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Changes in urinary volatiles and proteins in male goats: A possible clue for females during mate selection

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ABSTRACT

Urine is believed to be a potent source of chemo-communication among mammals. The semiochemicals in the urine are useful to advertise the physiological conditions among members of the same species. The female goats select the intact male goats for mating, but what drives this mate selection is not known. We assume that biochemicals in the urine of intact males are pivotal during sexual selection. Therefore, we investigated the pooled urine of intact, prepubertal and castrated goats (n = 6/group) for volatile analysis using Gas Chromatograph- Mass Spectrometer (GC-MS) and protein profiling adopting Sodium Dodecyl Sulphate-Polyacrlamide Gel Electrophoresis (SDS-PAGE). Among the total of 23 volatile compounds, each group revealed unique compounds in their urine; four compounds in intact goats, eight compounds in castrated goats, and two compounds in prepubertal goats. The urine of intact goats contain as many as proteins compared to prepubertal and castrated goats. This is positively supported by the reduced level of creatinine in the intact goats, and elevated level in the prepubertal and castrated goats. The present study offer a template for understanding the physiology of male goats and its influence on urinary biochemicals that may together serve for mate selection.

1. Introduction

Animals rely on chemical cues for inter-individual communication. Chemical signals dedicate the prime channel of communication among animals. The chemical signals are present in the body secretions and involved in the context of social or sexual communication. The body secretions studied in detail pertaining to animal communication includes urine, feces, saliva, vaginal mucus, glandular secretions, milk, tear etc. (Tirindelli et al., 2009). The mode of communication facilitated by these body secretions comprises of individual recognition, puberty acceleration, social organization, mother-young interaction, sexual communication and so on (Archunan et al., 2014). Among the secretions investigated, urine seems to be a prime and potent source of communication among many animals such as mice (Achiraman and Archunan, 2006), rat (Selvaraj and Archunan, 2002), cat (Miyazaki et al., 2006), blackbuck (Archunan and Rajagopal, 2013), cow (Archunan and Ramesh Kumar, 2012), buffalo (Rajanarayanan and Archunan, 2011), and elephant (Goodwin et al., 2006).

The chemical composition of urine determines its participation level (solely/partly) in animal communication. The volatile compounds in the urine, in part are crucial in communication. In general, the differences in physiological condition have influence over the volatile compounds that are excreted out. For instance, urinary volatile compounds were changed in lion under different physiological conditions (Andersen and Vulpius, 1999). In male mice, the urinary volatiles were studied and specific volatiles in intact male mice that are used by female for sexual selection were identified (Osada et al., 2009). The presence/absence of particular volatiles is likely to change the preference level of behavior (attraction/aversion) in animals (Achiraman et al., 2014). The urine has also been reported to contain proteins, which could also dependent on physiological condition of the animal. Notably, in rats, the expression of a specific urinary protein was significantly changed when the rats attain sexual maturity (Vettorazzi et al., 2013). The urinalysis of animals attributes to identification of

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physiological conditions in non-invasive manner, especially in endangered species (Dehnhard et al., 2006).

Hitherto, most of the urinalysis studies have been conducted on female animals, and knowledge on male urinary constituents are fragmentary. In goats, during mate selection processes, females particularly select sexually-intact male goats over castrated and prepubertal goats. The physiological factors that drive this mate selection by females are not identified. We presume that the difference in the physiology (sexual state), in part might be reflected through urinary constituents. Therefore, we planned to study urinary volatiles and proteins collected from male goats of different physiological condition (intact, castrated and prepubertal).

2. Materials and methods

2.1. Test animals

Male goats with three different physiological conditions have been utilized for urine collection. The groups were, intact (24 months old), prepubertal (30 days old) and castrated [post-pubertally castrated (wether)] (castrated after 18 months), in which each group consists of six animals. As per the protocol of Adrian and Arancon (2011), the urine samples were collected by free-catch method using sterile glass beaker, transferred immediately to screw cap vials and stored at -20 °C until analysis. During the experimental period, the prepubertal animals were fed milk from does, and rests of the groups were given with cultivated forage and green fodder while grazing and water *ad libitum*. The study was carried out at Veterinary College and Research Institute, Namakkal, India in accordance with Institutional Animal Ethics Committee (IAEC).

2.2. GC-MS analysis

The urine samples respective of each physiological condition of goats were pooled and extracted with dichloromethane (1:1). The extract was filtered using Whatman filter paper and used for analysis of volatile compounds using GC-MS (QP-2010 plus, Shimadzu, Japan). The methodology for GC-MS was adopted from SankarGanesh et al. (2014). Two micro-liters of extract was injected into the GC-MS at 220 °C. The GC had a 30 m capillary column with a film thickness of 0.25 μm (30 m × 0.2 mm i.d., coated with UCON HB 2000). The oven temperature program was 40 °C for 10 min and then rose to 220 °C at 5 $°C^{-1}$, and held for 5 min. Mass spectrometer was operated in electron ionization mode at 70 eV. Ammonia was used as reagent gas and unknown compounds were identified by probability-based matching, using WILEY Registry™, NIST05 and NIST05s based library.

2.3. Protein precipitation and SDS-PAGE analysis

The urine samples collected from intact, castrated and prepubertal goats were precipitated using trichloroacetic acid (TCA) method. The individual samples (n = 6/group) (1 mL) were precipitated with 100% TCA followed by 0.1% TCA by centrifugation at appropriate rpm, and the resulted pellet was washed with acetone. The pellet was dissolved in PBS (pH -7.2) (50 μL), and proteins in the samples were quantified adopting Bradford method (1976). An equal quantity of protein (50 μg of protein) with gel loading dye was heated at 95 °C for 3 min, followed by loading onto individual well along with molecular weight marker (Genei, Cat. No:105979) for SDS-PAGE analysis (12% SDS-PAGE gel). The localization of protein bands were visualized using Coomassie blue staining method. The molecular weight identification of unknown proteins was achieved using the marker proteins.

2.4. Creatinine level

The level of creatinine in urine samples was assayed by kit method (Catalogue No: COD 11502, Biosystems Diagnostics Pvt. Ltd. TN, India) using semi auto analyzer (Tulip semi auto analyzer, Japan).

2.5. Statistical analysis

For creatinine analysis, statistical comparisons were made with one-way analysis of variance (ANOVA) followed by Duncan's multivariate analysis using SPSS version 17 (SPSS Inc., Chicago, IL, USA). We calculated the number of animals based on power calculation analysis and it satisfies the statistical requirement (Zar, 1984). The values are expressed in mean ± SD of six animals. Values with different alphabets are significantly different ($p < 0.05$) and values with same alphabets imply insignificant change between the groups at 95% confidence interval.

3. Results

3.1. GC-MS analysis

GC-MS analysis revealed the presence of 23 compounds altogether in all the phases. Among which, five compounds were common to all, besides to unique compounds in each group (two in prepubertal goats, four in intact males, and eight in castrated goats) (Figs. 1,2 and 3; Table 1). The intact male specific compounds were identified as Propan-2-one, 2-Methyl-2-Butenal, 7-Hexadecene and 1- Pentadecene. The compound, 1-chlorohexadecene was found in the urine of intact and castrated goats, and absent in prepubertals. Cyclopropane was present in intact and prepubertal urine but absent in castrated male goats. The molecular weight of the identified compounds was between 52 and 354. The chemical nature of the identified compounds was mostly belonged to phenol, hydrocarbon, steroid, alcohol, alkenes and alkanes. Alkenes, alkanes and alcohol were prominent in intact urine.

3.2. SDS-PAGE analysis

The protein analysis of prepubertal, intact and castrated goat urine revealed considerable difference (Fig. 4). The intact male goat urine contains more protein, whereas prepubertal and castrated male goats excrete fewer proteins. The intact goat contained proteins at all molecular weight ranges, including 35, 48, 55, 60, 70, 80 and 100 kDa. In contrast, low molecular proteins were merely absent in prepubertal and castrated male goat urine. Few proteins at high molecular weight are present in all the groups; however, their intensity varies between the groups. For instance, the 100 kDa protein was present in all the urine, however its intensity was higher in intact than prepubertal and castrated goats.

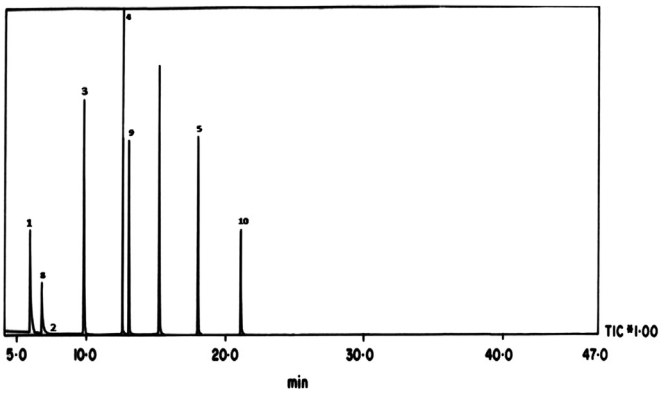

Fig. 1. GC-MS chromatogram of urine of prepubertal male goats. The number in each peak corresponds to the number of the compounds given in Table 1.

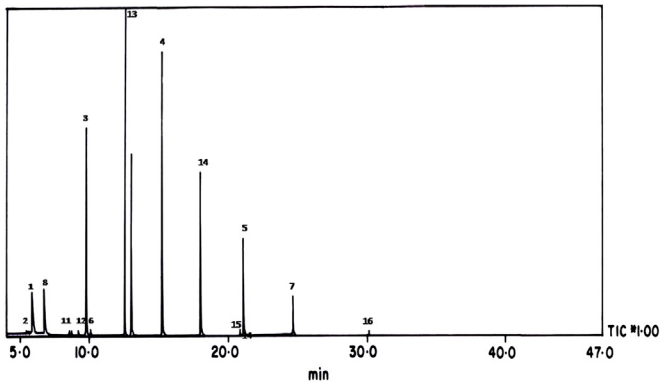

Fig. 2. GC-MS chromatogram of urine of intact male goats. The number in each peak corresponds to the number of the compounds given in Table 1.

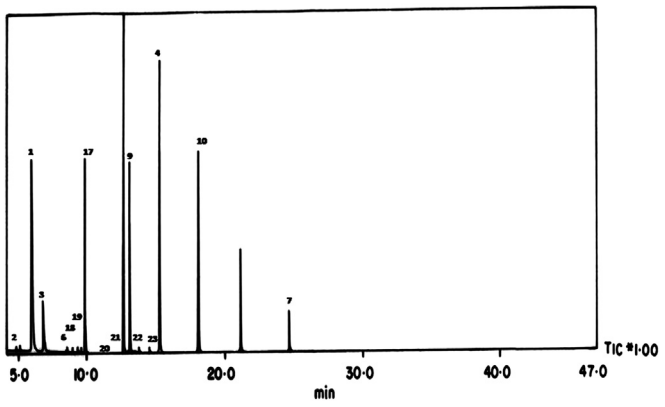

Fig. 3. GC-MS chromatogram of urine of castrated male goats. The number in each peak corresponds to the number of the compounds given in Table 1.

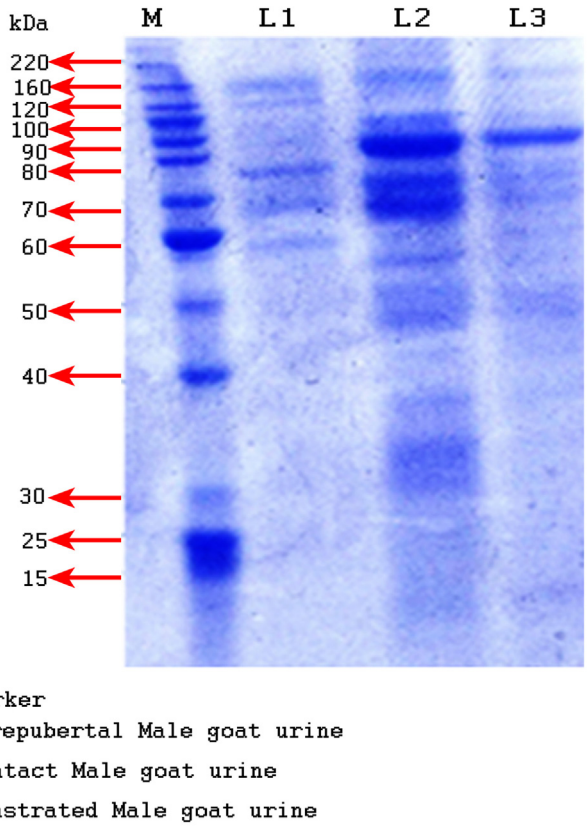

Fig. 4. SDS-PAGE analysis of urinary protein profile of prepubertal, intact, and castrated male goats. The urine of prepubertal goats revealed and intact male goats revealed high molecular weight proteins with consistent variation in the band intensity. The urine of castrated goats contain only fewer proteins also with less intensity bands.

3.3. Urinary creatinine level

The creatinine was found elevated in prepubertal urine compared to intact and castrated goat urine. The quantity of creatinine between the groups is statistically different between prepubertal and other groups, whereas the difference was insignificant in intact and castrated goats (Fig. 5).

Table 1
List of compounds identified in the urine of prepubertal, intact and castrated male goats.

S. No	Compound Name	Prepubertal male	Intact male	Castrated male	Molecular formula	Molecular weight (Da)
1	1-cyclopropyl-3-methyl-allene	✓	✓	✓	C_7H_{10}	94
2	1- Buten-3-yne	✓	✓	✓	C_4H_3CL	86
3	5- Tetradecene	✓	✓	✓	$C_{14}H_{28}$	196
4	5- Octadecene	✓	✓	✓	$C_{18}H_{36}$	252
5	1- Octadecene	✓	✓	✓	$C_{18}H_{36}$	252
6	1-Chlorohexadecene	X	✓	✓	$C_{16}H_{33}CL$	260
7	Cyclopropane	✓	✓	X	C_8H_{16}	112
8	2,6 bis (1,1- dimethylethyl) phenol	✓	X	✓	$C_{14}H_{22}O$	206
9	5- Eicosene	✓	X	✓	$C_{20}H_{40}$	280
10	Propan-2-one	X	✓	X	$C_{15}H_{12}$	72
11	2-Methyl-2-Butenal	X	✓	X	C_5H_8O	84
12	7- Hexadecene	X	✓	X	$C_{16}H_{32}$	224
13	1- Pentadecene	X	✓	X	$C_{15}H_{30}$	210
14	2- Dodecene	X	X	✓	$C_{12}H_{24}$	168
15	1-Phenyl- 2- Butanone	X	X	✓	$C_{10}H_{12}O$	148
16	Diflouromethane	X	X	✓	CH_2F_2	52
17	1,3 Pentadiene	X	X	✓	C_5H_8	68
18	3- Penten-1-ol	X	X	✓	$C_5H_{10}O$	86
19	Pyrazine	X	X	✓	$C_4H_4N_2$	80
20	2- methyl-1-pentanol	X	X	✓	$C_6H_{14}O$	102
21	1- Tetracosanol	X	X	✓	$C_{20}H_{50}O$	354
22	8-mehtyl-1- decene	✓	X	X	$C_{11}H_{22}$	154
23	Cyclopentane	✓	X	X	C_8H_{16}	112

✓- present; X- absent.

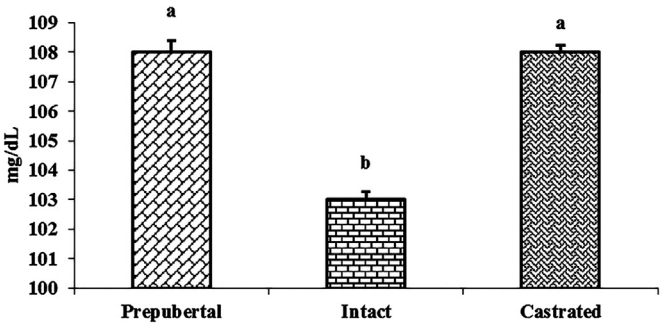

Fig. 5. The urinary creatinine level of prepubertal, intact, and castrated male goats. Values are expressed in Mean ± SD of six animals. Statistical comparisons were made with one-way analysis of variance (ANOVA) followed by Duncan's multivariate analysis. Values with different alphabets are significantly different and values with same alphabets imply insignificant change between the groups at 5% confidence level ($p < 0.05$).

4. Discussion

There have been considerable reports published regarding the sexual receptivity of female goats towards the male goats of different physiological condition. A study by Longpre and Katz (2011) corroborated that the attracting capacity of male goats may depend on the internal physiological conditions that reflected on external morphology. The physiological conditions analyzed in their study are prepubertal, intact, and castrated goats (wether (post-pubertally castrated goat) & stag (pre-pubertally castrated goat)). However, the actual role of physiology underscore the attracting capacity has not been elucidated. Prepubertal is a condition during which the role of testosterone is not fully established. In contrast, castration condition, prevent or inhibit the action of testosterone. Castration, in turn reduces the unpleasant smell in the meat, since the intact male gives the buck smell to the meat (Kebede et al., 2008). The concept of buck odor production has been studied in detail with castrated and intact goats by elucidating the difference in the pattern of volatile compounds (Murata et al., 2014). The study concluded with condition of physiology of the male goat, i.e. the castration and intact condition, in producing particular volatiles, which may confer the production of buck odor. Besides, intact male-specific molecules are pivotal during mate selection by the females. All these reports enforce to study the physiological variation of male goats. However, none of the study has been conducted to reveal the implication of physiology of male goats on body fluids. In order to differentiate the sexually intact males, female goats should have some clues, and we suspect these clues may be presented by the components of body fluids, specifically urine. Therefore, we subjected the urine of intact, prepubertal and castrated male goats to volatile analysis. We noticed a considerable variation in the number of volatiles during each condition of male goats, and found 5 volatiles as common to all the groups. A study in mice model by Harvey and Jemiolo (1989) revealed variation in urinary volatiles with physiological condition, and the compounds found at higher concentration in dominant males were absent in castrated males. Similarly, in our study we found four volatiles specifically in intact male urine that were absent either in intact or prepubertal's urine. The urinary volatile compounds present in intact mice were seems to be absent during castration and prepubertal condition, which were re-appeared when the castrated mice were administered with testosterone (Achiraman and Archunan, 2005). In line with this, we suggest the compounds unique to intact males may be of testosterone-dependent.

In higher animals, castration leads to the development of fat mass (Morand-Fehr et al., 2000). The volatile compounds excreted in the urine of animals are mainly resulted from the metabolism of fatty acids inside the body. Thus, the fatty alcohols such as 2-methyl-1-pentanol and 1-tetracosanol have been noted only in the urine of castrated animals of the present study may be resulted by high fat mass. It may also suggest that the increased fat mass could contribute to excretion of more volatile compounds in the urine of castrated goats. The report of Ahmad et al. (1996) clearly demonstrated that gradual increase in the testosterone level and surge in luteinizing hormone occur at the time of sexual maturation. In prepubertal condition of the present study, the signal from GnRH to testosterone may not be well pronounced. Ultimately, this could be resulted in the excretion of different volatile compounds with two specific compounds in the urine of prepubertal goat which were absent in intact or castrated goats. Since, the physiology of prepubertal goats closely resemble castration, both the urine contain two unique compounds in the present study, which were absent in intact goats.

The chemical nature and previous literatures suggest the compounds of the present study may have pheromonal property. For instance, the modified form of 1-octadecene was reported to have pheromonal property in the peach leafminer moth (Zhang et al., 2013). The compound, 2-methy-2-butanal identified in the present study has been well studied in provoking mother-young interaction in rabbit (Schaal et al., 2003). The study by Arnaud et al. (2002) reported that 1-pentadecene as a common pheromonal compound in flour beetles. It has been experimentally proved that the compound, 1-dodecne has pheromonal property which have good affinity with the pheromone binding protein in *Helicoverpa* species (Sun et al., 2012). The compound, 1-dodecne, has also been implicated in various insect chemical communication system (http://www.pherobase.com). Altogether, the volatile analysis of the present study was positively supported by the previous reports by conferring the pheromonal property.

The urine of animals contains considerable quantity of proteins that have biological significance and meaningful in communication. In mice and rats, the urinary proteins were studied in detail, and the participatory roles of the specific carrier proteins in chemical communication were described (Cavaggioni and Mucignat-Caretta, 2000; Achiraman and Archunan, 2002). In rodents, the urinary proteins are dependent on physiological conditions and found associated with volatile compounds (Rajkumar et al., 2009; Muthukumar et al., 2013). Further the urinary proteins were also useful in recognition of individuals (Hurst et al., 2001). We found differential expression of proteins based on the physiological conditions of the male goats. There were more number of proteins were found in intact goats, however the prepubertal and castrated goats exhibited few proteins. The low molecular mass proteins were exclusively present with over-expression of specific high molecular weight proteins in intact male goats, however this was not noted either in prepubertal and castrated goats. The urinary proteins of goats have been studied in other perspectives, however this is the first report elucidated the influence of physiological condition on urinary proteins of male goats. Moreover, the protein profiling results were highly correlated with urinary creatinine levels. For instance, intact male goats contain more proteins and significantly lower level of creatinine than prepubertal. The protein creatinine ratio in the urine serves as useful, simple and convenient method to assess proteinurea (Kumar et al., 2013). In this study, the urinary creatinine content of the goats suggests that the goats excreted normal level of proteins in their urine.

Testosterone is the key hormone regulating the male physiology. Knopf et al. (1983) demonstrated that the level of expression of liver mRNA responsible for a specific urinary protein has consistent variation between normal and castrated mice. The mRNA level was increased thousand fold when testosterone was administered exogenously to the castrated mice. Therefore, it is speculated that excretion of urinary proteins in male goats might be under the control of testosterone. We found more number of proteins in the urine of intact and less in prepubertal and castrated goats. This draw a conclusion that increased testosterone level may increase the expression of many urinary proteins.

5. Conclusion

The findings of the present study summarize the presence of specific volatile compounds during different physiological conditions of male goats. It could be due to the difference in the metabolism that indirectly controls the expression of unique volatiles in each physiological condition of male goats. Further, the protein analysis in the urine of male goats also gives a clue to denote the physiological difference. Together, this variation might be an additional element used by the females during mate selection. However, behavioral analysis of unique volatile compounds of intact male goats with female goats, and characterization of specific proteins using mass spectrometry are warranted to delineate the essential functions of these putative chemo-signaling molecules.

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Conflicts of interest

The authors declare no conflicts of interest.

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