Buck odor production in the cornual gland of the male goat, *Capra hircus*—Validation with histoarchitecture, volatile and proteomic analysis

Devaraj Sankarganesh, Rajamaniackam Ramachandran, Radhakrishnan Ashok, Veluchamy Ramesh Saravanakumar, Raman Sukirtha, Govindaraju Archunan, Shannugam Achiraman

1Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli-620 024, Tamil Nadu, India
2Department of Microbial Biotechnology, Bharathiar University, Coimbatore-641 046, Tamil Nadu, India
3Department of Livestock Production and Management, Veterinary College and Research Institute, Namakkal-637 002, Tamil Nadu, India
4Center for Pheromone Technology, Department of Animal Science, Bharathidasan University, Tiruchirappalli- 620 024, Tamil Nadu, India

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In many animals, glandular secretions or pheromones that possess biological moieties contain messages encoded by the intrinsic smell. In male goats, the cornual gland (a sebaceous gland), may synthesize and excrete relevant chemical components that are responsible for the ‘buck effect’. To test this, cornual glands from freshly-slaughtered male goats (N=6) were subjected to histoarchitecture analysis, to infer about the structural alignment, to the GC–MS analysis for volatile compounds and to SDS–PAGE for protein profiling followed by MALDI–TOF to characterize specific protein bands. The gland possesses sebum, vacuoles and hair follicles inferring its capability to synthesize and extrude the scent. We found 14 volatiles in GC–MS analysis, in which 1-octadecanol might be a putative pheromone of buck odor. We identified seven different proteins in SDS-PAGE. Two proteins, 28 and 33 kDa, were highly matched with DNA mismatch repair protein and Abietadiene synthase, respectively, as inferred from MALDI-TOF. Conclusively, the volatiles identified in the cornual gland suggest that the structural microelements of the gland may synthesize (sebum and vacuoles) and release the key volatiles through the hair follicles. The volatile(s) thus produced in male goats either solely or synergistically may confer the buck odor.

**Keywords:** GC–MS, Goaty odor, MALDI-TOF, Putative pheromone, Scent gland, Sebum

Physiological status of animal can be assessed by conspecifics through a variety of scent sources, including glandular secretions. Mammalian pheromones are characterized by multiple compounds used in intra-specific communication. In goats, ‘buck odor’ is believed to be produced in the skin/hair/gland of intact males and has the capacity to synchronize/regulate the reproductive cycle and ovulation in the female. The introduction of sexually naïve bucks to a group of non-ovulating females results in synchronization of estrus by increasing the LH pulse rate and leads to ovulation (buck effect). Perhaps all the stimuli (odor, sight, sound, and touch) are mutually involved; odor seems to be a key element in regulating the buck effect. Hamada *et al.* utilized the hair samples of males as an odor source and exposed it to the female goats for a short period and found an increase in GnRH pulses in females. Subsequently, Ichimura *et al.* exposed the female goats with the pooled hair samples of males in a muzzle-fitted mask relatively for a longer period (4 h) and observed greater GnRH pulses. The compound responsible for the buck odor was identified as 4-ethylcysteic acid.

Lancker *et al.* reported a sebaceous gland underneath the horns of the male goats. Earlier, the gland was swabbed and volatile compounds were identified in an attempt to validate the buck effect, but no conclusive information was derived as decades-old chemical separation (chromatography) techniques were adopted. Interestingly, the upper dermal layer extracts of sebaceous glands from intact and castrated
plus testosterone administered goats were shown to induce greater multiple unit activities in female goats. Further, it is also demonstrated that the extract of cutaneous gland possesses active ingredients to produce buck odor. Remarkably, Murata et al. identified a single olfactory molecule (4-ethylloctanal) that is responsible for the elicitation of buck effect by confirming the multiple unit activity and induction of LH pulses in female goats. However, hitherto no study was performed in the extracts of the cornual gland to identify the complete volatiles.

In many animals, the scent gland and the volatile compounds produced in it have pivotal roles in communication among conspecifics. For instance, Atta laevigata, an ant, has a mandibular gland (modified scent gland) and the concentration of the compound it synthesizes varies in relation to the castes. In male lizards, the femoral gland with granular cells is the major source of pheromones. The mammary gland of the rabbit secretes milk that contains 2-methyl but-2-enal, which is believed to be a mediator of mother-young interaction. The preputial gland of rodents has been reported to play a key role in communication between sexes. For instance, in an earlier investigation, we reported that the clitoral gland of female laboratory rats contains an estrus-specific compound which could be a mediator of sexual communication. In addition, synthesis of the key constituents of the preputial gland of male rat is testosterone-dependent, which indicates that there is a relationship between glandular secretions and sexual status of animals.

In hoofed mammals, (e.g., deer) the preorbital gland is a good source of chemical compounds that facilitate chemo-communication, and some of these compounds have been implicated in the expression of dominant status by a particular male animal in the colony. Considerable difference in the histoarchitecture of preorbital gland has been shown between territorial and non-territorial Indian blackbuck. In elephants, the temporal gland secretes a fairly high quantity of musth, which is reported to be androgen-dependent. The axillary gland secretion is one of the important sources of human pheromones. The secretory material has a peculiar smell due to microbial action and confers the message of maleness.

Considering the large population size and great diversity in mammals, reports are fragmentary in support of the glandular sources performing roles in chemical communication. In goat, previous reports clearly indicate that hair/skin samples of head area contain compounds of goaty odor, which is presumably due to the presence of sebaceous glands (cornual gland). However, the study of the cornual gland in the perspectives of histology, and volatile analysis in the extracts of the gland is not performed, which may further shed light on buck effect. Therefore, we embarked a study on the cornual gland to 1) Confirm the secretory and excretory nature of the gland by subjecting it to histological analysis, 2) Detect the complete volatiles (putative pheromones) in the extract of the gland by GC–MS analysis, and 3) To elucidate the protein profile to look for the presence of carrier proteins, if any.

**Materials and Methods**

**Test animals and collection of gland samples**

Adult male goats (N=6), Capra hircus fed with standard ration and water ad libitum were used for the present study. The inclusion criterion was sexually intact male goats and they were not castrated. The goats were sacrificed at the slaughter house during March–April when their age was <24 months, and 2 gland samples (2 from each) were obtained. The gland was located as described previously. The hairs in the caudomedial border beneath the horns were shaved and the gland was located as a distinct oval-shaped structure with 1–3 cm in length and 0.5–1.5 cm wide. We used one gland from each goat for extract preparation by homogenizing with PBS. This extract was used for volatile analysis in GC–MS and remnant of the extract was processed for SDS–PAGE analysis. Another gland from each goat was subjected to histological sectioning and representative of single gland structure was shown in results. The study does not involve live animals, and the glands were collected from the monitored animals after sacrifice at the slaughter house.

**Histoarchitectural analysis**

The glands were dissected and fixed immediately in 10% neutral buffered formalin, and subsequently embedded in paraffin. The sections (4 µm) were made, and stained with haematoxylin and eosin. The stained sections on glass slides were examined under a light microscope (Wetzlar Hund, Germany) and photographs were captured with real-time interpretations by a veterinary anatomist.

**Sample preparation**

The glands from six goats were sliced into small pieces using sterilized blades, which were
homogenized using a sterilized homogenizer with PBS (pH 7.2). A part of the homogenate (approximately 5 mL) was extracted with dichloromethane in 1:1 ratio in a sterilized glass vial. Briefly, the extract was vortexed and kept in the refrigerator to allow distinct separation of layers of the extract. After 30 min, the solvent layer (crystal-like) was transferred to GC–MS vial using a Pasteur pipette and used for analysis of volatile compounds in GC–MS.

Gas chromatography–mass spectrometry

The final extract was filtered with Whatman filter paper (Grade 1, 11 μm pore size, Merck Biosciences) before injected into GC–MS (QP-2010 plus, Shimadzu, Japan). Two micro-liters of the pooled extract was injected into the GC–MS using the auto-injector. The injector temperature was 200°C and operated in split mode. The GC–MS was fitted with 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 temperature regimens were as follows; initial oven temperature, 40°C for 10 min; ramp 1 to 150°C at a rate of 5°C/min; ramp 2 to 230°C at a rate of 10°C/min and finally held at 230°C for 4 min. The detection accuracy was 1 ng/peak. The mass spectrometer was operated in electron ionization mode at 70 eV, and ammonia was used as carrier gas at a flow rate of 1.0 mL/min. The identification of unknown compounds was made by probability-based matching, using WILEY Registry™, NIST05, and NIST05s based library.

SDS–PAGE analysis

The remnant of the gland extract (homogenized using PBS) was centrifuged at 10000 rpm for 10 min at 4°C (Remi Cooling Centrifuge). The resulted pellet was discarded and the supernatant was transferred to another tube. Primarily, the protein quantity in the supernatant was estimated adopting Bradford method by reading the formed blue color solution in a spectrophotometer at 595 nm. Sixty micrograms of protein sample were taken and processed with sample loading dye and used in SDS–PAGE analysis. The gel was stained with Coomassie brilliant blue and de-stained (ethanol, acetic acid, and water) to visualize the bands. We have used molecular weight marker of medium range (Genei, Bangalore, India, Cat. No: 105979), which helped us to speculate the molecular weight of the proteins present in our sample.

De-staining and in-gel digestion

The protein bands of interest were excised and the gel plugs were de-stained separately using 100 μL of 25 mM NH₄HCO₃ in 50% (v/v) acetonitrile (1:1) by incubation at 37°C for 30 min. Each gel plug was sliced into small cubes, and placed in 1.5 mL Eppendorf tube. After drying in a Speed–Vac (Savant), the gel was incubated in 100 μL of 2% β-mercaptoethanol/25 mM NH₄HCO₃ for 20 min at room temperature. The same volume of 10% 4-vinlypyridine in 25 mM NH₄HCO₃/50% acetonitrile was added for cysteine alkylation. After 20 min, the gel was soaked in 1 mL of 25 mM NH₄HCO₃ for 10 min, dried and incubated overnight with 25 mM NH₄HCO₃ containing 100 ng of modified trypsin (Promega). The tryptic digest was removed from the gel and the proteins were extracted first with 300 μL of 25 mM NH₄HCO₃ and then in 50% acetonitrile. The two fractions were pooled and dried in a Speed–Vac and then stored at −20°C for further analysis. When resumed, each fraction was re-suspended in 0.1% formic acid immediately before use.

MALDI–TOF MS analysis

The MALDI-TOF MS data were acquired using an Ultraflex TOF/TOF spectrometer (Bruker Daltonics, Billerica, MA, USA), equipped with 50 Hz pulsed nitrogen laser (337 nm), operated in reflectron mode (positive ion) at 25 kV accelerating voltage with 90-ns time delay. External calibration was done using peptide I calibration standards (angiotensin II, angiotensin I, substance P, bombesin, ACTH clip 1–17 and 18–39, and somatostatin 28) with molecular masses ranging 1000–3200 Da. The samples were prepared by mixing an equal amount of peptide (0.5 μL) with dihydroxybenzoic acid/o-cyano-4-hydroxycinnamic acid (CHCA) saturated with 0.1% trifluoroacetic acid (TFA) and acetonitrile (1:1) as matrices. The mass spectrum of the 33 and 28 kDa proteins was obtained and the mono-isotopic masses of the protein spectrum were analyzed. The masses below 50 m/z were not considered due to interference from the matrix.

Database analysis

The spectra were processed using Flex analysis software. Database search was accomplished with the assigned masses of the monoisotopic peptide. Mascot search engine (Matrix Science, London, UK) (http://www.matrixscience.com) was utilized for the protein identification. Mascot analysis was carried out.
using carbamidomethyl as fixed modification and oxidation (M) as variable modification. The search was made under the taxonomy of the Eukaryota for 33 kDa protein and under all taxonomical entries for 28 kDa protein. The score value was checked for the level of significance and matched sequences (bold and underlined) were identified.

**Results**

**Structural organization of cornual gland as revealed by histoarchitecture analysis**

The gland is located close to the apical region of the dermal papilla. It opens at the dermal pore and is surrounded by the roots of the hair follicles. The surrounding area is filled with smooth muscle fibers, running at a different direction, *i.e.*, cross, oblique and longitudinal. The contraction of the smooth muscle fiber between the lobules and the fibers located around the gland could be involved in the extrusion of sebum. Acini are serous, *i.e.*, formed of small lobules, and the acinar gland is lobulated (Fig. 1).

**Volatile compounds in the cornual gland extract and their features as revealed in the GC–MS analysis**

Fourteen volatiles were identified in the cornual gland extract, among which most of the compounds were alkanes and alkenes whereas, some were alcohols. The molecular weights of the compounds were between 84 and 280 Da. There were 4–20 carbon atoms, and 3–40 hydrogen atoms in the compounds (Table 1; Fig. 2).

**Proteins in the cornual gland extract as revealed in the SDS–PAGE analysis**

The protein extract of the gland was separated in 12% SDS–PAGE, which revealed the presence of seven different protein fractions, and their molecular weights ranged from 15 to 58 kDa. Among the different proteins, the intensity of 48 kDa protein fraction was the highest (Fig. 3).

**Table 1 — List of compounds identified in the cornual gland extract of male goat, *Capra hircus***

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Molecular formula</th>
<th>Molecular weight (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cis-1-Chloro Buten-3-yne</td>
<td>C₆H₁₂Cl</td>
<td>86</td>
</tr>
<tr>
<td>1-Cyclopropyl octane</td>
<td>C₁₁H₂₂</td>
<td>154</td>
</tr>
<tr>
<td>Tri cyclohexane</td>
<td>C₆H₁₂</td>
<td>84</td>
</tr>
<tr>
<td>Alpha Dodecene</td>
<td>C₁₂H₂₄</td>
<td>168</td>
</tr>
<tr>
<td>5-Octadecene</td>
<td>C₁₈H₃₆</td>
<td>252</td>
</tr>
<tr>
<td>1,2 Hydroxyethoxy tridecane</td>
<td>C₁₃H₂₈</td>
<td>184</td>
</tr>
<tr>
<td>2,4-bis 1,1 dimethyl ethyl phenol</td>
<td>C₃H₆O</td>
<td>94</td>
</tr>
<tr>
<td>1-Pentadecene</td>
<td>C₁₅H₃₀</td>
<td>210</td>
</tr>
<tr>
<td>5-Eicosene</td>
<td>C₂₀H₄₀</td>
<td>280</td>
</tr>
<tr>
<td>2,3 Dimethyl hexane</td>
<td>C₃H₁₈</td>
<td>114</td>
</tr>
<tr>
<td>1,2 Benzenedicarboxylic acid</td>
<td>C₇H₉O</td>
<td>164</td>
</tr>
<tr>
<td>2-Methyl 2-Butenal</td>
<td>C₃H₆O</td>
<td>84</td>
</tr>
<tr>
<td>1-Hepten-5-one</td>
<td>C₇H₁₅O</td>
<td>112</td>
</tr>
<tr>
<td>1-Octadecanol</td>
<td>C₁₈H₃₈O</td>
<td>270</td>
</tr>
</tbody>
</table>

**Fig. 1** — Histoarchitecture of the cornual gland of male goat (10 & 40 X). The picture depict the presence of Vacuoles (A), Hair follicles (B), and Duct (C) in the gland.

**Fig. 2** — GC–MS chromatogram of male goat cornual gland extract.
Identification of the protein as revealed in MALDI–TOF

Among the different protein fractions, two protein fractions (at 28 and 33 kDa) were subjected to MALDI–TOF analysis, which revealed high score matching with DNA mismatch repair protein and abietadiene synthase, respectively, in MASCOT analysis. The sequence coverage for DNA mismatch repair protein was 22% and that for abietadiene synthase was 20% (Figs. 4 & 5, matched sequences are indicated bold).

Discussion

The histological technique was adopted to have an idea about the structural organization of the cornual gland and the observation confirmed that the cornual gland is a sebaceous gland that produces sebum. Previous studies have shown that rat preputial gland produces volatile compounds and also some proteins involved in communication. It is an established fact that sebaceous gland produces sebum, which is an oily and waxy substance as documented in the humans. According to Abbasi et al., the interdigital gland of sheep produces sebum and is discharged by the hair follicles present adjacent to the lobules. We also made a similar finding, and the cornual gland is formed of numerous lobules, located adjacent to the hair follicles indicating that sebum is produced and it is extruded via the hair follicles. In the deer, the fluid secretion release is visible, whereas in the goat, the

Fig. 3 — SDS–PAGE profile of male goat cornual gland extract [L1: Marker and L2: Cornual gland extract]

Fig. 4 — Mass Spectra of 28 kDa protein obtained from MALDI–TOF.
A. Number in the mass spectrum gives precise m/z (M+H) values for the detected peptide ion signals.
B. The matched 22% sequence coverage shown in bold and underlined.

Fig. 5 — Mass Spectra of 33 kDa protein obtained from MALDI–TOF.
A. Number in the mass spectrum gives precise m/z (M+H) values for the detected peptide ion signals.
B. The matched 20% sequence coverage shown in bold and underlined.
extrusion of the secretory material is not visible, but one can sense the pungent odor of the glandular secretion, which indicates that the secretion is released as a volatile substance. However, on several occasions, the gland is ruptured by hitting against the wall of fence especially, when the buck was encountered with a female goat in estrus (Sankarganesh, personal communication). Thus, it is comprehensive that the gland does secrete a material, and when the buck desires to mate with a female in estrus, the gland is ruptured and the secretion discharged as a volatile substance.

A pioneering study on goat scent gland suggested that cornual gland lacks the capacity to produce any pheromones; however, the study did not speak about volatiles. To validate and support our hypothesis, we carried out some preliminary studies so as to conclude that cornual gland is one of the major scent glands and may be involved in buck odor production. Earlier, it was reported that the scent gland of insects has distinctive roles in producing the compounds, mostly volatiles, and these compounds have been connected with eliciting behavioural responses. In this context, we analyzed the volatile portion of the gland and found 14 volatile compounds through GC–MS analysis, of which most of the compounds were alkanes, but few belonged to alkenes, aldehyde, ketone, and alcohol groups. The compounds under the class alkanes, isoalkanes, alcohols, ketones, carboxylic acids, saturated- and unsaturated-aldehydes have been reported in interdigital secretions and suggested to have a role in the expression of the dominant status. Therefore, we have reason to believe that the volatile compounds identified in the cornual gland of goat in the present study appear to have some crucial role in goat communication. Thus, we propose that the compounds identified in the present study are putative pheromones that might be released via hair follicles. Interestingly, Murata et al. have shown that the head skin of male goats contains a compound, 4-ethylotanal, which they believed to be involved in buck effect. However, in our study, we found 1-octadecanol in the glandular extract. It could be postulated that there may be a possible derivatization of 4-ethylotanal to 1-octadecanol while released through the hair follicles. In addition, the other compounds belonged to ketone, alkanes, and alkenes reported by Murata et al. are similar to our findings. It is important to note from the present study that 1-octadecanol is present only in the glandular extract of intact male goat and not found in other body secretions (urine and feces) of female goats, and urine of male goats (unpublished data). Therefore, we strongly conclude that 1-octadecanol is a gland-specific volatile produced only in the cornual gland of intact male goats.

Most of the compounds identified in the present study are not new but have already been reported to act as pheromones in other animals and insects. The compound 2-methyl-2-butenal, identified in the cornual gland has been earlier reported in rabbit’s milk, where it facilitates typical nipple searching behaviour in rabbit pups. Characterization of the other compounds also revealed most of them to be pheromones but the exact role has not been evaluated. The database available on the website (pherobase) gives adequate details about each of the pheromonal compounds. Thus, some of the compounds identified in the present study (e.g., 5-Eicosene) have been reported in the pherobase.

The compounds produced from scent sources are generally volatile in nature and, therefore, they are associated with carrier molecules which helps in sustained release of the signal. Flower, in his review, has elucidated the structural and functional aspects of the lipocalin superfamily proteins, to which the carrier proteins generally belong. A number of original studies have explained the possible role of lipocalin proteins which are related to chemical communication. However, in goats, the presence of carrier protein in any body secretions or scent sources was not evaluated. Therefore, we looked for carrier molecules that would bind and deliver volatile compounds. Seven protein bands, in the molecular weight ranging from 15 to 58 were identified. To elucidate the functional aspect of the proteins, we subjected two of the proteins to MALDI–TOF analysis that were identified as DNA mismatch repair protein and abiedatiene synthase. However, these two proteins are not carrier proteins, but appear to be regular proteins and have other roles.

DNA mismatch repair protein (MMR protein) has been reported in the sebaceous gland of other animals, where its function is crucial in inhibition of tumor formation since the absence of this protein have been linked to Lynch syndrome. Tome et al. identified DNA mismatch repair protein in various tissues of mice and found its concentration to be higher in the proliferative tissues than non-proliferative tissues. As cornual gland is one among the proliferative tissues, the presence of MMR protein must associate with
some crucial role although its relative concentration in other tissues of goat needs to be verified. Alterations in MMR protein has been implicated in the development of Muir–Torr syndrome. Though the MMR protein has been implicated in repairing the errors that occur during DNA replication by avoiding mismatch pairing of nucleotides, how the defective protein is involved in the development of this syndrome is not yet clearly understood. We infer that cornual gland is an important organ that produces the sebum and plays role in the communication of goats, and MMR protein may be necessary to keep the gland in a healthy condition.

Conclusion
The cornual gland of goat is diversely lobulated, reflecting sebaceous gland organization. Inferring from the histo-architectural analysis, the gland has the capacity to produce and expel volatile compounds, which we evidenced as putative pheromones. We suggest 1-octadecanol might contribute to buck odor majorly, however further studies are required to apply 1-octadecanol in view of improving goat production (estrous synchronization and induction of ovulation). This is the first report proving the potential of the cornual gland in the production of volatiles.

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References


