse (D'Innocenzo et al., 2006) and human (Zeng et al., 1996). For instance, Major Urinary Proteins (MUPs, 19 kDa) have been identified in the urine of mice (Robertson et al. 1993), alpha₂-globulin (18 kDa) has been demonstrated in the urine (Rajkumar et al., 2009) and preputial gland of rat (Ponmanickam and Archunan, 2006; Ponmanickam et al., 2009), aphrodisin (17 kDa) in hamster vaginal mucous (Singer et al., 1986) and salivary lipocalin (20 kDa) in the boar salivary gland (Marchese et al., 1988). These proteins carrying hydrophobic ligands are involved in pheromone communication.

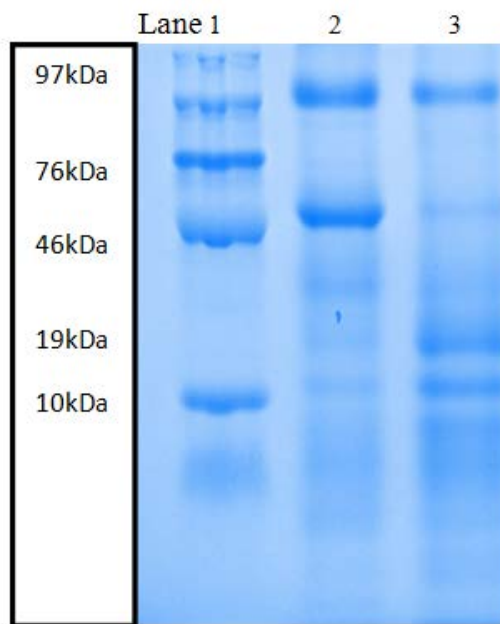


Figure 5. Separation of low molecular proteins (Lane 1 – marker; Lane 2 – Male urine sample; Lane 3 – Female urine sample)

Burger and Pretorius (1987) reported that the small globular albumin like protein (18kDa) is secreted in preorbital gland of African antelope. Another protein like aphrodisin (17kDa) is secreted in hamster vaginal fluid by vaginal tissue and Bartholin gland was also reported by Briand et al. (2004).

4. Conclusions

The present study can be used as a tool to identify the peculiar oestrus specific stage of female elephants and thereby allowing the male elephants to mate with it. This would result in good conception ratio of females.

Acknowledgements

The authors are thankful to the Management of Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi for providing necessary lab facilities.

REFERENCES

- [1] A.G.Singer, F.Macrides, A.N.Clancy, and W.C.Agosta, Purification and analysis of a proteinaceous aphrodisiac pheromone from hamster vaginal discharge. *J. Biol. Chem.* 261: 13323-13326, 1986.
- [2] C.Zeng, A.I.Spielman, B.R.Vowels, J.J.Leyden, K.Biemann, and G.Preti, A human axillary odorant is carried by apolipoprotein D. *Proc. Natl. Acad. Sci. USA.* 93: 6626-6630, 1996.
- [3] D.Robertson, D., Beynon, R., and Evershed, R., Extraction, characterization and binding analysis of two pheromonally active ligands associated with major urinary protein of house mouse *Mus musculus*. *J. Chem. Ecol.* 19: 1405-1416, 1993.
- [4] D'Innocenzo, A.M.Salzano, C.D'Ambrosio, A. Gazzano, A.Niccolini, C.Sorce, A.Scaloni, and P.Pelosi, Secretory proteins as potential semiochemical carriers in the horse. *Biochemistry.* 45: 13418-13428, 2006.
- [5] E.D.Plotka, U.S.Seal, F.R. Zarembka, L.G. Simmons, A.Teare, I.G.Phillips, K.C.Hinshaw, and D.G.Wood, Ovarian function in the elephant: luteinizing hormone and progesterone cycles in African and Asian elephants. *Biology of Reproduction.* 38: 309-314, 1988.
- [6] J.L.Brown. Reproductive endocrine monitoring of elephants: an essential tool for assisting captive management. *Zoo Biology: Published in affiliation with the American Zoo and Aquarium Association*, 19(5): 347-367, 2000.
- [7] L.Briand, F.Blouin, D.Troiter, Natural ligands of hamster aphrodisin. *Chem.Senses.* 29:425-430, 2004.
- [8] L.E.L.Rasmussen. Source and cyclic release pattern of (Z)-7-odecenyl acetate, the pre-ovulatory pheromone of the female Asian elephant. *Chemical Senses.* 26: 611-623, 2000.
- [9] M.S.Burger, and P.J.Pretorius, Mammalian pheromone studies VI. Compounds from the preorbital gland of the blue duiker, *Cephalophus monica*. *Z. Naturforsch.* 42:355-357, 1987.
- [10] P.Ponmanickam and G.Archunan, Identification of alpha₂-globulin in the rat preputial gland by MALDI-TOF analysis. *Indian J. Biochem. Biophys.* 43: 319-322, 2006.
- [11] P.Ponmanickam, G.Jebamercy, G.Archunan, and S.Kannan, Detection of Alpha-globulin in rat pup preputial gland by MALDI-TOF mass spectrometry. *Curr. Zool.*, 55: 296-300, 2009.
- [12] R.Mozuraitis, V. Buda, J.Kutra, A.K.B.Karlson, p- and m-Cresols emitted from estrous urine are reliable volatile chemical markers of ovulation in mares. *Anim. Reprod. Sci.*,

130: 51–56, 2012.

- [13] R.Rajkumar, R., Ilayaraja, C, Mucignat, A. Cavaggioni, and G. Archunan, . Identification of alpha₂-globulin and bound volatiles in the Indian common house rat (*Rattus rattus*). Indian J. Biochem. Biophys. 46: 319–324, 2009.
- [14] R. Sukumar. The Story of Asia's Elephants. 1st Edition, Mumbai, India, 2011
- [15] S. Marchese, D. Pes, A. Scaloni, V. Carbone, and P. Pelosi, Lipocalins of boar salivary glands binding odours and pheromones. Eur. J. Biochem. 252: 563–568, 1998.
- [16] S. Rajanarayanan, and G. Archunan, Identification of urinary sex pheromones in female buffaloes and their influence on bull reproductive behaviour. Res. Vet. Sci. 91: 301–305, 2012.
- [17] S. S. Bist. Elephant conservation in India - An overview. Gajah. 25: 27–37, 2002.
- [18] T. Rajagopal, and A. Divya, Identification of putative pheromone and molecular characterization of pheromone carrying protein in urine of Rajapalayam dog (*Canis familiaris*). M.Phil., thesis submitted to Ayya Nadar Janaki Ammal College, Sivakasi, 2012.
- [19] V. Buda, Mozuraitis, R., Kutra, J., and Karlson, A. K. B., p-Cresol: A sex pheromone component identified from the estrous urine of mares. J. Chem. Ecol. 38: 811–813, 2012.