

GENERAL INTRODUCTION

Animal communication in relationship to all behaviours be it sexual, aggressive or territorial, depends upon multiple sensory cues, the function of each is over-lapping extensively. Sexual interactions among mammals depend exclusively on one sense known as olfaction. It is an ancient sense, its precursors can be found in most primitive single-celled organisms, reflecting the need of every organism to sense its chemical milieu (Sheperd, 1988; Buck, 2000). The olfactory cues play an important role in mammalian sexual behaviour and have led to use the term ‘pheromone’ and ‘pheromone responses’ in defining such behaviours.

Pheromones are first defined in 1959 by Karlson and Butenandt, the term pheromone refers to air-borne chemical substances, that are secreted externally by an animal and cause a specific reaction in a receiving individual of the same species; the reaction involves either the release of a specific behaviour or physiological change in the recipient’s endocrine or reproductive system (Doty, 1976; Dominic, 1987; Rekot, 2001). Although originally the term “pheromone” introduced in the context of insect communication, the meaning of the word pheromone has been extended to include in the context of chemical communication in broader sense and also in relation to mammalian behaviours -(Keverne, 1998; Dominic, 1991; Vandenberg, 1999). The original discoveries of Bruce, Whitten and Vandenberg in the late fifties and middle sixties paved the way and attracted many scientists to involve in pheromonal communication in animals. Since then, progress in pheromone research has been rapid. The nature

and effect on the recipient of some pheromones in variety of animals have been elucidated. However, less progress has been shown in chemically identifying pheromones, even though several physiological functions related to reproduction and development has been shown to modify them. It is hoped that in the nearest future with the use of very precise analytical methods further development on pheromones will follow and their mechanism of action will be elucidated, which in turn allow to obtain pheromones synthetically.

Based on the type of response by the recipients the pheromones are classified as “primer”, “releaser” and “imprinting pheromone”. Releaser pheromones induce a rapid behavioral response in the recipients generally mediated through the central nervous system. The pheromones involved in sex attraction, evocation of aggression, recognition, alarming behavior and mother-young interactions are the citations of releaser pheromones. Occasionally, a releaser pheromone may act through a rapidly acting neurohumoral pathway e.g. the milk ejection reflex in mammals. It is suggested that the term “releaser pheromone” should be substituted by “signalling” pheromone to denote the transfer of information and not to describe the nature of the response.

Male mice urine contains releaser pheromones, which attract females, and organize aggressive activities in males. Similarly male rats investigate urine odours or receptive females, although sexual experience may be in some cases, necessary for this preference (Carr *et al.*, 1965; Dominic, 1978; Dominic, 1991). Males of certain mammalian species such as horse, bull etc. exhibit a distance grimace known as “flehmen”, following exposure to urine of conspecific females (Estes, 1972). Flehmen seems to be a motor pattern associated with olfactory detection of certain substances, especially the urinary metabolites (Dominic,

1991). The occurrence of Flehmen in cattle is due to signaling pheromones produced from females during specific period.

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 and established a concrete idea regarding their influence on reproductive functions. To the best of our knowledge, the influence of primer pheromones on mammalian reproduction are derived from studies carried out on a single species namely laboratory mice (Dominic, 1991; Archunan and Dominic, 1991). It is well known that individually housed female mice exhibit short and regular estrous cycle, however, unisexual grouping of the mice induces irregular estrous cycle: LeeBoot effect (Vander Lee and Boot, 1955).

Female mice continue to show estrous cycle disruption as long as they remain unisexually grouped. Pairing or previously grouped females with males induce a new estrous cycle: Whitten effect (Whitten, 1956). Social odours exert both acceleratory and decelaratory influence on the onset of puberty as indicated by vaginal opening: Vandenberg effect (Vandenberg, 1969; 1999). Exposure of newly inseminated female to a strange male caused pregnancy block: Bruce effect (Bruce, 1959; Brennan, 1999). These are the few examples of Primer pheromonal effects.

Imprinting pheromones organize the central nervous system of the preweaning offspring's at a critical period that cause permanent alterations of adult behaviour. Females of *Mus musculus domesticus* reared with their parents prefer to mate with males of different strains than its own. However, they display sexual preference for males of their sub-species relative to males of another species, if the choice is between two subspecies (Dominic, 1978). Hyashi and

Kimura (1978) documented that the imprinting like process may be involved in the acquisition of female preferences for male odours. Thus, females reared in the absence of males and exposed to an inaccessible male for only 4 days during the third week of age established a masked long lasting attachments to normal males, while those exposed before or after this critical period exhibited either weak or no preference when they become adults (Hyashi and Kimura, 1978).

Urine and faeces are the two most primitive as well as common source of mammalian pheromones (Dominic, 1976; Archunan, 1989; Rameshkumar *et al.*, 2000). Apart from urine and faeces, a number of specialized cutaneous gland secretions have evoked to elicit pheromonal responses in mammals (Albone, 1984; Kannan *et al.*, 1998; Kannan and Archunan, 2000). There are about 40 different specialized glands identified in mammals (Balakrishnan and Alexander, 1985a, b). Based on the specific body sites, they are named as armpit, flank, tarsal, ventral, anal, caudal, digital and inter-digital glands (Balakrishnan and Alexander, 1977 a, b; Mary and BalaKrishnan, 1991). Vaginal secretions are alsoa source of pheromones responsible for sexual attraction and arousal in male species like sheep (Signoret, 1991) hamster (Singer *et al.*, 1984) rhesus monkey (Michael, 1975) and bovine (Ma *et al.*, 1995; Sankar and Archunan, 2002).

In certain species like pig, saliva contains sex pheromones, which are involved in sexual arousal (Signoret, 1970). In human-beings, sweat plays a significant role in sexual behaviours by altering the menstrual cycle (Zeng *et al.*, 1996). It is widely believed that many female mammals advertise their readiness for mating through olfactory signals. The most likely sources of such signals are urine, which contains the metabolic products of many hormones and secretions of reproductive organs itself. The secretions of specialized scent glands may also be influenced by changes in the reproductive status of the females (Ebling, 1977).

In mammal, odorants are detected by the olfactory epithelium (OE) that lines the nasal cavity (Shepherd, 1988). Signals generated in olfactory sensory neurons in the OE in response to odorants are relayed through main olfactory bulb (MOB) to the olfactory cortex and then to other brain areas. Through these path ways odour signals ultimately reach higher cortical areas involved in conscious perception of odours as well as limbic areas, such as the amygdala and hypothalamus, that are involved in emotional and motivated responses (Buck, 2000). Most of the mammals have a second olfactory sense organ called the vomeronasal organ (VNO). The VNO is a tubular structure in the nasal septum that is connected to the nasal cavity by a small duct. Removal of the VNO or severing its connection to the brain interferes with pheromone effects but not with general odour sensing. Although some pheromones are sensed in the OE (Dorries *et al.*, 1997), many reports suggest that the VNO might be specialized to detect pheromones (Halpern, 1987; Wysocki and Meredith, 1987; Keverne, 1999).

It is likely that the two systems are specialized for receiving and responding two different kinds of molecules; the main olfactory system primarily handles relatively volatile chemical substances that are air-borne, whereas the vomeronasal organ (i.e accessory olfactory system) primarily deals with large, relatively non-volatile molecules that are contacted by the animal. The vomeronasal system is likely to be important in elicitation or facilitation of specific functional responses such as the initiation of mounting by a male or the release of leutinizing hormone from the pituitary. In contrast, the main olfactory system should have more analytic functions with a less specific to particular response. Hence, the two systems are likely to be involved in the detection and analysis of two different classes of signals, those with specific functions and those that just provide informations (Wysocki and Meredith 1987; Johnston, *et al.*, 1994; Ichikawa, 1996a,b; Keverne, 1999).

Adult female and male vertebrates often have a distinct hormone profiles that regulate sex-typical reproductive behaviours (Meisel and Sachs, 1994). It is generally believed that castration may differentially affect odour preference and production by altering aromatase activity (Powers *et al.*, 1985; Yahr and Stephen 1987). Earlier studies (Gregory *et al.*, 1975; Power *et al.*, 1985; Brown, 1985a, b; Ferkin *et al.*, 1992; Pfeiffer and Johnston, 1994) indicated that testosterone treatment increases the amount of time spent in investigating female urine. The role of gonadal hormones in the production of chemical signals is established in certain mammals (Pietras and Moulton, 1974; Ferkin and Johnston, 1995).

Pheromones have strikingly different chemical structures. It is as if this type of communication is so ancestral that many different molecules have evolved or been co-opted to serve pheromonal functions. Some pheromones also serve hormonal or physiological functions within the individual. Others are metabolites of hormonal and physiological pathways, and still others are unique compounds with no known function other than pheromonal communication. Some pheromones are a single compound (Rasmussen *et al.*, 1997 a, b) while others are cocktail of multiple compounds in the correct proportions. The important property of a compound used as a chemical signal is an appropriate volatility. This limits the upper molecular weights of an olfactory pheromone to about 300 and the effective lower molecular weight to around 50. Many different chemicals have been reported as pheromones i.e. alcohols, aldehydes, acids, 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).

Bio-communication by both volatile and non-volatile compounds is amply documented (Novotny *et al.*, 1984; Klemm *et al.*, 1994; Mucignat-Caretta *et al.*, 1995). There is a doubt, whether the volatile and non-volatile compounds show synergistic or individual effect in bio-communication. However, the probable suggestion is that once attracted to the vicinity of potential mate by volatile odours, ultimately animals may sniff and lick surfaces imbued with nonvolatile odour, which alone or together with sensory cues may execute mating behaviour (Sachs, 1996). However the experimental evidence for this suggestion is yet to be tested.

In addition to this, most of the workers believed that lipids and proteins are actively involved in olfactory communication in most of the mammalian species. Sebaceous glands of rat, rabbit and fishing bat have many kinds of fatty acids, glycerides that appear to be involved in individual recognition and scent marking behaviours. Mice and rats excrete great amount of protein (non-volatile) in their urine but their function remained unknown for many years. The apparent waste of large quantity of protein in urine first suggested the idea that it would be involved in some physiological function; its unique presence in the males was an indication that it would be involved in pheromonal communication (Pelosi, 1994).

Recent investigation in mouse and rat convincingly demonstrated that urinary proteins (major urinary protein, α -₂U globulin) play a significant role in the delivery of chemical messages (Cavaggioni and Mucignat-Caretta, 2000). It is believed that these urinary proteins bound with odorant molecules inside the blood stream and release them in urine. Most interestingly the time of dissociation of the odorant –protein complex is very long and urinary protein can store and slowly release the odorants in air for hours and days. In addition, urinary proteins are usually stable to drying and heating and are not likely to be quickly denatured

when released out of the body. For instance, the major urinary proteins (MUPs) of male mouse bind the signalling volatiles and release these volatiles from urine into the environment (Cavaggioni and Mucignat-Caretta, 2000). Recently it has been identified that the male mouse urinary protein itself acts as pheromone by accelerating the puberty in young female mice (Mucignat-Caretta *et al.*, 1995). It seems that this phenomenon of excreting large quantities of proteins in urine is typical for rodents (Cavaggioni *et al.*, 1999).

Chemical investigations on mammalian pheromones have been restricted largely to the species, which are of interest to perfume industry. It is a matter of great regret that less chemical investigation has been conducted on odour of mammalian species. However, in the last two decades there is a considerable study on the chemistry of mammalian pheromonal identification in mouse (Liebech *et al.*, 1977; Novotny *et al.*, 1985a), rat (Kannan *et al.*, 1998; Kannan and Archunan, 2000; Selvaraj and Archunan, 2002) bobcat (Mattina *et al.*, 1991) horse (Ma and Klemm, 1997) bovine (RameshKumar *et al.*, 2000) white-tailed deer (Jemiolo *et al.*, 1994), tiger (Brahmachary, 1996) elephant (Rasmussen *et al.*, 1997a) and human (Stern and McClintock, 1998). These findings indicate that mammalian pheromones may be a single compound or a mixture of compounds and that each of the major fractions is faithfully involved in conveying specific signals related to reproductive and social behaviours.

Several substances have been identified in the urine of adult male mice and are dependent upon high levels of testosterone (Novotny *et al.*, 1984). Some of these substances are volatile such as, 2-sec-butylthiazole (Liebech *et al.*, 1977), 2,3dehydro-exobrevicommin and 4-ethylphenol (Novotny *et al.*, 1985a). The male originated 2-sec-butyl-dehydrothiazole and dehydro-exo-brevicommin were found to be involved in eliciting intermale aggression (Novotny *et al.*, 1985a) and

attractiveness to females (Jemiolo *et al.*, 1985). The compound 2,5 dimethylpyrazine, a characteristic urinary volatile compound of grouped adult females (Novotny *et al.*, 1985b) reported to be involved in puberty acceleration in mice is an adrenal-mediated compound. It has further shown that adrenalectomy but not ovariectomy, eliminates the biological activity of excreted urine in causing a delay in sexual maturation of juvenile mice (Drickamer and McIntosh, 1980). Nishimura *et al.*, (1989) reported that the hormone-dependent amines in male mice urine viz., isobutylamine and isoamylamine are found to be involved in acceleration of puberty.

Mammals can distinguish an enormous diversity of odorants that vary in size, shape, functional groups and charge (Beets, 1970). In contrast, only a few mammalian pheromones have been identified though many different pheromone effects have been recorded that can be elicited by urine or other bodily secretions (Halpern, 1987; Wysocki and Meredith, 1987; Novotny *et al.*, 1990; Keverne, 1999). Mammalian pheromones are found to be involved in many reproductive behaviours such as sexual attraction (Kannan *et al.*, 1998), interference with puberty, estrous cycle and pregnancy (Dominic, 1991) as well as social behaviours namely territorial marking (Doty, 1980; Prakash and Idris, 1992), individual identification (Poddar-Sarkar and Brahmachary, 1999) mother-young interaction (Leon, 1983) and evocation of aggression (Mugford and Nowell, 1971).

The basic research on pheromones in animals is a beginning to result in some practical applications, particularly in insect and rodent pest management, breeding and rearing of farm animals and in the control of human fertility. As a result it will be further step to extend possibilities of controlling reproductive process in animals. The main problem in endangered species is that communication between their partner is affected by pollution and disturbance

caused by humans. However, the communication in animals for reproduction mainly depends on pheromones. Hence, investigating the role of chemical signals in these species and make a suitable environment with such odours, which may enhance their sexual activity and increase the productivity of animals. In fact, learning the communicatory behaviour in laboratory animals will give a new insight in chemo signalling, which can be further applied to the wild animals.

Estrus detection by the male partner is a classical example for pheromonal communication. The male smells the urine, vaginal mucus of female in estrus and makes the claim to be ready for mating (Johnston, 1990). The odour of the most mammalian species probably varies with their reproductive state; the chemical constituents of scent glands, vaginal secretions and urine may all vary with the estrous cycle (Ebling 1977; O'Connell *et al.*, 1981). Many studies indicate that males were attracted to the estrous females than to diestrus or non-estrus females. It is surprising that only a few species have been investigated, especially considering the belief of that nearly all male mammals can detect estrus on the basis of female odours. Other male behaviours such as mounting, Flehmen should also be differentially influenced by the odour of females in different reproductive status. But little information is available.

In a rather complex sequence of olfactory communication process that occurs in mice (Vandenbergh, 1983), the urine of estrous animals may carry a variety of messages to both male and females. The physiological and behavioural phenomena that are readily observed in association with the estrus of rodents include an increase in activity and phase advance in circadian rhythm (Cushing, 1985) and a distinct change in urination patterns (Wolff and Powell, 1979). Moreover, male mice are sexually attracted by estrous odours. Like-wise, in some rodent species e.g *Mus musculus* (Brown, 1985 a, b), *Peromyscus maniculatus*

(Terman, 1984), *Microtus pinetorum* (Boyer et al., 1988), pheromones that inhibit reproduction have been found in the urine of grouped females.

Occurrence and length of estrous cycle are known to influence certain olfactory signals originating from other females in a social group. While the onset of the first estrus is delayed (Vandenbergh *et al.*, 1972; Archunan and Dominic, 1990b) by a signal from grouped adult females, the urine from estrus, pregnant or lactating animals is reported to accelerate the sexual development of young females in addition to promoting estrus in adult conspecific (Drickamer, 1999). According to Drickamer, the chemosignals involved are influenced by population density and dominance (Drickamer, 1999) of the donor females. These regulating mechanisms are probably not a laboratory artifact since it was observed in natural and semi natural populations of house mice (Massey and Vandenbergh, 1980) and in some vole species *Clethionomys glareolus* (Bujalska, 1970), *C. rutilus* (Gilbert *et al.*, 1986) *C. rufocanus* (Kawata, 1987).

Several signalling phenomena have been observed to be associated with female mouse urine, including sex attractant, an aggression-reducing signal and an ultrasound-eliciting signal (Davies and Belgamy, 1972). The attractant signal appears to be related to the estrus state of the female, while the other two are not. Male mice are more interested in sniffing tubes containing urine from estrous females than from diestrus females (Whitten, 1966). Female mouse urine may be the source of a primer pheromone responsible for the Lee-boot effect (Vander Lee and Boot, 1955) that reflects the shortened periods of diestrus and prolongation of estrus in isolated females. Although the source and nature of the primer pheromones have not been deduced once again urine is suspected. Maruniak *et al.*, (1975) also observed that female marking behaviour increases in the presence of male.

The above literature clearly establishes that female mice pheromone have significant function in male reproduction as well as in female reproduction. The chemical substances that are involved in these functions are not yet explored. Up to now most pheromonal characterization study have been carried out in most male species rather than in females. Chemical characterization of male mice urinary pheromones is well reported. Further, characterization of female urinary pheromones is somewhat difficult due to the hormonal change during reproductive cycle. A more direct approach to identify the compounds used as chemical signals is to identify and fractionate the biological materials repeatedly and attempt to isolate the active compounds with the bioassay of the fractions. Few attempts have been made in different mammals to isolate estrus-specific compounds and partial success has been obtained.

Even though the role of olfactory stimuli in mammalian reproduction and behaviours have been known for a long time, our knowledge of mammalian pheromones is not as exhaustive as that of insect pheromones. Moreover, chemical characterization and experimental investigation into this field have been made only during the last few decades with special reference to male laboratory mice and in some field animals. The chemistry and pathways of mammalian pheromone remain to be explored. The female to male and female to female olfactory signals have not been chemically characterized at present, but attempts have been made by Schwende *et al.*, (1984) and Andreolini *et al.*, (1987) to find out the estrus indicating volatile compounds but it did not succeed.

Unlike the production of male pheromones, female pheromones especially estrus specific pheromones are produced in a specific period, that too in a very short span of time. Therefore, it is difficult to collect the sample and characterize the pheromone. This may be one of the reasons that many scientists show less

interest in identifying the estrus specific pheromonal compounds. But this study is very important for rodent pest management. This technology may further be applied to farm and wild animals.

The present study has been focussed on chemical characterization of volatile compounds of mice (*Mus musculus*) urine, to address which component(s) of the volatile fraction is specific to estrus and to analyse its influence on social and reproductive behaviour in mice. Further, the present investigation proposes to examine the possibility of endocrine dependency in urinary volatile constituents.

Objectives of the present study:

- i) to find out the changes of biochemical urinary constituents in various reproductive stages of mice
- ii) to know the chemistry of urinary volatiles with special reference to estrous cycle
- iii) to understand the role of identified volatile compounds in pheromonal communication by behavioural analysis
- iv) to evaluate the involvement of olfactory-vomeronasal system in detecting estrus phase and
- v) to compare the identified compounds with the chemistry of other mammalian pheromones to give support for convergent evolution.