General Introduction
Blackbuck (*Antelope cervicapra* Linn. 1758) is the only living representative of the genus *Antelope* Pallas 1766. The present geographical distribution of this species is restricted to some states of India. This unique animal has figured prominently in Indian ancient literature, art and also in the memories of British colonials and the hunters' note book (Schaller, 1967; Nair, 1977; Mungall, 1977). Blackbuck has been categorized as vulnerable by the IUCN endangered commission (ZSI, 1994). In 1967 a first “Deer Conservation Unit” was started with a small population of 11 Blackbuck and 8 spotted deer in Ballavpur wildlife sanctuary, West Bengal, India (Bhattacharya and Chattopadhy, 1984). The Blackbuck is typically Indian subcontinent in distribution. It is quite abundant in Rajasthan, Madhya Pradesh and Gujarat (Ranjitsingh, 1989).

The Blackbuck is a medium sized (23-45 kg, 120-130 cm length, 60-80 cm height), and sexually dimorphic species in which males are larger than females (Mungall, 1978). It is striking colored in black and white, and sports a magnificent pair of spiraling horns. It is found in a wide range of habitats, from grasslands to open woodlands. It is a selective grazer living in groups that range from two to several hundred individuals (Ranjitsingh, 1989). The social units in Blackbucks are (1) solitary males (territorial), (2) all-male or bachelor groups, composed of two or more juvenile, sub-adult or adult males, (3) females in group, composed of females of all age-classes, fawns, and juvenile and sub-adult females, and (4) mixed groups, in which the whole range of age classes of both sexes may be represented (Mungall, 1978).

The systematic position of *Antelope cervicapra* may be summarized as follows:

Kingdom: Animalia  
Phylum: Chordata  
Class: Mammalia  
Order: Artiodactyla  
Sub-order: Ruminantia  
Family: Bovidae  
Sub-family: Antilopinae  
Genus: *Antilope* Pallas, 1766  
Species: *cervicapra*
Wild Blackbuck population is likely to continue to decline as a consequence of poaching, hunting, shrinkage of natural habitats and deforestation, with probably fewer than 25,000 individuals in their native range (Sudhakar Kar, 2001). The cheetah is a prime native predator for Blackbuck, and has also been considered one of the reasons for decline of the population of Blackbuck. Further, the main problem in endangered species appears to be lack of communication between their partner which is affected by pollution and disturbance caused by humans. In the forest habitat blackbuck acts as an indicator for natural disturbances. If the Blackbuck population is high that reflects that the ecosystem is not severely disturbed by human activities and vice versa.

Blackbuck shows a regular estrous cycle similar to domestic ewe and cow with the cycle length of 15 to 21 days. Besides, the receptive period is about 24 h which is very short (Backhaus, 1958; Schmied, 1973). Therefore, the possibility of mating chance is comparatively less and they are slow colonizers. Moreover, under semi-natural conditions, during estrus period the high frequency of inter-male competition occurs and the breeding males miss out the coitus at particular times. During dominant hierarchy determination, the dominant and predominant fights occur. This time the animal is injured in different parts of body (ear pinna, leg, eye, jaw, horn broken etc.), or sometimes it leads to death. This is also considered one of the reasons for decline of the Blackbuck population in captive condition.

Nowadays, there are about thousands of Blackbucks living in captivity for free-ranging situation all over the world. The behaviour, growth and reproduction of the captive Indian Blackbuck vary from place to place around the world. The reasons for such variation are yet to be identified (Ranjitsingh, 1989; Sudhakar Kar, 2001). Therefore, one of the conservation methods to help the prevention of the extinction of the Indian Blackbuck is to increase the breeding in zoos. In such cases, artificial insemination may be an alternative method to carry out fertilization processes in success.

The use of artificial insemination represents the most economical and suitable strategy to overcome the low production of wild zoo animals in captivity. Artificial insemination has been proved beyond doubt in zoo animals such as African elephant (Schwammer et al., 2001), African rhinoceroses (Hildebrandt et al., 2002) and Indian Blackbuck (Holt et al., 1988). The knowledge of the female reproductive status to
predict the time of ovulation is a prerequisite for artificial insemination. Ovulation is often characterized in terms of its duration and intensity. Ovulation as in ungulate occurs 15 to 18 hrs after the end of estrus or about 35 to 45 hrs after the onset of estrus (Jainudeen and Hafez, 1992). Artificial insemination in particular, for which the exact timing of insemination is the major limiting factor, would be much easier if estrus could be determined.

There is a fundamental need to develop strategies to assess accurately the endocrine status of wild-zoo animals. The determination of the reproductive status is most important for the effective reproductive management and for assisted reproductive techniques such as artificial insemination, in-vitro fertilization and embryo transfer for non domestic animals. These techniques have helped the conservation of many critically endangered animals. Endocrine studies normally require the collection of repeated blood samples for hormone evaluation, which is extremely difficult in wild animals especially cervids and antelopes. Capturing, restraining or chemical immobilization are particularly stressful to the animals and might therefore affect the steroid levels (Welsh and Johnson, 1981). Non-invasive urinary and faecal steroid metabolites evaluations are used in the captive exotic animals (Peter et al., 1996, Schwarzenberger et al., 1996, Hart and Leedy, 2004). Non-invasive reproductive monitoring in few non-domesticated species was well developed using estrogen determination in urine samples in wild broodstock of Senegalese sole (Garcia-Lopez et al., 2006), Chinese water deer (Mauget et al., 2007), Asiatic lion (Umapathy et al., 2007) and giraffe (Del Castillo et al., 2005).

Consequently, non-intrusive urinary pheromonal studies are now being used in a variety of disciplines viz. animal science, behavioural ecology, pest management and conservation biology, this field is a new avenue for research on pheromone biology proven valuable to determine reproductive status of female (pre-ovulatory, ovulatory, post-ovulatory) in both domestic and non-domestic animals (Rasmussen, 1998; Rajanarayanan and Archunan, 2004; Sankar and Archunan, 2008). Pheromones are powerful species-specific chemical signals that organized both social and reproductive conduct of all living organisms i.e. micro-organisms, insects, reptiles, birds and mammals (Archunan, 2003).
Chemical signals that convey information between members of the same species are commonly termed "Pheromones". This term was first used by Karlson and Luscher (1959) and defined as substances secreted on the outside of an individual and received by a second individual of the same species in which they release a specific reaction involving either the release of a specific behaviour or physiological change in the recipients’ endocrine or reproductive system (Dominic, 1987; Rekwot et al., 2001; Archunan, 2009). Based on the types of response by recipients, the pheromones are classified as “primer”, “releaser” and “imprinting”. Primer pheromones induce a delayed response to prolonged stimulation mediated through central nervous system and endocrine system. A number of primer pheromonal effects have been studied leading to the establishment of concrete ideas regarding their influences on reproductive functions. The original discoveries of ‘Bruce’, ‘Whitten’ and ‘Vandenbergh’ in the late fifties and the middle sixties on primer pheromones paved the way and attracted many scientists to involve in pheromonal research in animals. Releaser pheromones induce a rapid behavioural response in the recipients, generally mediated through the central nervous system. The pheromones involved in sex attraction, evocation of aggression, recognition, alarming behaviour and mother-young interactions are the citations of releaser pheromones. Imprinting pheromones organized the central nervous system of the pre-weaning offsprings at a critical period and cause permanent alterations of adult behaviour. Hyashi and Kimura (1978) documented that the imprinting like process may be involved in the acquisition of female preferences for male odours. Female of *Mus musculus domesticus* reared with their parents prefer to mate with males of different strains than its own.

In mammals, the olfactory systems are classified into two types based on structurally and anatomically, such as main olfactory system (MOS) and accessory olfactory system (AOS) specialized for the detection and transmission of pheromonal information (Wysocki and Meredith, 1987; Halpem and Marinez-Marcos, 2003). The sensory organ of main olfactory system is the main olfactory epithelium which lines part of the nasal cavity and is associated with central circuits. The peripheral receptor of the accessory olfactory system is the vomeronasal organ (VNO). This is a closed tubular structure, entirely separated from the main olfactory epithelium and connected to the nasal cavity in the mice through the narrow vomeronasal duct (Wysocki and Meredith, 1987). The main olfactory epithelium is in position to detect volatile odorant
carried by air stream, whereas the VNO is harnessed to detect non-volatile water soluble molecules (Ichikawa, 1996a, b; Cavagioni et al., 1999).

Urine, faeces, and vaginal secretions are reported to be common sources of mammalian pheromones for sexual attraction and arousal in male species (Carr et al., 1965; Leon, 1974; Mykytowycz and Goodrich, 1974; Dominic, 1976; Signoret, 1991). Apart from that a number of specialized cutaneous glands secretions have evoked to elicit pheromonal responses in mammals (Albone, 1984; Archunan, 2009). In certain species, the pig saliva contains sex pheromones, which are involved in sexual arousal (Signoret, 1970). In human, sweat displays a significant role in sexual behaviour by altering the menstrual cycle (Zeng et al., 1996).

In the last two decades, there has been a considerable study on the chemistry of mammalian pheromone identification in mouse (Liebich et al., 1977; Novotny et al., 1984), rat (Kannan et al., 1998; Kannan and Archunan, 2000), rabbit (Schaal et al., 2003), cat (Mattina et al., 1991), hamster (Singer et al., 1986), pig (Dorries et al., 1997), tiger (Brahmachary, 1996), white tailed deer (Jemiolo et al., 1995), horse (Ma and Klemm, 1997), cow (Rameshkumar et al., 2000; Sankar et al., 2007; Sankar and Archunan, 2008), buffalo (Rajanarayanan, 2004), elephant (Rasmussen et al., 1997), monkey (Michael et al., 1975) and human (Stern and McClintock, 1998). These findings indicate that mammalian pheromones may be single compound or a mixture of compounds and that each of the major fraction is faithfully involved in conveying specific signals related to reproduction and social behaviour. The molecular weight of the olfactory pheromones center around 50 to 300. Many different chemicals have been reported as pheromones i.e. alcohols, aldehydes, or saturated or unsaturated aliphatic or aromatic compounds from non-polar molecules such as alkanes and alkenes to very polar compounds which may be acidic (acids or phenols) or basic (amines) (Dominic, 1991; Kannan et al., 1998; Rameshkumar et al., 2000; Kannan and Archunan, 2001; Selvaraj and Archunan, 2002; Achiraman and Archunan, 2006; Sankar and Archunan, 2008).

Chemical cues (pheromones) are found to be significant in the induction of ovulation, as well as, in identifying the receptivity of the female (Keverne, 1997; Dominic, 1991). Communication of the time of the physiological events of ovulation
and co-ordination of sexual behaviour are important for ensuring successful fertilization (Michael, 1975; Ziegler et al., 1993; Rajanarayanan and Archunan, 2004).

**Behavioural effects of mammalian pheromones**

Mammals can distinguish among an enormous diversity of odorants that vary in size, shape, functional groups and charge (Beets, 1970). Nevertheless, only a few mammalian pheromones have been identified, though many different pheromonal effects have been recorded and their sources have been described (Halpern, 1987; Wysocki and Meredith, 1987; Novotny et al., 1990; Keverne, 1999).

For example, in rabbit, the pheromone shown to be a single molecule, 2-methylbut-2-enal, which is produced in milk and is sufficient to elicit nipple searching and grasping behaviour when presented on its own (Schaal et al., 2003). In pig, the androgen derivatives 5α-androst-16-en-3-one and 5α-androst-16-en-3-ol are present at high concentrations in male saliva: each component elicited pheromonal effects to attract estrus sows and cause them to adopt a characteristic "mating stance" known as standing (Dorries et al., 1997). Urine from adult mice contains high levels of specific volatiles like (R,R)-3,4-dehydro-exo-brevicomin (DB), (S)-2-sec-butyl-4,5-dihydrothiazol (BT), E,E α-farnesene, E β-farnesene and 6-hydroxy-6-methyl-3-heptanone. These volatile productions are dependent on testosterone and they can therefore be viewed as signaling the presence of a reproductively active mate (Schewende et al., 1986). These volatile compounds accelerate the attainment of puberty in pre-pubertal females when presented individually (Novotny, 2003), whereas a mixture of DB and BT induce estrus and synchronizes cyclicity in adult females (Jemiolo et al., 1986).

In elephant, the adult male releases high level of frontalin during musth, and the females have more response to this compound during the follicular stage. During ovulatory period, the female releases (Z)-7-dodecen-1-yl acetate in the urine to which male elephant is attracted and exhibited Flehmen (Rasmussen et al., 1997). The female horse shows significant levels of faecal pheromonal compounds like tetradecanoic and hexadecanoic during estrus than non-estrus (Kimura, 2001). The cow urinary volatiles like 1-iodoundecane acted as sexual attractants in bull (Rameshkumar and Archunan, 2002). Notably, four estrus-specific salivary pheromone in cow namely trimethylamine,
acetic acid, phenol-4-prophyl, pentanoic acid and propionic acid have been detected. Among these compounds, trimethylamine is more attractive than other compounds (Sankar et al., 2007). Moreover, three estrus-specific faecal pheromones in cow such as acetic acid, propionic acid and 1-iodoundecane, are reported to attract the bull and influence several pre-copulatory behaviours (Sankar and Archunan, 2008). The volatile compounds like 1-chloro octane, 4-methyl phenol and 9-octadecanoic acid were present exclusively in the buffalo estrus urine. The volatile 4-methyl phenol is found to be involved to attract the bull on the other hand, 9-octadecanoic acid stimulates bull mounting behaviour (Rajanarayanan, 2004).

In primates, the volatile fatty acids present in the vaginal secretions of rhesus monkey involve sex attractions for male monkey (Michael et al., 1975). In human, concentration of C2-C5 aliphatic fatty acids that secreted from the vaginal barrel referred as “Couplins”, vary with the menstrual cycle and hence Couplins are preferred as Pheromones (Michael, 1975).

The above reports clearly documented that the female produced a specific odour during estrus through urine, faeces, saliva, vaginal secretion, which constitute a major source of chemical communication in mammals.

**Pheromonal communication via proteins**

Chemical communication in vertebrates often utilizes soluble proteins, called pheromone-binding proteins (PBP) (Cavaggioni and Mucignat-Caretta, 2000; Pelosi, 2001). They are present in high concentration in biological fluids involved both in the perception and in the delivery of chemical messages of pheromonal significance. These proteins expressed in chemosensitive organs, and to carry hydrophobic molecules across aqueous mucus towards receptor neurons and consequently, are suggested to play a key role in pheromonal activation (Lazar et al., 2002).

For volatile pheromones to be transported efficiently in aqueous body secretions they need to be bound to proteins known as lipocalins. These are a large class of proteins, typically around 17-20 kDa that have a high level of homology and including odorant-binding proteins (OBPs) produced by vertebrate chemosensory epithelia. It has been proposed that OBPs and pheromone-binding proteins play complementary roles in chemical communication, the first in the perception of pheromones and the second in
their delivery (Finlayson et al., 1965; Pelosi, 1994). These proteins have a common β-barrel structure enclosing a cup-like, hydrophobic ligand-binding pocket (Flower, 1996).

The pheromone binding proteins have been studied in several sources, including mouse and rat urinary proteins (Cavaggioni et al., 1990; Bacchini et al., 1992; Hurst et al., 2001), hamster vaginal secretory proteins (Singer et al., 1986; Briand et al., 2000), pig salivary proteins (Marchese et al., 1998; Scaloni et al., 2001), and human sweat protein (Zeng et al., 1996). Major urinary (MUPs) and α2u-globulin proteins are the lipocalins that were first described in mouse and rat respectively, which are synthesized in the liver and excreted in the urine (Finlayson et al., 1965; Shaw et al., 1983). The role of MUPs in chemical communication between sexes is further confirmed by the selective expression of these proteins, whose synthesis is under hormonal control, only in adult males, being absent in females as well as young or castrated individuals.

Proteins of the same family are abundantly secreted from the submaxillary glands of the adult mature boar, being completely absent in the sow and in castrated pigs. The salivary lipocalins (SALs) contains two compounds of the boar sex pheromonal endogenous ligands, namely, 5α-androst-16-en-3-one and 5α-androst-16-en-3-ol (Marchese et al., 1998). Aphrodisin is another lipocalin secreted in the vaginal fluid of the hamster (Singer et al., 1986; Briand et al., 2000; Vincent et al., 2001). In this case, a bound volatile molecule, 1-hexadecanol, has been purified from the same fluid and confirmed to be the proteins endogenous ligand (Briand et al., 2004). A potent odorant of human sweat, 3-methyl-2-heptenoic acid, is carried by another lipocalin called apolipoprotein-D, abundantly produced in the sweat under certain conditions (Briand et al., 2000).

**Scent marking / dominance hierarchy via chemical signals**

Many social ungulates are characterized by well-defined stable dominance hierarchies (Cassinello, 1995; Cote, 2000). These hierarchies in turn determine the animals access to resources such as space, food, and mating opportunities. Interactions leading to the establishment and maintenance of hierarchies can be stressful to subordinate animals (Clarke and Faulkes, 1997; Grandin, 1999). Dominance hierarchy
systems are common among male animals and most of their interactions are mediated through the use of social odours, principally scent marking is involved (Gosling and Roberts, 2001).

Scent marking is a common behaviour among ungulates, which involves the deposition of secretory substances \textit{(i.e. non-volatile chemicals)} on substrate, intended to elicit a response from conspecifics. These chemical cues have been used as deterrent signals by territory holders (Gorman and Stone, 1990; Zuri \textit{et al.}, 1997) and provide information about the sex of animal, sexual advertisement, aggression, spatial orientation, physical condition, physiological state, age, social ranking, reassurance, to assess competitive ability and territoriality (Thiessen and Rice, 1976; Gosling, 1987; Vila \textit{et al.}, 1994; Ferkin and Johnston, 1995; Gosling and Roberts, 2001; Hurst and Beynon, 2004).

Various hypothesis of possible functions of scent marking in mammals have been put forth and they fall into five categories (Gosling and Roberts, 2001; Wolff \textit{et al.}, 2002). (1) The “territorial demarcation” suggests that scent marks are placed around territorial boundaries and act as a delineation of territory (Gorman, 1990; Schulte, 1998; Roberts and Dunbar, 2000). (2) The “ownership” suggests that animals scent mark to identify food resources within a home range or to indicate a priority of use of the resources (Kruuk, 1992). (3) According to the “mate-attraction” females may scent mark as a way to advertise reproductive state and to incite male- male competition (Heymann, 1998; Wolff \textit{et al.}, 2002). (4) The “stink fight” suggests that scent marking is a form of non-combative fighting (Gosling and Roberts, 2001). (5) The “self-advertisement” has been asserted for rodents, where hamsters and prairie voles were found to scent mark simply to advertise their presence and identity (Wolff \textit{et al.}, 2002).

The different specialized scent glands are involved in the pheromonal communication in different mammalian species (Balakrishnan and Alexander, 1985). They are common in carnivores, rodents and in most ungulate groups, but are also found in some primates. The glands may be situated in many different parts of the body. These glands generally consist of sebaceous and/or apocrine glands, the proportion of which varies greatly among animals species (Sokolov, 1982; Flood, 1985). The glandular secretions have volatile compounds, which serve as chemical signals if they trigger responses in conspecific receivers (Wilson, 1975). Mykytowycz
(1970) reviewed the role of scent glands in communication and Eisenberg and Kleiman (1972) have dealt with olfactory communication in mammals.

Since 1971 International symposium on ungulate behaviour and management in Calgary, Canada, our understanding of behavioural mechanisms of chemical communication in this group has progressed from description to experiments and modelling. Primer pheromones in few domestic and non-domestic ungulates communication have been identified. In our laboratory we have successfully identified pheromonal compounds by gas chromatography linked mass spectrometry from urine, saliva, faeces and scent glands of farm animals like cow, buffaloes and sheep and some rodent species of mouse and rat. These reports above gave an idea to the chemical ecological (Pheromone and Behavioural biology) studies in Indian Blackbuck.

The identification and synthesis of pheromone is the start of process, which may result in the development of economic and ecologically sustainable wild and zoo animal breeding management and conservation in captivity. As urine, faeces and scent glands are the major sources involved in pheromonal communication it might contain information for communication.

To date, data describing the social and reproductive behaviour of Indian Blackbuck are scarce. Only limited information is available about the behaviour of these animals in zoological collections (Ranjitsingh, 1989; Isvaran and Jhala, 1999). No study has yet been reported on the chemical characterization of the pheromonal compounds in the Indian Blackbuck. Therefore, the studies were undertaken to investigate the reproductive and, social behaviours and pheromones of an endangered species of Indian Blackbuck (*Antelope cervicapra* L.) to enhance captive breeding and conservation.
The objectives of the present study are

- To observe the effect of zoo visitor density on animal behaviour and faecal cortisol concentration in adult male Blackbuck.
- To study various reproductive behaviours during estrus and non-estrus period.
- To assess the Flehmen behaviour of dominant male in relation to estrous cycle.
- To examine the chemical profiles of female Blackbuck urinary volatiles and faecal steroids across the estrous cycle so as to detect estrus specific compounds. Chemical communication in Blackbuck can be exploited and manipulated for the breeding in captivity through artificial insemination.
- To evaluate the social hierarchy (dominant hierarchy) in adult male Blackbuck herds.
- To test the hypothesis of duration of dominancy in relation to hormonal production/ holding potential/ pheromonal production/ frequency of mating.
- To investigate the chemical profiles of urinary and preorbital gland secretions of territorial vs bachelor males in order to characterize the dominancy specific compounds.
- To study the architecture of the preorbital gland and to determine its morphology and anatomical location.
- To examine the low molecular mass proteins in preorbital gland secretion of territorial vs bachelor males by SDS-PAGE and a complimentary approach of LC-MS/ MS analysis.