Do Urinary Volatiles Carry Communicative Messages in Himalayan Snow Leopards [*Panthera uncia*, (Schreber, 1775)]?

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Abstract. Felids urinate and spray 'Marking Fluid' for territorial maintenance and to transmit messages of their reproductive status. The very rare Himalayan snow leopard also utilises these two primary modes for chemical communication. The present paper is the first report on the volatiles in urine of snow leopards which were analysed with the help of headspace solid phase micro extraction gas chromatography mass spectrometry. Chemical profiles revealed the presence of numerous low molecular weight compounds with different functional groups like alcohols, aldehydes, ketones, sulphur containing compounds. Many monoterpene alcohols, which are common secondary metabolites of plants, are abundant in the urine collected during the months of October to December, the typical reproductive season of the snow leopard in the Darjeeling hills of the Eastern Himalaya. 6-Methyl-5-hepten-2-one was identified from this felid which has a characteristic odour perceptible by the human nose. Among many sulphur containing compounds, Dimethyl disulfide and Dimethyl trisulfide were common in all urine samples of both sexes. Saturated, monounsaturated and polyunsaturated fatty acids were also identified from the lipid fraction of the urine which, in nature, may play an important role by increasing the durability of the volatiles.

1 Introduction

'Chemical signals' which regulate a variety of physiological phenomena in many felids are the primary mode of information transfer related to the reproductive behaviour of these carnivores. (Albone 1984; Brahmachary and Dutta 1981, 1984; Wyatt 2014). All cat species, in general, have two modes of pheromonal communication, ordinary Urination and the spraying of Marking Fluid (MF) (Brahmachary and Dutta 1979, 1984, 1987; Brahmachary 1996; Brahmachary and Poddar-Sarkar 2015; Poddar-Sarkar and Brahmachary 2014). Alongside visual, auditory and tactile cues, members of the cat family predominantly use these two behavioural modes to mark their territory and to

C. D. Buesching (Ed.): *Chemical Signals in Vertebrates 14*, pp. 27–37, 2019.
https://doi.org/10.1007/978-3-030-17616-7_3

inform other individuals about their reproductive status. Thus, 'scent marking'—differently termed as MF by us, plays a significant role in social interactions among snow leopards. Controversy regarding the origin of urine and MF in big cats existed for many years in international literature, however, it is now concluded that both these secretions are ejected through the urinary tract of these feline species (Poddar-Sarkar and Brahmachary 2014). In the present article, we try to identify those volatile and less/non-volatile chemical compounds in the urine of snow leopard, *Panthera uncia* syn. *Uncia uncia* (Schreber 1775) which may act as putative pheromones, although substantial evidence is yet to be required at this stage. Due to the extreme climatic conditions of the Darjeeling hills, unusual logistic constraints for work and strict zoo regulations hinder the authors' intention for exhaustive work on this highly threatened animals. However, the authors intend to project their findings based on urinary volatiles which might be the first report on chemical communication of snow leopard in the Himalayas.

P. uncia is a crepuscular felid and the native to the North Eastern Himalayan mountain range of the Indian subcontinent (Fig. 1). Snow leopards generally lead a solitary lifestyle, rarely use audible sounds and exist in a very low population density in the Eastern Himalaya. In order to communicate with each other, snow leopards scrape the ground with their hind legs and spray urine against rocks to leave markings on the landscape (Sharma et al. 2006). Although the chemistry of urinary volatiles from other big cats such as lion, *Panthera leo* (Andersen and Vulpius 1999), tiger, *Pathera tigris tigris (*Poddar-Sarkar and Brahmachary 2014), bobcat, *Lynx rufus* (Mattina et al. 1991) and cheetah, *Acinonyx jubatus* (Poddar-Sarkar and Brahmachary 1997; Burger et al. 2006) were reported by many authors, little is known about the composition of snow leopard urine and the investigation of volatiles by headspace might be informative in the context of this species' olfactory communication.

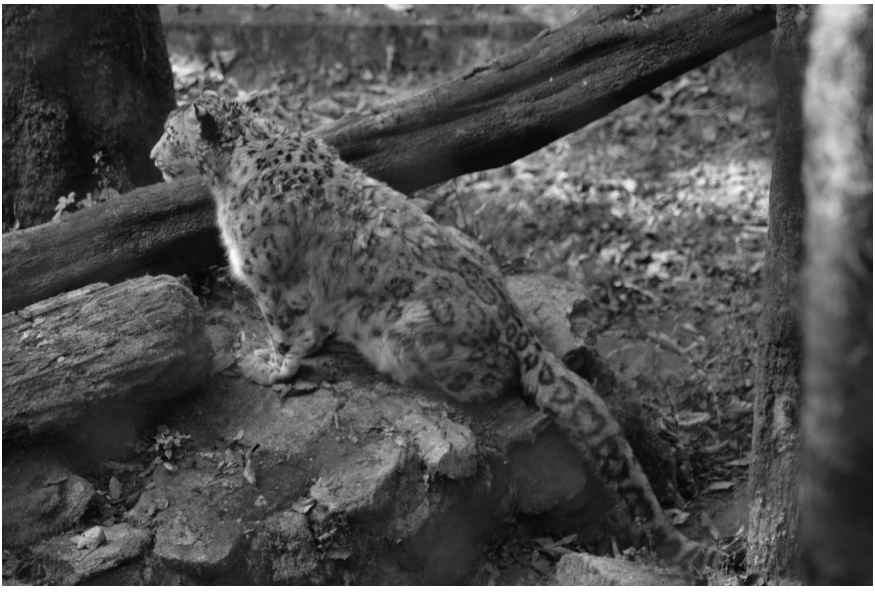

Fig. 1. Snow leopard *Panthera uncia* [Zoo name-Tista; f2—Studbook No: 2399]

2 Materials and Methods

2.1 Collection of Samples

Urine was collected from snow leopards kept in open-air enclosures in the Padmaja Naidu Himalayan Zoological park (PNHZP), Darjeeling, West Bengal, India (27° 03W′30.1″N 88°15′14.4″E). PNHZP is situated within the Darjeeling hill range (altitude 6,700 ft.) in the designated Eastern Himalaya Biodiversity Hotspot that has an undulated topography, an average temperature range during winter to summer of ∼4–15.6 °C, a humid climate during the monsoon season with an average rainfall of ∼309 cm and occasional snowfall during winter. After close observation of snow leopard behaviour, the schedule of urine collection was decided to be at dawn and dusk when the animals were fully active. Urine was collected from three female snow leopards (f1 = Studbook No: 2540, DOB 25.05.2004, f2 = Studbook No: 2399, DOB 29.03.2002, f3 = Studbook No: 2538, DOB 11.03.2004) on 17 occasions and from one male (m = Studbook No: 2404. DOB 08.07.2002) on 10 occasions following the procedure previously adopted by our team (Brahmachary and Dutta 1987; Poddar-Sarkar and Brahmachary 2014) over the years. Just before collection, animals were moved to a closed enclosure. Sampling schedule was rationalised for maximum collection opportunity and to maintain uniformity in experimental design covering both reproductive and non-reproductive seasons throughout the year during 2017–2018. Samples were pipetted out from the precleaned floor (only with distilled water) of the enclosure into 10 ml airtight Teflon-coated glass vials (Agilent, India), crimped immediately and transported to the laboratory under the ice. Samples for headspace volatiles (HSVs) were processed at the earliest convenience (i.e. between 72 and 96 h after collection) and samples for lipid work were kept at −20 °C for future analysis.

2.2 Chemical Analysis

Absorption of headspace volatiles (HSV) was optimised by attaching a 1 cm 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane [(DVB/CAR/PDMS); (Supelco, USA) stableflexTM, 24 Ga] SPME fibre with manual assembly holder (Supelco, USA) over the sample vials. Equilibration for absorbing the vapour phase was maintained at room temperature for 2 h in each case. Chemical analysis of HSV was performed using gas chromatography mass spectrometry [GCMS; Agilent 7890A, USA- triple axis MS-5975C] with a DB-WAX (30 m × 0.25 mm × 0.25 µm) column. Samples were desorbed for 10 min at the injector port at a temperature of 260 °C. The column temperature was maintained at 35 °C for 2 min initial hold, then ramping at a rate of 4 °C/min up to 210 °C with 3 min hold. The identity of the compounds was assigned by matching their retention time with authentic standards, whenever available [Sigma (USA) and by Dr. Ehrenstorfer GmbH, (Germany)] (Table 1) as well as by co-chromatography and by comparison of their respective mass spectral data obtained from the NIST (2011) library, by calculating Linear Retention Index (LRI) in relation to n-alkanes of C11–C19, considering the EI-MS fragmentation pattern and from previous records of this laboratory. The flow rate of the carrier gas (Helium) was maintained at 1 ml/min. The front inlet temperature was 250 °C. The temperature of

Table 1. Head Space Volatiles (HSV) identified by SPME-GCMS from urine of male (m) and three females (f1, f2 and f3) of Snow leopards

Volatile compounds	Rt	LRI (DB-WAX)	Identification mode[a]
Alcohols			
1-pentanol[f2, f3, m]	13.206		i, ii, iv, v, vi
1-hexanol[f1, f2, m]	16.457	1359	iii, iv, v, vi
3-Octanol[f3, m]	17.711		i, ii, iv, v
2-Hexen-1-ol, (Z)-[f2, m]	18.041		iv, vi
1-Heptanol[f1, f2, f3, m]	19.677		i, ii, iv, v, vi
2-Ethyl-1-hexanol[f1, f2, f3, m]	20.707	1496	iii, iv, v
Linalool[f1]	22.348		iv, vii
cis-Dihydro-alpha-terpineol[f3, m]	22.787		iv, vi
1-octanol[f1, m]	22.788	1565	i, ii, iii, iv, v, vi
Cyclohexanol,3,5-dimethyl-[f2, m]	23.856		iv, vii
Terpineol-4-ol[f1, m]	23.914		iv, vii
1-nonanol[f1]	25.854		i, ii, iv, v, vi
alpha-terpineol[f1, f3, m]	26.738	1652	iii, iv, vi
gamma- terpineol[f1, f3, m]	26.827	1655	iii, iv, vii
Trans-2-pinanol[f1, m]	26.834		iv, vii
Phenethyl alcohol[f1, f2, f3, m]	32.413	1913	i, ii, iii, iv, v
Phenol[f1, f2, f3, m]	34.735		iv, v
p-cresol[f3, m]	36.574		i, iii, iv, v
Ketones			
4-Heptanone[f1, f2, f3, m]	8.561	1118	i, ii, iii, iv, v
3-octanone[f1, f2, f3, m]	12.748		i, ii, iv, v
2-octanone[f1]	13.987	1284	iii, iv, v
8-Hydroxy-2-octanone[f1, f2]	14.091		iv, vii
2-hexanone,3,4-dimethyl-[f1, f2, f3, m]	14.313		iv, v
Cyclohexanone, 2,2,6- trimethyl-[f2, f3, m]	14.682		iv, viii
3-Ethylcyclopentanone[f2]	15.28		iv, vii
2,3-octanedione[f1]	15.385	1323	iii, iv, v
6-Methyl-5-hepten-2- one[f1, f2, f3, m]	15.568	1337	i, ii, iii, iv, v
2-nonanone[f1, f2, f3, m]	17.227	1387	i, ii, iii, iv, v, vi
Trans-3-Nonen-2-one[f1]	19.207		iv, vii
2-decanone[f1]	20.707		i, ii, iii, iv, v, vi
2-Undecanone[f1]	23.882		iv, v
Acetophenone[f1, f2, f3]	25.186	1645	i, ii, iii, iv, v
2-piperidinone, 1-methyl-[f2, f3]	29.957	1815	iii, iv, vii
Aldehydes			
Pentanal[f2, f3]	4.974		iv, v
Hexanal[f2, m]	9.911	1186	iii, iv, v, vi
Heptanal[f1]	10.738	1182	iii, iv, v, vi

(*continued*)

Table 1. (*continued*)

Volatile compounds	Rt	LRI (DB-WAX)	Identification mode[a]
Octanal[f1, f2, m]	13.855	1287	i, ii, iii, iv, v, vi
Nonanal[f1, m]	17.195	1391	i, ii, iii, iv, v, vi
Decanal[m]	20.548		i, ii, iv, v, vi
Benzaldehyde[f1, f2, f3, m]	21.362	1516	iii, iv, v, vi
Phenylacetaldehyde[f2]	23.373		iv, v
Sulphur containing compounds			
Dimethyl disulphide[f1, f2, f3, m]	7.156		i, ii, iv, v
2,4-Dithiapentane[m, f3]	13.639	1280	iii, iv, vii
Dimethyl trisulfide[f1, f2, f3, m]	16.667	1371	i, ii, iii, iv, v
Thiophene, 2-pentyl-[f1, f2, f3]	19.231		iv, vii
Ethanol,2-(metylthio)-[m, f3]	21.852	1534	iii, iv, vii
2,4,5-Trithiahexane[f2]	25.377		iv, vii
Nitrogen containing compounds			
Hexanenitrile[f2, m]	14.218		iv, v
Pyridine,2,4,6- trimethyl-[f2]	17.036		iv, vii
Pyrazine,tri methyl[f2, f3]	17.94	1405	iii, iv, vii
Oxime-, methoxy- phenyl-_[f1, f2, f3, m]	28.92		iv, vii
Indole[f2, f3, m]	44.386		iv, vii
Hydrocarbons			
Ethylbenzene[f1, m]	8.473	1120	iii, iv, v
beta-ocimene[f1, f2]	8.836		iv, vii
p-xylene[m]	9.189	1134	i, ii, iii, iv, v
Benzene, tert-butyl-[f1, f2, m]	12.869		iv, vii
Terpinolene[f1]	12.92		iv, vii
p-cymene[f3, m]	12.984		iv, vii
benzene,1,2,3- trimethyl[f1, m]	13.156		iv, vii
p-cymenene[f1, f3, m]	18.64		iv, vii
Benzene, 1,3-dichloro-[f1, f2, f3, m]	18.779	1436	iii, iv, vii
1-Phenyl-1-butene[f1, f3]	18.865		iv, vii
Azulene[f1, f2, f3, m]	27.546	1729	iii, iv, v
Acids			
Acetic acid[f1, f3, m]	19.562	1427	i, ii, iii, iv, v
Butanoic acid, 4- hydroxy-[f1, m]	24.55		iv, v

[a]Compounds were identified by (i) comparing retention time (Rt) with authentic compounds; (ii) Co-chromatography with authentic compounds; (iii) Linear retention index (LRI) relative to C11–C19 n-alkanes and compared with published LRI data (PubChem); (iv) comparing mass fragmentation pattern with NIST library (2011); (v) analysis of characteristic features of MS fragments; (vi) Comparison with published mass spectrometric data from our group; (vii) Absolute configuration not determined

the MS source, quadrupole and auxiliary heater were set at 230 °C, 150 °C and 280 °C, respectively. The electron energy was 70 eV (vacuum pressure-2.21 e-0.5 torr). The mass fragment scan range was 50–450 amu at 0.5 s/scan.

Lipids were extracted from the urine by using the Bligh and Dyer's (1959) method. An aliquot of chloroform extract was taken in a pre-weighed glass vial for gravimetric estimation of lipids. The solvent was evaporated to dryness with a stream of N_2 and the residue weighed again by precision balance. For the analysis of fatty acids (FA), the chloroform phase was used. The fatty acids present in urine were derivatized to fatty acid methyl ester (FAME) by acid catalysed esterification (Poddar-Sarkar 1996). FAME was recovered with n-hexane and finally dried over anhydrous sodium sulphate. The volume of the n-hexane was reduced under a stream of nitrogen and subjected to GCMS. 1 μl of hexane extract of FAME was injected to HP5-MS column (30 m 0.25 mm × 0.25 μm) of GCMS (Agilent Technologies, USA; 7890A GC system with 5975C triple axis detector MS) apparatus. The programme was set at 70 °C initial hold for 1 min for column temperature, ramping at 4 °C/min. up to 260 °C with a final hold for 3 min. FAME were identified by calculating their relative retention time (RRt) and comparison with authentic mixture of 37 FAME and PUFA (Supelco, Lot No: LB80556 and LB77207, USA). Identification was confirmed by comparing mass fragmentation pattern of the compounds from the NIST (2011) data base.

2.3 Statistical Data Analysis

A total number of 27 samples from three females and one male were analysed by GCMS. For quantitative analysis, each peak was normalised by calculating the relative percentage considering total ion count from the chromatogram. Chemical compounds identified by mass fragmentation pattern were grouped into different classes on the basis of their functional group or nature of backbone. Summation of nine classes of compounds from all females during the reproductive season (RS) and non-reproductive season (NRS) were plotted in Fig. 2. In addition, a comparative assessment on the basis of such 35 identified volatile compounds which were present in all urine samples of four leopards were done by heat map (Fig. 3). For heat map generation, successive steps were followed: Step (i) the sampling events were segmented into two seasons: RS and NRS for each animal; (ii) A table was developed by considering average amount for each identified compounds taking all females in a pool and for data in male in separate pool; (iii) Values were converted to the percentage of the row sum of each compound considering NRS & RS separately for female pool as well as for male pool; (iv) Derived values were used to form the final matrix (Fig. 3). Statistical data were processed using past software (3.21 version) for generating the heat map.

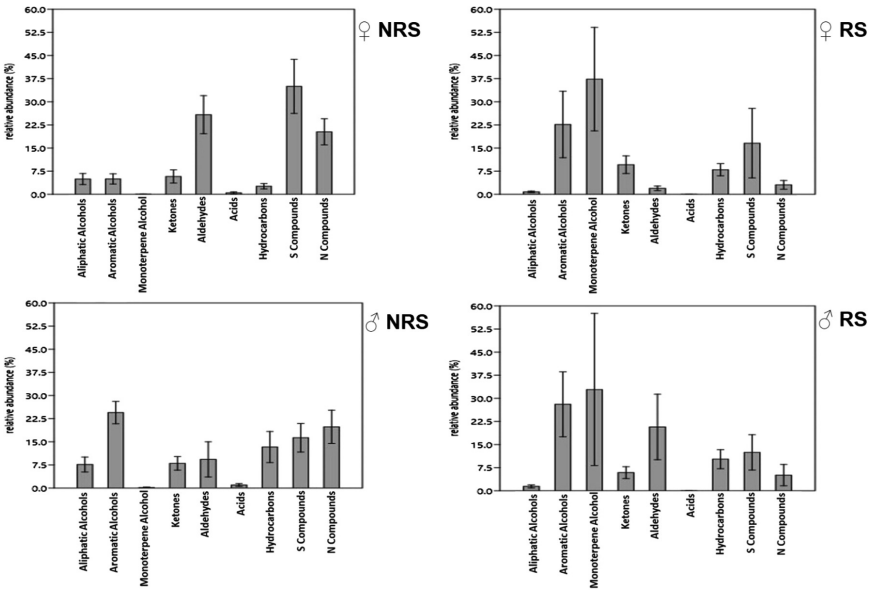

Fig. 2. HSV profile of nine classes of compounds distinctively separated from urine of male and female during non-reproductive (NRS) and reproductive seasons (RS) of snow leopard; S—sulphur containing compounds, N—nitrogen containing compounds

3 Results

A number of volatile organic compounds (VOCs) with different functional groups like alcohols, aldehydes and ketones were identified from the HSVs of urine of snow leopard of both the sexes (Table 1) The most interesting compound 6-*Methyl-5-hepten-2-one* which has a characteristic aroma, perceptible by the human nose was emitted from the fresh urine of snow leopard. However, no such distinctive aroma was perceptible from the distilled water washings of the floor processed in the same manner as a control, and 2-acetyl-1-pyrroline, the aroma molecule responsible for the characteristic smell of 'Basmati rice' present in MF of tigers and Indian leopards (Brahmachary et al. 1990; Brahmachary 1996; Poddar-Sarkar and Brahmachary 2014) was not detected in the urine of snow leopards. *Dimethyl disulphide* and *Dimethyl trisulfide*, two sulphur compounds were also identified from urine of snow leopard (Table 1). Low boiling straight chain alcohol of carbon number 5, 6, 7, 8 and aldehyde of 6, 8, 9 were common HSVs present in the urine of both male and females. Two carboxylic acids, such as acetic acid and 4-hydroxy butanoic acid were identified from both sexes. Some urinary constituents like phenol, benzaldehyde, p-cresol, acetophenone were also identified from urine. Some compounds which are very common secondary metabolites of plants like azulene, 1-methyl-2-piperidone, beta-ocimene, p-cymene, p cymenene were also identified in urine of snow leopard. HSV profile of the urine collected from reproductive season showed significant presence of some terpenoids such as alpha-terpineol, gamma-terpineol, terpineol-4-ol, cis-dihydro-alpha-terpineol and terpinolene

(Table 1). We found distinctive variations in the relative abundance of some compounds in both sexes during RS and NRS. During RS, high amounts of monoterpene alcohols and aromatic alcohols were identified in contrast to the lower amounts of sulphur and nitrogen containing compounds as well as aliphatic alcohols (Fig. 2). RS and NRS differed between male and female urine (Fig. 3).

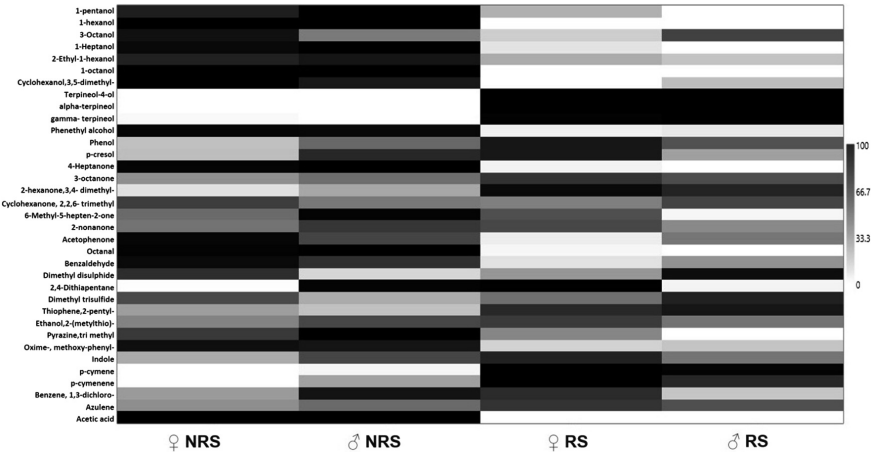

Fig. 3. Heat map generated on the basis of the statement mentioned in Sect. 2.3 of the text. Thirty five compounds of the Y-axis with different functional groups and structural backbones segregated the chemical profile of urine during NRS and RS from both sexes

Lipids present in the urine of snow leopard of both sexes ranged from 0.95 to 1.42 mg/ml. The Lipid fraction of snow leopard urine mostly contained Saturated Fatty Acids (SFA) of even and odd carbon number. Palmitic acid (16:0) is the most abundant in all cases (Fig. 4). In addition to even carbon number SFA such as Decanoic acid (10:0), Dodecanoic acid (12:0), Tetradecanoic acid (14:0), Octadecanoic acid (18:0), Eicosanoic acid (20:0), Docosanoic acid (22:0) and Tetracosanoic acid (24:0) some SFA with odd carbon number, such as Tridecanoic acid (13:0), Pentadecanoic acid (15:0), Heptadecanoic acid (17:0), Nonadecanoic acid (19:0) and Heneicosanoic acid (21:0) were also identified. In addition, Benzeneacetic acid is also detected in the urine of snow leopard. Five monounsaturated FAs (7-Hexadecenoic acid, 9-Hexadecenoic acid, 9-Octadecenoic acid,11-Eicosenoic acid and 13-Docosenoic acid) and two polyunsaturated FAs (9,12-Octadecadienoic acid and 5,8,11-Eicosatrienoic acid) were identified (Fig. 4).

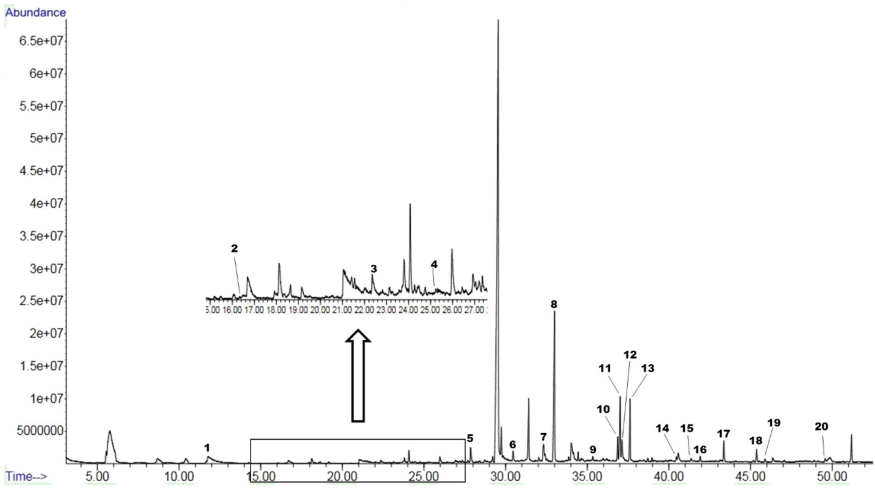

Fig. 4. Fatty Acids identified from lipid part of Urine of Snow leopard: Peak No 1. Benzeneacetic acid methyl ester 2. Decanoic acid methyl ester 3. Dodecanoic acid methyl ester 4. Tridecanoic acid methyl ester 5. Methyl tetradecanoate 6. Pentadecanoic acid methyl ester 7. 9-Hexadecenoic acid methyl ester 8. Hexadecanoic acid methyl ester 9. Heptadecanoic acid methyl ester 10. 9,12-Octadecadienoic acid methyl ester 11. 13-Octadecenoic acid methyl ester 12. 9-Octadecenoic acid methyl ester 13. Methyl stearate 14. 5,8,11-Eicosatrienoic acid methyl ester 15. 11-Eicosenoic acid methyl ester 16. Eicosanoic acid methyl ester 17. Heneicosanoic acid methyl ester 18. 13-Docosenoic acid methyl ester 19. Docosanoic acid methyl ester 20. Tetracosanoic acid methyl ester

4 Discussion

One of the volatiles of fresh snow leopard urine is sulcatone (*6-Methyl-5-hepten-2-one*), which imparts a characteristic odour perceptible to the human nose. Urine of snow leopards contains many characteristic low molecular weight compounds with diverse functional groups such as pentanol, hexanol, heptanol, 3-octanone, nonanal, indole, etc. which might play a role in chemical communication. Similar types of compounds have been shown to moderate and govern a variety of specialised behaviours related to kin recognition, choosing potential partners and maintaining social standings in many mammals including other felids and canids (Andersen and Vulpius 1999; Burger et al. 2006; Raymer et al. 1984; Soso and Koziel 2017; Wilson 1980). Dimethyl disulphide (DMDS), a male attractant compound of Hamster Vaginal Secretions (Singer et al. 1976) and one of the most important constituents of MF in lions (Soso and Koziel 2017) as well as Cheetah urine (Burger et al. 2006) was also confirmed in the urine of Snow leopard. Many secondary metabolites of plant systems were identified from the urine of snow leopard, and we observed fragmented leaves in their scats. Interestingly, we found significant variation in urinary terpenoidal compounds during their reproductive season. Hexanal, Octanal, Nonanal, 4-heptanone and benzaldehyde, identified from urine of both female and male snow leopard were considered as common urinary volatiles of many mammals such as the house mouse

(Novotny et al. 1999), white-tailed deer (Miller et al. 1998) and elephant (Rasmussen and Greenwood 2003), coyote (Schultz et al. 1988), ferret (Zhang et al. 2005) and MF of lions (Soso and Koziel 2017). Other low carbon alcohols and aldehydes are also common urinary volatiles of many mammals (Albone 1984). Phenethyl alcohol, detected in urine of snow leopard, is one of the major volatiles of lion MF (Soso and Koziel 2017). Fatty acids, identified from the urine of snow leopard are similar in nature to other big cats such as tiger, leopard, lion and cheetah (Poddar-Sarkar 1996; Poddar-Sarkar and Brahmachary 2014). It can be assumed that lipids may be delaying the dissipation of urinary volatile constituents which may facilitate animals to mark vast areas for territorial maintenance (Brahmachary and Dutta 1987; Poddar-Sarkar and Brahmachary 1996; Poddar-Sarkar 1996; Poddar-Sarkar and Brahmachary 2004). Brahmachary and Dutta observed previously that steam distillation separates the smell of volatiles which rapidly vanishes after being liberated from the heavier lipids (Brahmachary unpublished; Brahmachary and Choudhuri unpublished; Brahmachary and Dutta 1979; Poddar-Sarkar and Brahmachary 2014). As urine is one of the major sources of pheromone in other felids, it can be presumed that it might play a similar role in snow leopards. Nevertheless, extension of this work may add some new findings in the future. Therefore, by analysing the VOCs of urine throughout the year, the physiological status of the animal can be assessed and could form an important basis for the planning and management of future breeding programmes of this rare species as well as being utilised for zoo management and conservation purposes.

Acknowledgements. Author SD [CSIR sanction no-09/0289(0996)/2017-EMR-1], SM [09/0289(1004)/2017-EMR-1] and PD [108(Sanc.)/ST/P/S&T/1G-24/2014] are grateful to Council of Scientific & Industrial Research (CSIR), Government of India and Govt. of West Bengal respectively for providing their fellowships during this work. We would also like to acknowledge the Department of Science and Technology (Fund for Infrastructure development in Science and Technology programme) Govt. of India for extending GCMS facility in the Department of Botany, University of Calcutta. We also acknowledge the kind help and assistance from Principal Chief Conservator of Forest (wildlife), Govt. of West Bengal, and Director of Padmaja Naidu Himalayan Zoological Park, Darjeeling, West Bengal.

Note:

We dedicate this paper to the memory of our mentor Late Prof. R.L. Brahmachary with our deep grief and sorrow. He corrected our initial draft of this manuscript but passed away on 13 February 2018 when we were submitting the final version of this paper.

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