Indian Journal of Experimental Biology
Vol. 53, December 2015, pp. 771-778

Identification of pheromone-carrying protein in the preorbital gland post in the endangered Indian male Blackbuck *Antelope cervicapra* L.

T Rajagopal[1,3], R Rajkumar[1,4], P Ponmanickam[1,5], S Achiraman[1,2], P Padmanabhan[6] & G Archunan[1]*

[1]Center for Pheromone Technology, Department of Animal Science; [2]Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India.

[3]Department of Zoology and Microbiology, Thiagarajar College, Madurai 625 009, Tamil Nadu, India.

[4]Institute of Medical Biology (IMB), Singapore 138 648.

[5]Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi 626 124, Tamil Nadu, India

[6]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore-637 553

Received 30 April 2014; Revised 01 November 2014

In mammals, a low molecular mass protein (17-20 KDa) reported from the pheromone sources such as urine, saliva, glandular secretion, etc., as ligand-carrier (pheromone carrier) has been associated with chemo-communication. Since the preorbital gland post is one of the major pheromone sources in Indian Blackbuck, an endangered species, we assumed thta it possibly contains low molecular mass protein for chemical communication. Hence, we investigated the preorbital gland post in territorial and non-territorial male blackbucks for such low molecular mass proteins adopting SDS-PAGE and LC-MS/MS analysis. The total content of protein was higher in the post of territorial males than non-territorial males of adult and sub-adult. In fact, the protein profiles such as 17, 21, 25, 42 and 61 kDa were noted in the gland secretion of territorial and non-territorial males. The intensity of the 17 kDa protein band was higher in territorial males than non-territorial males. In-gel trypsin digestion of the 17 kDa band was processed and subjected to LC-MS/MS and SEQUEST analyses. The results of LC-MS/MS and SEQUEST search showed the presence of α_{2u}-globulin in the 17 kDa band. In addition, the identified α_{2u}-globulin sequence possessed GDW residues, which are the characteristic signature for lipocalin family. Since the α_{2u}-globulin has been reported from the pheromone-carrying proteins in some mammals, this protein may carry the volatiles (pheromone compounds) in male Blackbucks preorbital gland to evoke the scent marking for maintaining territoriality (home range) and attraction towards female, through the secretion of glandular protein.

Keywords: Chemo-communication, Endangered species, α_{2u}-Globulin, Olfactory communication, Scent marking, Territoriality.

The Indian blackbuck, *Antelope cervicapra* L., a member of the acelphin group of antelopes (Fam. Bovidae, Ord. Artiodactyla) is a highly endangered and endemic species of India. Wild Blackbuck population is on decline as a consequence of poaching, hunting, shrinkage of natural habitats and deforestation[1], with probably fewer than 25,000 individuals in their native range[1]. Blackbuck has been categorized as vulnerable by the IUCN endangered commission[2]. The social units in Blackbucks are: (i) solitary males (territorial), (ii) all-male or bachelor groups, composed of two or more juvenile, sub-adult or adult males, (iii) females in group, composed of females of all age-classes, fawns, and juvenile and sub-adult females, and (iv) mixed groups, represented by the whole range of age classes of both sexes[3]. Adult males generally establish territories that are consistently occupied round the year. Territorial advertising involves visual display, defecation, urination and preorbital gland marking[3,4]. Burger *et al.*[5,6] reported that antelopes have specialized skin gland, the preorbital gland, which has a ubiquitous distribution and secrete various components that are employed as chemical signals for pheromonal communication. Preorbital gland marking plays an important role in both reproduction and social behaviour with special reference to territorial activity of many ungulates. This would provide a mechanism to assess the proven competitive ability of potential mates[3-6]. For such complex information to be conveyed, scent marks must contain information about the species, sex, individual identity of the scent owner, colony or group membership, social status (dominant

*Correspondence:
Phone: +91 431 2407040; Fax: +91 431 2407045
E-mail: garchu56@yahoo.co.in

territory owner or subordinate), and the freshness of the scent relative to any adjacent scent marks from competitors[7-12].

Lipocalins are extracellular proteins with a molecular mass of ~20 kDa, commonly called pheromone-binding proteins (PBPs), responsible for olfactory communication in mammalian species[13-15]. They display binding with high selectivity and affinity for small hydrophobic molecules[15,16]. The PBPs have been reported in a few pheromone sources of mammals *viz*., urine of mouse[14] and rat[14,17], rat preputial gland[18,19], pig salivary gland[20], hamster vaginal discharge[21,] and human sweat[22].

They are present at high concentration, both in the perception (odorant-binding proteins) and in the delivery of chemical message (PBPs) of olfactory significance and strongly associated with ligands. The PBPs facilitate the slow release of odorants into the air[23], and thereby play a crucial role in protecting these pheromones from being rapidly lost by evaporation or degradation extending the 'shelf-life' of the scent mark[24,25]. In addition, these proteins are unusually resistant to drying and heating and are not likely to be quickly denatured when released into the environment[26].

Pheromone-carrying proteins (PCPs) in various pheromonal sources are meant for carrying the volatiles and performing the functions as scent marking, sex attraction, mother-young interaction, etc. Preorbital gland marking behaviour exhibited by territorial male Blackbuck is high compared to non-territorial males of adults and sub-adults[27-29]. In addition, more number of volatile compounds (39) were identified in the territorial males than non-territorial males of adult (29) and sub-adult males (13)[28]. Since the preorbital gland secretion plays a vital role in maintaining territory even in the absence of the emitter, there is a possibility of presence of slow releaser (low molecular mass protein) in the glandular secretion. But there is no study on PCP in Indian Blackbuck preorbital gland post. Hence in the present study, we attempted to identify the PCP in the preorbital gland post of territorial and non-territorial male Blackbucks adopting LC-MS/MS.

Materials and Methods

Study area— The study was conducted in the conservation and breeding center of Arignar Anna Zoological Park (AAZP) (13°16S, 79°54E altitude MSL+ 10 m to 100 m), Vandalur, Chennai, Tamilnadu, South India. The habitat of AAZP is a tropical evergreen shrub, degraded forest mostly consisting of thorny bushes. Average annual rainfall is about 250 mm. Annual average temperature is 26°C.

Study animal— In the zoos, Blackbucks were housed in an outdoor enclosure of about 3.5 acres within a dry moat. The Blackbuck enclosure consists of three zones: (i) the edge zone, the visitor area in which the distance between visitors and the blackbuck is about 7 m; (ii) the rear zone, the access area for zoo employees for the purpose of cleaning and feeding (twice daily); and (iii) the enrichment zone, a central area with trees, grass, food and water troughs and wooden sheds. Water was offered *ad libitum*, whereas food was served twice a day (10.30 a.m. and 4.00 p.m.). Application of invasive methods such as sample collection of tissue and blood even for academic investigation on the Blackbuck is strictly prohibited. Only the carcass of the Blackbuck is available for examination.

Social status of male Blackbuck— We adopted the nomenclature of the male social status as: (i) harem masters (i.e., territorial) which hold harems; (ii) challengers (i.e., predominant males) without their own harems, but they challenge the harem master and try to hold females; and (iii) the bachelor groups which stay away from the female groups[4,28,30]. Indian Blackbucks change their social status from 'bachelor' to 'challenger' and become 'harem masters', but some of the bachelors may never reach the highest social rank during their lifetime[30]. During the hierarchy period, the territorial male (dominant male) coat is velvet-black in colour compared to non-territorial males. In addition, the dominant male spends more time with the female and never allows the entry of other males into this territory[3,4,30].

Sample collection— Preorbital gland posts were collected from identified 15 male Blackbucks which included 5 each of territorial males 4-7 year old (a); non-territorial adult males 4-5 year old (b); and non-territorial sub-adult males 2-3 year old (c). All male Blackbucks deposit the secretion (gland rubbing) on substrates, such as tree bark, sticks, grass, bushes, etc. The post materials were collected immediately after rubbing and the samples were placed in glass vials with Teflon-lined stoppers and saved for further analysis (Fig. 1a-c). The samples were collected with the help of a zoo veterinarian following standard guidelines of Central Zoo Authority, New Delhi.

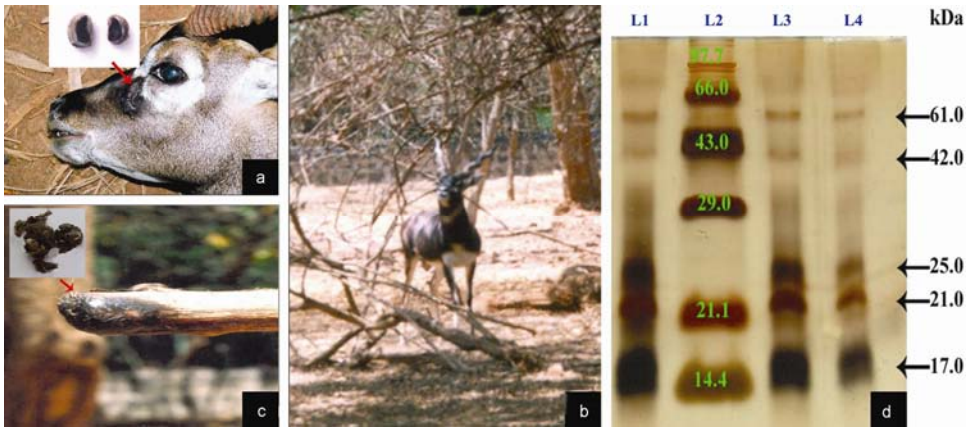

Fig.1— (a) Opening of an excretory duct on the Indian Blackbuck preorbital gland skin surface (Arrowheads bean shape of the preorbital gland); (b) Postures of male Blackbuck while preorbital gland scent marking on plant substrate secretion of the preorbital gland; (c) Resinous appearance of preorbital gland post on twig (Arrowheads indicate preorbital gland post); and (d) Electrophoretic distribution of total proteins analysed by 12% SDS-PAGE. [Lane 2 represent molecular weight markers. Lane 1, 3 and 4 represent extract from preorbital gland secretion of territorial males, non-territorial males of adult and sub-adult respectively]

Preparation of extract— Portions (1 g) of the post materials were homogenized with PBS (pH 7.2) separately for each category. The homogenate was clarified by centrifugation at 12500 × g for 15 min and the supernatant was removed. Further, the samples (proteins) were desalted from the supernatant by size exclusion chromatography (SEC) on spun columns of Sephadex G25. Total proteins in the preorbital gland secretion were estimated adopting the method of Bradford[31].

SDS-PAGE analysis— SDS-PAGE (one-dimensional) was performed as described by Laemmli[32]. All samples were run under reducing condition in 12% SDS-PAGE. The protein (25 µg) was loaded on the gel for determination of molecular mass. Protein standard (protein molecular weight marker- medium range, Genei, Bangalore), 4 µl, was applied on the gel separately. The gels were run for 3 h at 50 V, and the proteins were stained with silver nitrate.

Destaining and in-gel digestion— Specific protein band from SDS-PAGE gel was excised and the gel plug was destained using 100 µl of 25 mM NH_4HCO_3 50% (v/v) acetonitrile (1:1) by incubation at 37°C for 30 min. This step was repeated until no stain was visible in the protein band. After drying in a Speed-Vac (Savant, Germany), the pooled gel pieces were incubated in 100 µl of 2% β-mercaptoethanol/25 mM NH_4HCO_3 for 20 min at (room temperature) in the dark. Equal volume of 10% 4-vinylpyridine in 25 mM NH_4HCO_3/50% acetonitrile was added for cysteine alkylation. After 20 min incubation, the pieces were soaked in 1 ml of 25 mM NH_4HCO_3 for 10 min, dried and then incubated overnight (~18 h) in 25 mM NH_4HCO_3 containing 100 ng of modified trypsin (Promega). The tryptic digest was removed from the gel and dried in a Speed-Vac and then kept at −20°C until further analysis. The preparation was re-suspended in 0.1 % formic acid immediately before use.

Mass spectrometry analysis— All mass spectrometry analyses were performed using an LTQ-Orbitrap (Discovery) hybrid mass spectrometer with a nanoelectrospray ion source (ThermoElectron, San Jose, CA, USA) coupled to a nano-flow HPLC (Agilent Technologies 1200 series, Germany). A 100 × 0.075 mm Agilent C18 column (3.5 µm particle diameter), with mobile phases of A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile), was used. The pump flow rate was set at 0.5 µl/min, and peptide elution was achieved using a linear gradient of 5%-35% B for the first 30 min followed by a rapid increase to 95% B over the next 10 min. The conventional MS spectra (Survey Scan) were acquired at high resolution (M/ΔM, 60,000 full width half maximum) over the acquisition range of m/z 200-2000, and a series of precursor ions were selected for the MS/MS scan. The former examined the accurate mass and the charge state of the selected precursor ion, while the latter acquired the spectrum (CID spectrum or MS/MS spectrum) for the fragment ions generated by collision-induced dissociation.

Database searching – SEQUEST (*TURBO*)— The mass spectrometry dataset was analyzed using Xcalibur software (version 2.0 SR1). Product ion scans obtained from the tandem mass spectrometry experiments were investigated using the database search software SEQUEST (TURBO). Further, the

mass spectra of OBP were searched in order to allow a wide range of modifications using our PTM finder-in-house program. Furthermore, all the modified spectra identified in this study were manually examined and the fragmented ions (from the nano-LC-MS/MS) labeled as b, y, and y-NH_3, and b-H_2O ions.

Results

Protein concentration in the preorbital gland secretion of the territorial and non-territorial males of adults and sub-adults differed considerably. One-way ANOVA with post hoc comparison and DMRT test clearly showed that the average protein concentration was significantly higher ($F=_{0.001;\ 2,17}=55.905$) in the preorbital gland secretion of territorial male Blackbucks (163.54 µg/ml) than non-territorial males of adults (123.16 µg/ml) and sub-adults (112.55 µg/ml) (Tables 1 & 1a).

The SDS-PAGE profiles of preorbital gland posts revealed the presence of different molecular mass proteins, 17, 21, 25, 42 and 61 kDa, respectively (Fig. 1d). The staining intensity of all major polypeptides in preorbital gland secretion of territorial and non-territorial male was found to differ (Table 2). Among these fractions, the 17 and 21 kDa polypeptides appeared to be prominent in the gland secretion of all males. The electrophoretic profile showed the 17 kDa mass protein intensity to be relatively high in territorial males as compared to non-territorial males, adults as well as sub-adults (Table 2). These observations were confirmed from the band areas as determined by densitometric scanning. Thus, the intensity of low molecular mass protein i.e., 17 kDa, prompted us to investigate this protein further, adopting LC-MS/MS.

Mass spectrum of 17 kDa protein was obtained by LC-MS/MS and the mono-isotopic numbers of spectrums were analyzed with SEQUEST algorithm (Fig. 2). The results of SEQUEST search showed the presence of α_{2u}-globulin in the first hit in the search

Table 1— ANOVA with post hoc comparison (one-way) of proteins concentration in preorbital gland secretion of territorial and non-territorial male Blackbucks

VAR00001	SS	df	MS	F	Sig.
Between Groups	10011.760	2	3337.253	55.905	0.000*
Within Groups	1193.892	14	59.695		
Total	11205.652	17			

[The means were compared using DMRT (Duncan Multiple Range Test). The means gain score of protein concentration in preorbital gland secretion of territorial males as compared with non-territorial male Blackbuck is statistically significant. *High statistically significant (P <0.001)]

Table 2— Densitometric pattern of preorbital gland protein intensity profiles

Band (kDa)	Band Intensity (pixel)		
	Territorial male	Non-territorial males of	
		Adult	Sub-adult
61	152.85±1.02[a]	116.93±0.45[b]	113.79±0.11[c]
42	134.54±0.21[a]	120.51±0.62[b]	108.17±0.59[c]
25	243.68±1.25[a]	185.37±1.64[b]	173.34±0.47[c]
21	254.65±0.25[a]	204.41±0.33[b]	185.58±1.28[c]
17	286.52±1.09[a]	216.90±0.55[b]	192.96±0.72[c]

DMRT, Duncan Multiple Range Test

[Values are articulated in Mean±SE of 6 experiments. Means with same superscripts are not significant (P <0.05) to DMRT]

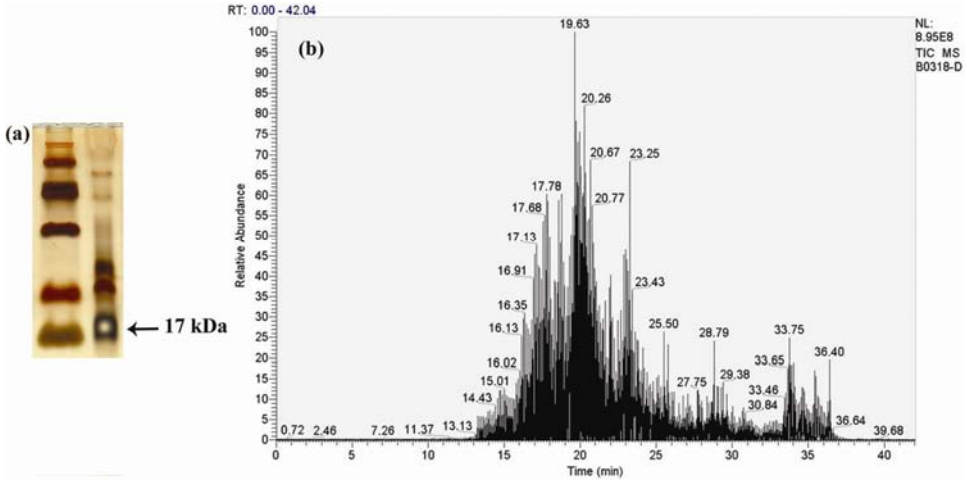

Fig. 2— (a) Protein spot selection from 17 kDa of territorial male preorbital gland secretion for LC-MS/MS analysis; and (b) Mass spectrum by LC-MS/MS analysis. [Protein spot from 17 kDa was subjected in gel digestion with trypsin]

Table 3— Nature of the protein identified in the male Blackbuck preorbital gland secretion by SEQUEST algorithm search

[Table shows α_{2u}-globulin fragmented with trypsin and map out of total peptides using LC-MS/MS. Matched peptides shown in **Bold Red**]

MKLLLLLLCLGLTLVCGHAEEASSVRGNLDVDKLNGDWFSIVLASDKREKIEENGSMRVFMQHIDVLENSLGFKFHIKK NGECREVYLVAYKTPKDGEYFVEHDGGNTFTLKTDYDRYVMIHLVNVKNGETFQLMLLYGRTKDLSSDIKEKFEKLCV AHGITRDNIIDLTKTDRCLKARG

#	zBP File	z dM	MH+	Xcorr dCn	Sp	RSp	Ions	Reference	( )Sequence
27	02003	2 - 0.0	1877.0	5.22 0.56	2168	1	24/30	uptr:q9jjh9_rat+9	(-)VFMQHIDVLENSLGFK
26	01979	2 - 0.0	1877.0	5.13 0.47	2088	1	24/30	uptr:q9jjh9_rat+9	(-)VFMQHIDVLENSLGFK
33	02093	3 - 0.0	2462.3	4.16 0.43	1312	8	31/84	uptr:q9jjh9_rat	(-)GNLDVDKLNGDWFSIVLASDKR
17	01523	3 - 2.0	1867.9	2.45 0.04	1044	43	25/56	uptr:q9jjh9_rat	(-)TDYDRYVMIHLVNVK
18	01707	2 - 1.9	2043.9	2.38 0.07	792	42	17/34	uptr:q9jjh9_rat	(-)DGEYFVEHDGGNTFTILK
21	01844	2 - 2.0	1878.9	2.14 0.11	1367	3	18/30	uptr:q9jjh9_rat+9	(-)VFMQHIDVLENSLGFK

list. In addition, the search result showed that the identified protein matched with 6 residue peptide of α_{2u}-globulin, with GxW motif, a common characteristic signature of lipocalin proteins (Table 3).

Discussion

Pheromones are important molecules for chemical communication used in animal breeding, territoriality and conspecific recognition[33-36]. In Blackbuck, the preorbital gland has been reported to be one of the major pheromone sources involving scent or territorial marking and conveys various signals such as sex attraction and aggression[4]. In pheromonal sources, the low molecular mass proteins have been reported as ligand carriers for the processes of pheromonal communication in several mammals[16,37]. In the present study, the total protein content of territorial male gland post was higher than that of non-territorial males of adults and sub-adults. An earlier study showed high levels of preorbital glandular volatile compounds and faecal testosterone concentration in the territorial males compared to the non-territorial males of adults and sub-adults[28]. The high concentration of protein seen in the urine of dominant male mice reflects augmented secretion of steroid hormones[37]. Asa *et al.*[38] reported that the production of glandular protein depends upon the androgen status that is higher in the dominant males than the subordinates. Thus, the present findings are in agreement that males use preorbital glandular proteins for territorial marking to attract the opposite sex and to repel member of the same sex conspiring to reach the higher social hierarchy.

The SDS-PAGE profiles of preorbital gland posts revealed the presence of different molecular mass proteins, among which the 17 and 21 kDa proteins appeared prominent in the gland secretion in all males. Nevertheless, the concentration of protein secretion in the sub-mandibular gland of adult male hamsters is several times higher than in the females[39]. Comparable to this, in the present study the 17 kDa protein was identified in preorbital gland post in territorial and non-territorial males of adults and sub-adults, wherein the intensity of the band was relatively high in the territorial males as compared to non-territorial males. The high concentration of these proteins in the gland of territorial males reflects a high rate of synthesis of these proteins. The presence of low molecular mass proteins in the preorbital glandular posts are consistent with the earlier reports on PBPs with molecular mass of 20 kDa in the boar salivary gland that can bind with steroid pheromone[40]. Burger *et al.*,[41] reported that a small globular albumin-like protein (18 kDa) is secreted in preorbital gland of Klipspringer antelope. Another protein, aphrodisin (17 kDa), secreted in hamster vaginal fluid by vaginal tissue and Bartholin gland was also reported to be a pheromone-carrier and facilitate the male copulatory behavior[21,42]. The pheromone-carrying protein, α_{2u}-globulin (18 kDa) was reported in the preputial gland[43,44] as well as urine[45] of both adults and pups[46] of rat. While the intensity of this protein was higher in the preputial gland of adult rat than the prepubertal rat[47]. Interestingly, odorant-binding proteins (OBPs) of vertebrates have the molecular weight around 20 kDa[48]. Muthukumar *et al.*[49] found that a lipocalin protein (14.5 kDa) appeared in the rat urine prominently during the estrus and metestrus phases compared to proestrus and diestrus phases.

The LC-MS/MS results revealed the major low molecular mass protein 17 kDa as α_{2u}-globulin, a protein belonging to lipocalin family. The presence of

α_{2u}-globulin in the preorbital gland posts of Blackbuck is consistent with the earlier reports pertaining to adult male rat and mouse, which have shown this protein to serve as a pheromone-carrier[45,50]. The lipocalin proteins in the pheromone sources have been reported as pheromone-carrier in several mammalian species[51,52]. For example, aphrodisin, a lipocalin protein abundantly secreted from the vaginal fluid of the hamster, has five volatiles (*i.e.* hexadecanol, 1-octadecanol, z-9-octadecen-1-ol, E-9-octadecen-1-ol and 1-hexadecanol) bound to it, which facilitates the mating behaviour of the male[21]. Similarly, salivary lipocalins (SAL) secreted from the submaxillary glands of the adult male boar contains two endogenous ligands, 5α-androst-16-en-3-one and 5α-androst-16-en-3-ol as components of the boar sex pheromonal system[40,53]. Lipocalin EquC1, the abundant protein in horse sweat, contains a putative pheromone, oleamide[54]. In humans, the components of axillary odour are associated with apolipoprotein-D and the potent odoriferous substance was identified as 3-methyl-2-heptenoic acid[22,55]. It is reported the bound form pheromones such as farnesol 1 and 2 in the alpha α_{2u}-globulin (18.54 kDa) of preputial gland of Indian commensal rat[44] and further its binding efficiency with farnesol is confirmed by molecular docking and fluorescence analysis[56]. Similarly, the volatiles 1-chlorodecane, 2-methyl-N-phenyl-2-propenamide, hexadecane and 2,6,11-trimethyl decane were found to bind with the α_{2u}-globulin of urine of Indian common house rat[45]. In our previous study, we reported four volatile compounds *viz*., 2-methyl propanoic acid, 2-methyl-4-heptanone, 2,7-dimethyl-1-octanol and 1,15-pentadecanediol in the preorbital posts of the territorial male particularly during the hierarchy period[28]. Therefore, the present study strongly suggests that the α_{2u}-globulin in the preorbital gland may be carrying these volatile compounds for released into the environment, where these compounds may be used in defense of the home range and also suppress the daily activities of subordinate males, and thereby maintain the dominance hierarchy.

In general, lipocalins have conserved tertiary structure containing anti-parallel beta barrel with an alpha helix and share a conserved sequence motif "GxW" which is a characteristic signature for lipocalin family[15,57]. Further, the conserved tryptophan (W) has been shown to play a role in maintaining structure, stability and ligand affinity[58]. As shown in table 2, SEQUEST search showed α_{2u}-globulin as the first hit. Further, this first hit possesses GxW motif which is a characteristic signature for lipocalins. The result further suggests that the identified protein belongs to lipocalin family.

This is the first report as far as identification of α_{2u}-globulin in preorbital glandular secretion of territorial and non-territorial male Blackbuck. In the preorbital gland posts, the volatile compounds were identified in the adult and sub-adult male Blackbucks[28]. It is hypothesized that these volatile compounds may bind with this low molecular mass protein (α_{2u}-globulin)-carrier and involve in pheromonal communication. Since the α_{2u}-globulin is reported as pheromone carrying protein in rodents, this protein may perform the same role in male Blackbuck preorbital gland to evoke the scent marking for maintaining territoriality (home range) as well as hierarchy and attraction towards female. Further studies are required to confirm the protein in the range 17 kDa to be a lipocalin family protein and its binding affinity with the pheromonal compounds which may throw light on the role of pheromone-protein complex in Blackbuck olfactory communication.

Acknowledgements

Thanks are due to the Chief Wildlife Warden and the Director, Arignar Anna Zoological Park (AAZP), Vandalur, Chennai, for facilitating this study; Dr K Senthilkumar (Veterinary Assistant Surgeon) and Dr PN Khan (Zoo Veterinarian), AAZP for assisting collection of preorbital gland secretion. This investigation was supported by grant from the UGC by way of Rajiv Ghandi National Fellowship to one of the authors, TR, and UGC-SAP and DST-PURSE.

References

1 Kar S, *Balipadars Blackbuck*, (Wildlife Wing Forest Department, Government of Orissa), 2001, 1.

2 Ghosh AK, *The Red Data Book on Indian Animals*: *Part-I Vertebrates*, (Zoological Survey of India, Kolkata), 1994, 534

3 Rajagopal T, Archunan G, Geraldine P & Balasundaram C, Assessment of dominance hierarchy through urine scent marking and its chemical constituents in male blackbuck *Antelope cervicapra*, a critically endangered species. *Behav Proc*, 5 (2010) 58.

4 Rajagopal T, Manimozhi A & Archunan G, Diurnal variation in preorbital gland scent marking behaviour of captive male Indian Blackbuck (*Antelope cervicapra* L.) and its territorial significance. *Biol Rhy Res*, 42 (2011) 27.

5 Burger BV, Nell AE, Spies HSC, Le Roux M & Bigalke RC, Mammalian exocrine secretions. XII. Constituents of preorbital

gland secretions of bontebok *Damaliscus dorcas dorcas* and blesbok *D. d. phillipis*. *J Chem Ecol*, 25 (1999a) 2085.

6 Burger BV, Greyling J & Spies HSC, Mammalian exocrine secretions. XIV: Constituents of preorbital secretion of steenbok, *Raphicerus campestris*. *J Chem Ecol*, 25 (1999b) 2099.

7 Gosling LM, A re-assessment of the scent marking in territories. *Z Tierpsychol*, 60 (1982) 89.

8 Roberts SC, Behavioural responses to scent marks of increasing age in klipsringer, *Oreotragus oreotragus*. *Ethology*, 104 (1998) 555.

9 Idris Mohd & Tripathi RS, Behavioural responses of desert gerbil, *Meriones hurrianae* after removal of scent marking gland. *Indian J Exp Biol*, 49 (2011) 555.

10 Achiraman S, Ganesh DS & Kannan S, Response of male mice to odours of female mice in different stages of oestrous cycle: Self-grooming behaviour and the effect of castration. *Indian J Exp Biol*, 52 (2014) 30.

11 Lawson RE, Putnam RJ & Fielding A, Chemical communication in Eurasian deer (Cervidae): Do individual odours also code for *attributes*?. *J Zool* (Lond.), 253 (2001) 91.

12 Archunan G, Vertebrate pheromones and their biological importance. *J Exp Zool India*, 12 (2009) 227.

13 Novotny MV, Pheromones, binding proteins and receptor responses in rodents. *Biochem Soci Trans*, 31 (2003) 117.

14 Beynon RJ & Hurst JL, Urinary proteins and the modulation of chemical scents in mice and rats. *Peptides*, 25 (2004) 1553.

15 Flower DR, The lipocalin protein family: structure and function. *Biochem J*, 318 (1996) 1.

16 Cavaggioni A & Mucignat-Caretta C, Major urinary proteins, α2u-globulins and Aphrodisin. *Biochem Biophy Acta*, 1482 (2000) 218.

17 Dinh BL, Trembly A & Dufour D, Immunochemical study of rat urine proteins: Their relation to serum and kidney proteins. *J Immunol*, 95 (1965) 574.

18 Archunan G, Kamalakkannan K, Achiraman S & Rajkumar R, Identification of glandular (preputial and clitoral) proteins in house rat (*Rattus rattus*) involved in pheromonal communication. *Indian J Exp Biol*, 42 (2004) 1032.

19 Kamalakkannan S, Achiraman S, Rajkumar R, Rameshkumar K & Archunan G, Identification of sex-associated protein in the preputial gland of house rat: a new insight in RPM. *Acta Physiol Hung*, 93 (2006) 145.

20 Booth WD & White CA, The isolation, purification and some properties of pheromaxien, the pheromonal steroid-binding protein, in porcine submaxillary glands and saliva. *J Endocrinol*, 118 (1988) 47.

21 Singer AG, Marcides F, Clancy AN & Agosta WC, Purification and analysis of a proteinaceous aphrodisiac pheromone from hamster vaginal discharge. *J Chem Ecol*, 261 (1986) 1323.

22 Zeng C, Spielman AI, Vowels BR, Leyden JJ, Biemaan K & Preti G, A human axillary odorant is carried by apolipoprotein D. *Proc Nat Acad Sci USA*, 93 (1996) 6626.

23 Hurst L, Robertson DHL, Tolladay U & Beynon RJ, Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Anim Behav*, 55 (1998) 1289.

24 Lazar J, Greenwood DR, Rasmussen LE & Prestwich GD, Molecular and functional characterization of an odorant binding protein of the Asian elephant, *Elephas maximus*: implications for the role of lipocalins in mammalian olfaction. *Biochemistry*, 41 (2002) 11786.

25 Lazar J, Rasmussen L, Greenwood DR, Bang IS & Prestwich GD, Elephant albumin: a multipurpose pheromone shuttle. *Chem Biol*, 11 (2004) 1093.

26 Stopková1 R, Hladovcová D, Kokavec J, Vyoral D & Stopka P, Multiple roles of secretory lipocalins (Mup, Obp) in mice. *Folia Zool*, 58 (2009) 29.

27 Rajagopal T & Archunan G, Scent marking by Indian Blackbuck: characteristics and spatial distribution of urine, pellet, preorbital and interdigital gland marking in captivity. In: *Biodiversity and Wildlife Conservation*, (Ed. Reddy MV, Daya Publishing House, New Delhi), 2008, 71.

28 Rajagopal T, *A study on the reproductive behaviour and pheromones of an endangered Indian Blackbuck (Antelope cervicapra L.) to enhance captive breeding and conservation*. Ph.D. thesis, Bharathidasan University, Tiruchirappalli, India, 2009.

29 Rajagopal T & Archunan G, Histomorphology of preorbital gland in territorial and non-territorial male blackbuck *Antelope cervicapra*, a critically endangered species. *Biologia*, 66 (2011) 370.

30 Mungall EC, Social development of the young blackbuck antelope (*Antelope cervicapra*). In: *AAZP Regional Conference Proceeding*, American Association of Zoological Parks and Aquarium, Washington, USA, 1977, 100.

31 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem*, 72 (1976) 248.

32 Laemmli UK, Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature*, 227 (1970) 680.

33 Rekwot PI, Ogwu D, Oyedipe EO & Sekoni VO, The role of pheromones and biostimulation in animal reproduction. *Anim Reprod Sci*, 65 (2001) 157.

34 Sillero-Zubiri C & Macdonald DW, Scent-marking and territorial behavior of Ethiopian wolves *Canis simensis*. *J Zool*, 245 (1998) 351.

35 Archunan G, Stud male-originating chemosignals: a luteotrophic agent. *Indian J Exp Biol*, 52 (2014) 5.

36 Pelosi P, The role of perireceptor events in vertebrate olfaction. *Cell Mol Life Sci*, 58 (2001) 503.

37 Novotny MV, Harvey S & Jemiolo B, Chemistry of male dominance in the house mouse, *Mus domesticus*. *Experientia*, 16 (1990) 109.

38 Asa CS, Peterson EK, Mech LD & Seal US, Deposition of anal sac secretion by captive wolves (*Canis lupus*). *J Mammal*, 66 (1985) 89.

39 De PK, Sex hormone regulation of 20.5 and 24-kDa major male specific proteins in Syrian hamster sub-mandibular gland. *J Steriod Biochem Mol Biol*, 58 (1996) 183.

40 Marchese S, Pes D, Scaloni A, Carbone V & Pelosi P, Lipocalins of boar salivary glands binding odours and pheromones. *Eur J Biol Chem*, 252 (1998) 563.

41 Burger BV, Yang TP, Le Roux M, Brandt WF, Cox AJ & Hart PF, Mammalian exocrine secretions. XI: Constituents of the preorbital gland secretion of klipspringer, *Oreotragus oreotragus*. *Chem Ecol*, 23 (1997) 2383.

42 Briand L, Trotier D & Pernollet JC, Natural ligands of hamster aphrodisin. *Peptides*, 25 (2004) 1545.

43 Ponmanickam P & Archunan G, Identification of alpha-2u globulin in the rat preputial gland by MALDI-TOF analysis. *Indian J Biochem Biophy*, 34 (2006) 319.

44 Rajkumar R, Ilayaraj R, Liao CC, Archunan G, Achiraman S, Prakash S, Ng WV & Tsay YG, Detection of alpha(2u)-globulin and its bound putative pheromones in the preputial gland of the Indian commensal rat (*Rattus rattus*) using mass spectrometry. *Rapid Comun Mass Spectrom*, 24 (2010) 721.

45 Rajkumar R, Ilayaraja R, Mucignat C, Cavaggioni A & Archunan G, Identification of alpha 2u-globulin and bound volatiles in the Indian common house rat (*Rattus rattus*). *Indian J Biochem Biophys*, 46 (2009) 319.

46 Ponmanickam P, Jebamercy G, Archunan G & Kannan S, Detection of α_{2u}-globulin in rat pup preputial gland by Maldi-Tof mass spectrometry. *Curr Zool*, 55 (2009) 296.

47 Ponmanickam P, Palanivelu K, Govindaraj S, Baburajendran R, Habara Y & Archunan G, Identification of testosterone-dependent volatile compounds and proteins in the preputial gland of rat *Rattus norvegicus*. *Gen Comp Endocrinol*, 167 (2010) 35.

48 Pelosi P, Odorant-binding proteins. *Crit Rev Biochem Mol Biol*, 29 (1994) 199.

49 Muthukumar S, Rajesh D, Saibab G, Alagesan A, Rengarajan RL & Archunan G, Urinary lipocalin protein in a female rodent with correlation to phases in the estrous cycle: A experimental study accompanied by In silico analysis. *PLOS ONE*, 8 (2013) 1.

50 Timm DE, Baker LJ, Mueller H, Zidek L & Novotny MV, Structural basis of pheromone binding to mouse major urinary protein (MUP-I). *Prot Sci*, 10 (2001) 997.

51 Cavaggioni A, Findlay JBC & Tirindelli R, Ligand binding characteristics of homologous rat and mouse urinary proteins and pyrazine binding protein of the calf. *Comp Biochem Physiol*, 96 (1990) 513.

52 Bacchini A, Gaetani E & Cavaggioni A, The pheromone binding proteins of the mouse, *Mus musculus*. *Experientia*, 48 (1992) 419.

53 Scaloni A, Paolini S, Brandazza A, Fantacci M, Marchese S, Navarrini A, Fini C, Bottiglieri C, Ferrara L & Pelosi P, Purification, cloning and characterization of novel odorant-binding protein in the pig. *Cell Mol Life Sci*, 58 (2001) 823.

54 D'Innocenzo B, Salzano AM, Ambrosio DC, Gazzano A, Sorce C, Dani FR, Scaloni A & Pelosi P, Secretory proteins as potential semiochemical carriers in the horse. *Biochemistry,* 45 (2006)13418.

55 Spielman AI, Zeng XN, Leyden JJ & Preti G, Proteinaceous precursors of human axillary odor: Isolation of two novel odor-binding proteins. *Experientia*, 51 (1995) 40.

56 Ilayaraja R, Rajkumar R, Rajesh D, Muralidharan, AR, Padmanabhan P & Archunan G, Evaluating the binding efficiency of pheromone binding protein with its natural ligand using molecular docking and fluorescence analysis. *Sci Rep*, 4 (2014) 5201.

57 Flower DR, North AC & Sansom CE, The lipocalin protein family: Structure and sequence overview. *Biochem Biophys Acta*, 1482 (2000) 9.

58 Gasymov OK, Abduragimov AR, Yusifov TN & Glasgow BJ, Binding studies of tear lipocalin: The role of the conserved tryptophan in maintaining structure, stability and ligand affinity. *Biochem Biophys Acta*, 1433 (1999) 307.