



Oestrus pheromones in farm mammals, with special reference to cow

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ABSTRACT

Pheromones are defined as chemical signals that are released from one individual and induce specific endocrine or behavioural reactions in another individual of the same species. Odours play a significant role in signalling the stage of reproduction of the cow, and the bull can use a combination of factors displayed by the female to determine its receptivity. During oestrus, females release olfactory molecules, thus signalling the stage of their cycle and stimulating sexual behaviour and functions of males. Attempts were made to review the chemical nature of olfactory signals of oestrus in milk, urine, blood, saliva, skin gland secretion during the prooestrous and oestrous cycle. Several behavioural studies indicated that bovine milk from different stages of the oestrous cycle had different odours. Bulls have also been found to detect pheromone odours and differentiate between oestrus and non-oestrus urine. Bioassay involving rats revealed the presence of maximal pheromone activity during prooestrus. Vaginal fluid is also reported to act as a chemical signal. Saliva, serum, skin gland secretions and faeces have also been studied for their pheromonal properties. Systemic evaluation of either urine or vaginal secretions over the whole cycle has yet to be investigated. The use of artificial olfaction could enable more accurate detection of oestrus and potentially increase fertility in cows. The current status of chemical signals (pheromones) of oestrus and their identification in farm animals is reviewed in this article with emphasis on cows.

Key words: Cattle, Farm Animals, Oestrus, Pheromone

The term pheromone was introduced by Karlson and Lüscher (1959) as substances secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for instance a definite behaviour or developmental process. Afterwards this term was extended to any form of innate intraspecific chemical communication i.e. to define chemicals emitted by living organisms to send messages to individuals of the same species. Most pheromones consist of blends of two or more chemicals, which need to be emitted at exactly the right proportions to be biologically active and to prevent mismating between species. These pheromones are associated to binding proteins and very specific receptors responsible for olfactory perception in the target animal (Ha and Smith 2006). This perception induces stereotyped behaviour (releaser pheromone) and/or physiological changes (primer

pheromone). The pheromones are different from the recently coined term “signature mixtures” which are defined as a variable chemical mixture (a subset of the molecules in an animal’s chemical profile) learned by other conspecifics and used to recognize an animal as an individual (e.g. Lobsters, mammals) or as a member of a particular social group such as a family, clan or colony (e.g. ants, bees, mongoose). A key difference between pheromones and signature mixtures is that in all taxa so far investigated it seems that signature mixtures need to be learnt (Wyatt 2009 and 2010). Both pheromones and signature mixtures are detected by main olfactory system or accessory olfactory system or both depending on pheromone and species. The accessory olfactory system consists of a sensory organ, the vomeronasal organ, and its central projection areas– the accessory olfactory bulb, which is connected to the amygdala and hypothalamus, and also to the cortex (Mucignat-Caretta 2010). Most of our knowledge on pheromones relies on insects, due to the clear-cut and readily identifiable response that insects have to these molecules, which has allowed the identification of pheromones from over three thousand species (www-pherolist.slu.se/pherolist.php) and has led to new concepts of pest control. Contrary to the large number of known insect pheromones, very few were characterised

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in mammals, despite the importance of these chemical cues in regulating social and reproductive behaviours. Chemical communication plays an important role in mammalian sexual behaviour and reproductive processes (Brennan and Zufall 2006). Animals communicate information concerning reproduction to conspecifics in order to co-ordinate reproductive activities (Rekwot *et al.* 2001).

In farm animals, the generalised use of artificial insemination (AI) has made reproduction more effective. Efficiency of AI partly depends on the precise detection of female receptivity, which is made by breeders on visual cues or with the help of detector males. Novel 'pheromonal cues' based methods are required, which could not only help in detection of oestrus in the farm animals but also satisfy the social requirements of sustainable environment and animal welfare. Thus, it is necessary to understand fundamental biology of the target livestock species, in particular the identification of chemical signals and their precise involvement in oestrus detection by the males. The present article attempts to review the literature available on the pheromones of oestrus and their identification in farm animals, especially cows.

Types of pheromones

If the original definition of pheromones relies on insects, it can easily be applied to mammals– it refers to chemical cues that are exchanged between congeners and either elicits a stereotyped and innate behaviour or induce a physiological change in the recipient endocrine or reproductive system (Doty 1976, Izard 1983). The major divergence with the original definition is the chemical nature of pheromone components, which were originally supposed to be air-borne volatiles, suggesting a long-range attraction. But increasing knowledge on the chemical nature of pheromones in both insects (cockroach, Korchi *et al.* 1999) and vertebrates (mice, Morè 2006) has demonstrated that proteins or peptides could act in the transfer of intraspecific information. Pheromones can be classified into following types:

Releaser pheromones: These pheromones elicit an immediate behavioural response. Rabbit milk contains a pheromone 2-methyl, but-2-enal which elicits stereotyped nipple-search behaviour in rabbit pups, and is vital for them to locate the nipples during brief daily period of suckling (Schaal *et al.* 2003). In the pig, the male stimulates the immobilization reflex of the sow by using sex pheromones contained in its saliva (Signoret and du Mesnil du Buisson 1961, Signoret and Mauleon 1962). These pheromones were identified as the steroids 5 α -androst-16-en-3-one and 5 α -androst-16-en-3 α -ol (Patterson 1966, Patterson 1968), which are synthesized in the testes and released in boar saliva (Claus 1979).

Primer pheromones: These pheromones mediate slow developing and longer-lasting changes to the endocrine state or development. A testosterone dependent constituent of male

mouse urine, a-farnesene, is a primer pheromone. It accelerates puberty of pre-pubertal female mice (Novotny *et al.* 1999, Novotny 2003). In domestic mammals, especially small ruminants, priming pheromones from the male seems to have an influence on the induction of puberty, the termination of seasonal anoestrus and shortening of postpartum anoestrus (Gelez and Fabre-Nys 2004).

Signaller pheromones: These pheromones convey the information about the sender, such as individual or group identity, which are important for parent-offspring recognition and mate choice (Potts *et al.* 1991, Yamazaki *et al.* 2000).

Modulator pheromones: These pheromones affect mood and thought processes in humans. This category has not gained wide acceptance (Wysocki and Preti 2004).

Major urinary proteins (MUPs) and Odorant binding protein (OBPs): Major urinary proteins (MUPs) and α 2u proteins are lipocalins that were first described in mouse and rat, which are synthesized in the liver and excreted in the urine (Finlayson *et al.* 1965, Shaw *et al.* 1983). Their roles are 1) to transport the pheromone in biological fluids, 2) to extend the period of bioavailability of the pheromone by delaying its liberation, and 3) to modulate the pheromone activity (Hurst and Beynon 2004). Odorant binding protein (OBPs) discovered in the nasal tissues of several vertebrates have strong similarity with MUPs (Pelosi *et al.* 1982, Cavaggioni *et al.* 1987). They are believed to shuttle odorants from the environment to the underlying odorant receptors, for which they could potentially serve as odorant presenter (Pugalthi *et al.* 2007). In some cases, the same genes have been found to be expressed in both the nose and in the liver (Utsumi *et al.* 1999). Presence of endogenous ligands only in MUPs, make them different from OBPs. These endogenous ligands have been recognised as specific pheromones (D'Innocenzo *et al.* 2006).

The boar's salivary gland (submaxillary gland) also secretes lipocalins which contain two components of the boar sex pheromonal system as endogenous ligands, namely, 5 α -androst-16-en-3-one and 5 α -androst-16-en-3 α -ol (Marchese *et al.* 1998). These proteins are also expressed in the nasal tissues of both sexes in pig but in this case these are devoid of ligands (Scaloni *et al.* 2001).

A general scheme of olfactory coding has hypothesised that pheromones are detected by sensory neurons of the vomeronasal organ (VNO), while general odours are detected by the main olfactory epithelium (MOE) sensory neurons (Dulac and Torello 2003, Brennan and Keverne 2004). But there is growing evidence that the coding of olfactory signals is more complex. Some pheromone-mediated behaviours are still effective after VNO lesions (Hudson and Distel 1986, Dorries *et al.* 1997, Lévy *et al.* 2004). Conversely, mouse VNO neurons can be stimulated by odorants not emitted by the same species, such as floral and woody smelling compounds (Sam *et al.* 2001).

Role and importance of olfactory compounds and pheromones in reproductive processes

The odoriferous compounds, potentially pheromones, may be released by males to influence female ovarian functions and oestrus behaviour as shown for sheep (Knight *et al.* 1983, Signoret 1991), goats (Chemineau *et al.* 1986, Claus *et al.* 1990), cattle (Izard and Vandenberg 1982) and pigs (Signoret and Mauleon 1962, Claus and Schams 1990, Claus 1994). However in cattle, the role of pheromones, especially the “oestrus pheromones” in cattle reproduction is not as clearly defined as it is in other species such as sheep, goat and pig, possibly due to nutritional and other environmental stresses (Rekwot *et al.* 2001). Mating within a narrow (12 to 22 h) period on the day of oestrus is a prerequisite for optimal fertilization in cow (Schams *et al.* 1977). These time requirements are mainly regulated by female lordosis behaviour, which allows copulation. In unrestrained cows, mating is preceded by a complex sequence of bull-cow interactions that starts few days before oestrus (day 0). In pro-oestrus, bulls separate cows from the rest of the herd (Schloeth 1961, Reinhardt 1983). To determine female’s receptivity, a bull uses a combination of factors displayed by the female, including visual, tactile, auditory and olfactory stimuli (Izard 1983, Zalesky *et al.* 1984). Visual and acoustic stimuli from cow were found important for optimal detection of oestrus by a bull (De Vuyst *et al.* 1964, Blaschke *et al.* 1984). In contrast, there are several reports that clearly show that there is an olfactory component, independent of vision, in the stimulation of male reproductive behaviour (Sambraus and Waring 1975, Paleologou 1977, Jacobs *et al.* 1980, Izard and Vandenberg 1982, Klemm *et al.* 1987). Indeed, females seem to release olfactory molecules during oestrus, thus signalling the stage of their cycle, and stimulating sexual behaviour and endocrine functions of males. Such effects were shown for horse (Stahlbaum and Houpt 1989), sheep (Lindsay 1965, Schanbacher *et al.* 1987, Gonzales *et al.* 1991, Walkden-Brown *et al.* 1993), goat (Ladewig and Hart 1980, Blissitt *et al.* 1994) and bovine species (Hradecký *et al.* 1983, French *et al.* 1989). Presence of pheromones was indicated in equine urine which may act as markers for detection of oestrus (Ma and Klemm 1997). For cows it was demonstrated by use of a bioassay (involving rats and not bulls) that maximal pheromone activity is present in urine prior to the onset of behavioural oestrus (Dehnhard *et al.* 1991).

The behavioural pattern of bulls and a possible involvement of the olfactory cues or pheromonal substances, in a group of oestrus-synchronised cows have been established (Masaki and Ohta 1990). Bulls routinely investigate the anogenital region or urine of females (Hafez and Bouissou 1975). Sniffing, licking and nuzzling of the anogenital region usually elicits urination from females under investigation. The male then puts its nose and mouth directly in the stream of urine or sniffs and licks the urine-soaked

substrate. The sniffing and licking behaviours follow with the male showing a typical posture of raised head with open mouth and curled upper lips, called “flehmen”. This behaviour is seen in many ungulate species in both males and females in response to several odours (Estes 1972). Oestrus-signalling compounds from urine and vaginal secretions were suggestive to lead bulls to flehmen reaction and later to a more intensive mounting behaviour (Hradecký *et al.* 1983, Garcia *et al.* 1986). The vaginal mucus and urine of oestrus cows, when rubbed on the vaginal membranes of non-oestrus cows was reported to attract and stimulate bulls through olfaction and a volatile odour in the cervico-vaginal mucus of oestrus cows was believed to be a source of sexual attraction for bulls (Hart *et al.* 1946).

The presence of maximal pheromone activity during pro-oestrus suggests that it is more important to signal the imminence of oestrus to the bull, than for stimulating precopulatory male sexual behaviour test in male rats and not in bulls (Dehnhard *et al.* 1991). Similarly, investigation of olfactory stimuli in a dairy herd revealed that it reaches a maximum in pro-oestrus (French *et al.* 1989). In contrast, Hradecký *et al.* (1983) observed that the bull flehmen reaction is most frequent on the day of oestrus. Similarly, the odour intensity was maximal on day 0, as detected by trained dogs (Kiddy and Mitchell 1981). This discrepancy was explained by a field study (Blazquez *et al.* 1988a) on a grazing dairy herd, which showed that cows attracted bulls the day before oestrus and that this attraction is maintained throughout oestrus. Dehnhard *et al.* (1991) observed that pheromone activity still remained, though there was a decline in the activity on the day of oestrus. If all sensory stimuli contribute to the finding of a partner and to sexual behaviour, the olfactory stimuli, however, seem to be more important for attracting partners rather than for stimulating the mating.

Assays for the oestrus pheromones

Analytical assays: The identification of the oestrous pheromones is attempted by analytical techniques comprising first, extraction of compounds using organic solvents (Ramesh Kumar *et al.* 2000) or without organic solvents i.e. Solid Phase Micro Extraction (SPME) (Guiraudie-Capraz *et al.* 2005), followed by Gas Chromatography-Mass Spectrometry (GC-MS) (Ramesh Kumar *et al.* 2000, Guiraudie-Capraz *et al.* 2005).

Although several sequence based search methods were exploited for protein family prediction, less effort was devoted to the prediction of OBPs from sequence data and this area is more challenging due to poor sequence identity between these proteins. Recently Pugalenthi *et al.* (2007) have proposed a new algorithm that uses Regularized Least Square Classifier (RLSC) in conjunction with multiple physicochemical properties of amino acids to predict OBPs from sequence derived properties irrespective of sequence similarity. It was reported that this method predicts 92.8%

of 56 OBPs non-homologous to any protein in the Swissprot database and 97.1% of the 414 independent database proteins.

Bioassays: The availability of a highly practicable bioassay is essential for assessing the biological activity of the oestrus-related pheromones characterized by physicochemical techniques. Several attempts were undertaken to characterize the biological and chemical properties of an oestrus-specific odour in cow using bulls (Hradecky 1986), dogs (Kiddy *et al.* 1978) or rats (Ladewig and Hart 1981, Dehnhard and Claus 1988) for bioassays. The species of the detecting animal has tremendous importance for the validation of biological activity. Thus, the use of conspecific males is relevant to characterize the pheromonal nature of a chemical signal emitted by females of the same species. In this case the behavioural response is expected to be stereotyped (courtship sequence) and non-learned, even if it is well known that adult males mate more successfully than young naïve males (Reinhard 1983). Besides, the use of trained detector animals such as dogs or rats strongly suggests that one or several odours could be common to the oestrus specific secretions of these species. In that case, the perception of one or several odours being part of the pheromone of their female can evoke one or several reactions associated with the sexual behaviour (e.g. penile erection). As a matter of fact, none of these studies reported the observation of a full stereotyped courtship behaviour, which could be obtained in response to conspecific females in oestrus or their body fluids (Dehnhard and Claus 1988, Rampin *et al.* 2006).

Nevertheless, the increase of Flehmen frequency is only one of the behavioural reactions that are expected in response to the perception of a pheromone. The oestrus specific odours can be ultimately qualified of “pheromone” at the condition they evoke stereotyped, non-learned behavioural sequence in conspecific males (Rivard and Klemm 1989).

In a bioassay, using rats trained to detect olfactory differences between estrous and diestrous cow urine. The oestradiol administration resulted into estrus symptoms observed by trained herdsman but urine from such cows were

not identified by rats as estrous urine. This suggests that the presence of the ovary seems to be necessary for oestrus-specific odour detected by trained rats used in the study (Dehnhard *et al.* 1991). It is not clear, if the odour identified by rats in the oestrus urine from cyclic cows is caused by molecule other than the “pheromone specific to estrus” because the same was not identified by rats in urine from oestradiol treated ovariectomised cows expressing signs of estrus.

The identification of a pheromone specific of a physiological stage in one species therefore must satisfy two conditions: (i) the molecule can be identified in that stage and (ii) molecule induces a physiological effect in this species. Tests in other species do not indicate that the molecule is a pheromone.

Physiological sources of pheromones

The chemical nature of pheromones is not precisely known, but several body fluids have been reported as potential sources of oestrus signalling compounds. The volatile compounds termed or suggestive of pheromonal properties in different bodily fluids of animals are being summarised in Table 1.

Oestrus pheromones in milk: Milk volatiles can be transferred directly from forage via the rumen (Honkanen *et al.* 1964) and the respiratory tract (Shipe *et al.* 1962) or produced by metabolism (Virtanen and Lampila 1967, Dumont and Adda 1978). The volatile constituents of bovine milk are of great interest to food scientists because those constituents contribute to flavour (Gordon and Morgan 1972, Dumont and Adda 1978, Badings 1991, Shiratsuchi *et al.* 1994). The volatile compound γ -12:2 lactone, identified in cow's milk was shown to be an active odorant (Bendaall 2001) and the lower rumen pH of cows fed on a concentrate diet might facilitate the production of such lactones (Urbach and Stark 1978). Since many flavour qualities are due to odour detection (Gordon and Morgan 1972), some volatile flavour compounds may possibly function as pheromones for olfactory communication. Indeed, the components of the

Table 1. Volatile compounds termed or suggestive of pheromonal properties in different bodily fluids of animals

Animal	Pheromonal compounds	Biological fluid	Reference
Boar	5 α -androst-16-en-3-one	Saliva	Patterson (1966, 1968)
Rabbit	5 α -androst-16-en-3 α -ol,	milk	Schaal <i>et al.</i> (2003)
Male mouse	2-methylbut-2-enal.	urine	Novotny <i>et al.</i> (1999)
Cow	α -farnesene	Oestrus blood	Klemm <i>et al.</i> (1994)
	Acetaldehyde	milk	Bendall (2001)
	γ -12:2 lactone	Oestrus urine	Ramesh Kumar <i>et al.</i> (2000)
	n-propylphthalate	Oestrus urine	Ramesh Kumar <i>et al.</i> (2000)
	1-iodoundecane	Oestrus faeces	Sankar and Archunan (2006)
	Acetic acid	Oestrus faeces	Sankar and Archunan (2006)
	Propionic acid	Oestrus faeces	Sankar and Archunan (2006)
Elephant	(Z)-7-dodecen-1-yl-acetate	Oestrus urine	Rasmussen <i>et al.</i> (1996)

pig appeasing mixture (Pageat 2001) were characterized in milk by Solid Phase Micro Extraction (SPME)-GC-MS (Guiraudie-Capraz *et al.* 2005). Using a combination of PAGE and mass spectrometry for protein identification evidence of a putative odorant binding protein was obtained in bovine colostrum. Its biological function is unclear, but pheromone transport could be considered (Fukuda *et al.* 2009).

The headspace sampling technique (GC-MS) and consequent reduction of sample pre-treatment allowed the identification of low-molecular weight volatile compounds in milk samples (Toso *et al.* 2002). Acetaldehyde, which had previously been found to be a marker of oestrus from headspace GC of bovine blood (Klemm *et al.* 1994) and vaginal secretions (Ma *et al.* 1995), was not detected in the milk samples because this compound has a shorter retention time than the solvent (diethyl ether). However, the results suggested that the property of three types of milk (pro-oestrus, oestrus and di-oestrus) samples were distinctly different from one to another (Weidong *et al.* 1997). Only one behavioural study was conducted and has failed to support a pheromone role for acetaldehyde in bovine (Prescicce *et al.* 1993).

Oestrus pheromones in urine: The role and importance of urine chemical signals in reproductive behaviour was clearly established in several species of mammals, especially rodents (Xia *et al.* 2006). In cattle, the bulls can detect pheromone odours and differentiate between oestrus and non-oestrus urine (Sambraus and Waring 1975). Urine is known to contain a large array of compounds that may confound the isolation of pheromones (Albone *et al.* 1986) and it is also believed to be an important source of oestrous signals (Crowell-Davis and Houpt 1985).

Several attempts were made to isolate and characterize the oestrus-signalling pheromones from cow's urine. Ramesh Kumar *et al.* (2000) analysed the GC-MS profiles of the volatile compounds of cow's urine extracted at three different stages: preovulatory (3–5 days before oestrus), ovulatory and postovulatory (2–4 days after oestrus). In a preliminary study, nine organic solvents were used to extract the compounds from pooled urine (n-hexane, acetone, methanol, ethanol, petroleum ether, diethyl ether, chloroform, dichloromethane and benzene). The maximum response was obtained when dichloromethane (DCM) was used for extraction, and the chemical profile of oestrus urine was distinguished by the presence of two specific compounds, di-n-propylphthalate and 1-iodo undecane, that were not found in other samples. As oestrous urine was found to elicit sexual behaviour in cattle, these two compounds may represent important chemical compounds that elicit signals that allow the bull to detect 'oestrous odours'. However the role of such compounds identified in the oestrus phase needs to be confirmed by their effects on the behaviour of bulls.

Oestrus pheromones in vaginal mucus: Though urine is considered to be primary source for chemical communication,

vaginal fluid is also reported to act as a chemical signal. Moreover, it is possible that urine and vaginal fluid may act together for completion of the pre-copulatory behaviour and successful mating (Klemm *et al.* 1987). Evidence for a pheromone was indicated by studies where dogs or rats were trained to detect oestrus by smelling cervico-vaginal secretions or urine (Kiddy *et al.* 1978, Ladewig and Hart 1981). Klemm *et al.* (1987) identified over 20 compounds: alcohols, diols, alkenes, ethers, diethers, ketones, primary amines, and aromatic alkanes. Nine compounds were found in samples that had been validated as being sexually stimulating; six of these compounds were validated as positive with another bull when randomly tested after 1 year of storage.

The dialyzable fraction of vaginal mucus and the neutral fraction prepared by ion-exchange chromatography of the dialyzable solution of vaginal mucus had a mounting inducing activity on the herd mates, as did the application of an animal's own vaginal mucus, suggesting that mounting-inducing pheromones are relatively low molecular weight, neutral substances (Nishimura *et al.* 1991). Another experiment suggested that vaginal mucus might act as an additional/ secondary source along with urine in eliciting copulatory behaviour and executing coitus in bulls during oestrus (Sankar and Archunan 2004).

Oestrus pheromones from faeces: The faeces is also suggested to act as pheromone cues in bovine bio-communication. The chemical profiles of oestrus faeces (bovine) were found to be distinguished significantly from other phases by the presence of three specific substances, viz. acetic acid, propionic acid and 1-iodo undecane. Bulls were observed to exhibit significantly higher ($P < 0.001$) repeated flehmen and mounting behaviour when the mixture of these compounds was applied to genital region of non-oestrus (dummy) cows (Sankar and Archunan 2008).

The levels of fatty acids, aldehydes, amines and alkenes in the faeces of oestrus mares were found to be significantly higher than their respective levels in the faeces of non-oestrus mares (Kimura 2001). Faeces of oestrus mares, foxes and rats were demonstrated to elicit more penile erection in rats than faeces of di-oestrus females of the same species did not (Rampin *et al.* 2006).

Oestrus pheromone from skin glands: The increased proportion of bull olfactory behaviours during an experiment, elicited by an increased perineal skin gland discharge, adds support to hypothesis that the perineal skin glands are also source of an oestrous pheromone in the cow (Blazquez *et al.* 1988b).

Oestrus pheromone from serum and other body fluids: The serum may act as transport medium of pheromone in body from its origin to place of excretion. The serum taken at oestrus was observed to evoke sexual behaviours such as flehmen and penis protrusion in bulls (Rivard and Klemm 1989). The saliva was observed to be an important oral cue

used by females in the selection of socio-sexual partners in cows (Sankar and Archunan 2004). Similarly adult female Mongolian gerbils were found preferentially attracted to saliva from adult non-sibling males when paired with saliva from their male siblings (Smith and Block 1991).

Apparently, these sources, regardless to the site of the production are distributed throughout the cow's bodily fluids, probably by the circulatory system. It is reported that the hormones, such as, FSH, LH and steroids which used to be measured in blood have now been quantified in human urine (Shimizu *et al.* 2003) and saliva (Loewit *et al.* 1987). The steroid androstenol, was identified in both human urine and sweat and believed to act as pheromone (Gower and Ruparelia 1993). These reports indicate that compounds present in one source are likely to be present in other sources as well. Therefore, the source of the oestrus pheromones may originate in organs other than the genitals, such as skin or mouth. However it is not known whether the olfactory signals present in various bodily fluids, which help in oestrus detection, are of the same nature.

Physiological regulation of pheromone secretion

In cow, both the inhibitive effect of progesterone and a stimulating effect of oestrogen for the occurrence of oestrus are well known (Dozier and Pritt 1987, Thomas *et al.* 1988). A highly significant correlation exists between progesterone concentrations and rat responses, which demonstrates that the synthesis of pheromone is blocked by high progesterone concentration and increases with the onset of luteolysis (Dehnhard *et al.* 1991). The optimal response of rats in a bioassay could only be obtained when the original pH of the urine was not altered. A transient shift of either acid or basic pH values had irreversible effects on the pheromone activity and thus the reaction of the rats. The degree of loss of activity depended on the pH, which was maintained for 3 h. Thus, a transient change of pH below 7.0 or above 9.5 was sufficient to abolish any specific reaction of the rats. Therefore, all attempts to isolate the pheromones should be carried out at a pH in the order of native urine as suggested by Dehnhard and Claus (1996). An assumed function of a cow pheromone which is to inform bulls of imminent oestrus one day prior to mating (Dehnhard *et al.* 1991), explains the instability of the pheromones as shown by a transient shift of pH in this study. Thus, it appears that the intrinsic instability limits the information to a short stage of the cycle and thus avoids confusion. In contrast, male pheromones in various species have to provide long-lasting information on dominance and on territories (Johnston and Lee 1976) and are consequently more stable substances (Claus 1979). The mechanism of this phenomenon was explained by exhaustive studies conducted by Rasmussen and collaborators on the Asian elephant oestrus pheromones. The oestrus pheromone was identified as (Z)-7-dodecen-1-yl-acetate (Rasmussen *et al.* 1997) and is present at high concentrations in the pre-ovulatory urine. This

compound is able to elicit in males the same range of behavioural responses the pre-ovulatory urine (Rasmussen *et al.* 1982 and 1996). This molecule is a part of hundred pheromones in Lepidoptera species, which indicates a strong evolutionary convergence between animal classes. Moreover, this work shed light on the implication of carrier proteins in pheromone liberation, which occurs via conformational modifications of the protein during the hormone-controlled pH decrease of urine (Rasmussen 2001, Lazar *et al.* 2002). Most of mammal pheromones are secreted in association with carrier proteins of the lipocalin family. The best-studied system is the association in mouse urine of small volatile pheromones and Major Urinary Proteins (Hurst and Beynon, 2004). Such information is not available in farm mammals, and it is not known if oestrus pheromones are bound and released by proteins in urine.

Molecular mechanism of olfactory perception

Buck and Axel (1991) cloned and characterized 18 different members of an extremely large multigene family that encodes seven transmembrane domain proteins whose expression is restricted to the olfactory epithelium. Members of this novel gene family were supposed to encode a diverse family of odorant receptors. A family of genes encoding candidate pheromone receptors was expressed in rat vomeronasal neurons. These receptors (V1R) consisted of seven transmembrane receptors. Individual V1R genes are composed of 1,000 nucleotides without introns and are expressed in vomeronasal sensory neurons whose cell bodies are located in the apical part of the VNO epithelium (Dulac and Axel 1995). Another type of pheromone receptor, V2R was reported simultaneously by three research groups (Herrada and Dulac 1997, Matsunami and Buck 1997, Ryba and Trinidelli 1997).

In rodents, two super families of seven transmembrane G protein-coupled receptors, V1Rs and V2Rs, serve as pheromone receptors (Dulac and Torello 2003). These cells also express a G protein subunit named G α 2. In contrast, the multiexon V2R genes are characterized by a long, highly variable N-terminal domain and are coexpressed with G α 0 in sensory neurons whose cell bodies are basally located. V2Rs are related to the Ca²⁺ sensing receptor and metabotropic glutamate receptors. V2Rs are expressed at high levels in small subpopulations of VNO neurons. V2Rs are primarily expressed in a different layer of VNO neurons from V1Rs, thus both gene families are likely to encode mammalian pheromone receptors (Ryba and Tirindelli 1997).

The V1R repertoires in cow and dog are substantially smaller than those in mouse and rat, which contain 187 and 102 putatively functional genes, respectively (Shi *et al.* 2005). Dog and cow have only 8 and 32 intact V1R genes respectively (Grus *et al.* 2005). Similarly Young *et al.* (2005) also found that dog, human and chimpanzee have very few intact V1Rs (8, 2, and 0 respectively). These findings were

unexpected because cows (Salazar *et al.* 2008) and dogs (Dennis *et al.* 2003) like mouse and rat, possess a functional VNO. Further, humans and chimpanzees appear to have suffered even more extreme deterioration of their vomeronasal organ and pheromone signalling components, perhaps as a result of dominant visual system. It was proposed that other gene families, such as V2Rs in the VNO or olfactory receptors in the nose, might be much more important than V1Rs in pheromone perception in dogs and other mammals (Young *et al.* 2005). It is unlikely that the small V1R repertoire could be compensated by a large V2R repertoire because all V2Rs identified from dogs and cows were pseudogenes (Grus *et al.* 2005). Furthermore, all V2R genes identified from the goat genome are pseudogenes and may not act as receptors (Wakabayashi *et al.* 2002). Since V1Rs are expressed in *Gxi2* –positive neurons and V2Rs are expressed in *Gao* –positive neurons (Mombaerts 2004) it is possible that functional V2Rs exist only in rodents and opossums among mammals. This suggests that mouse and rat may be atypical mammals in terms of their pheromone receptor genes and pheromone sensitivities.

Importance of detection of oestrus behaviour in cattle

The main problem in cattle reproductive management is the high frequency of silent and weak oestrous symptoms (Williamson *et al.* 1972, Bulman and Lamming 1978, Claus *et al.* 1983). Failure to detect oestrus or erroneous diagnosis of oestrus results in an enormous economic loss to the dairy industry worldwide. It is generally agreed that detection efficiency is <50% in most dairy herds (Bozworth *et al.* 1972, Barr 1975). Furthermore, research using milk and blood progesterone assays indicates that between 5 and 30% of all inseminations occur in cows that are not in oestrus (Appleyard and Cook 1976, Senger 1994). The single most important problem limiting the high reproductive efficiency in the national dairy herd (cows only) of America is reported to be poor detection of oestrus (Senger 1994). This is also true for Indian Dairy herd (cows and buffalo) because silent oestrus or poor expression of oestrus is more common in buffaloes (Awasthi *et al.* 2007, Perera 2011). As individual dairy herd increased in size, the problem of poor detection of oestrus has amplified due to a decrease in manpower input. There is an urgent need to develop and to apply new technologies that will provide highly effective automated methods for identifying cows and buffaloes in oestrus, thus eliminating the need for visual observation and marginally effective facilitators for detection (Senger 1994, Singh *et al.* 2000). The success of any new technology for oestrus detection depends on three factors: 1) the technology must solve the problem at a very high rate of effectiveness 2) the technology must be genuinely cost effective and contribute to increased profitability of the dairy enterprises and 3) the technology must make life easier for the management team. Technology that satisfies these three requirements will make

a long-term positive impact worldwide.

One interesting way of oestrus marker detection is the possibility to use trained detector animals such as dogs or rats (Kiddy and Mitchell 1981, Ladewig and Hart 1981, Dehnhard and Claus 1988, Rampin *et al.* 2006) to detect cycle-dependent odour differences in vaginal secretions and in urine of cows. For example, acetaldehyde is one compound associated with oestrus that the dogs might be able to detect by smelling. Published studies on blood (Klemm *et al.* 1994), vaginal secretions (Ma *et al.* 1995), and milk (Weidong *et al.* 1997) indicated that concentration of acetaldehyde increased prior to oestrus. Contrary to these results, Dehnhard and Claus (1988) observed that several classes of chemical substances or even individual compounds from urine and cervico-vaginal mucus, which were suggested as candidates for the cow pheromone (Nishimura *et al.* 1984, Preti 1984, Hradekcky 1986, Klemm *et al.* 1987) did not alter the sexual behaviour of trained rats. These results demonstrate that choice of the molecule as oestrus marker in such bioassays is of crucial importance for the efficiency of oestrus detection.

The use of artificial olfaction could enable more accurate detection of oestrus and has the potential to increase fertility in cows. Changes in perineal odour as oestrus marker were monitored through the use of an electronic nose (Lane and Wathes 1998). Twelve conducting polymer sensors were used to quantify odours in terms of a change in sensor resistance. Oestrus was identified using ovarian ultrasound, behavioural observations, and plasma assay for progesterone and oestradiol. Samples were taken from the dorsal lateral perineal (perivulval) area using cotton bud swabs and presented to an electric nose. It was revealed that odour signals between the luteal phase and oestrus could be distinguished for a group of five cows. In another experiment, samples were obtained daily from eight cows during the midluteal phase and from day 2 to day 8 of the cycle (day 0 as day of oestrus, induced with cloprostenol). Of the 12 sensors, 7 showed a significant change in resistance that was dependent on the day of the oestrous cycle. Basal values were those taken in the luteal phase; values peaked on day 1, rose transiently on day 3, and returned to baseline on days 5 to 6. The pattern was strongly correlated with plasma oestradiol concentration.

Involvement of pheromonal cues in oestrus detection by the bull is well assessed by bioassays. Females release the olfactory molecules during oestrus, thus signalling the stage of the cycle, and stimulating sexual behaviour and endocrine function of males. Efforts were made to characterize the pheromone in milk, urine, vaginal mucus, serum and skin gland secretion during the different phases of oestrous cycle but the chemical nature of pheromone components is still unknown. Failure to detect oestrus or erroneous diagnosis of oestrus results in an enormous economic loss to the dairy industry worldwide. New technologies for the solutions of this problem must be more effective than visual observation

and aids currently used to detect oestrus. Therefore, it is conceivable to use artificial noses or other biotechnologies to detect specific markers of oestrus, the pheromone components and first to gain knowledge on the chemical nature of oestrus-indicating pheromones. Moreover, oestrus-related chemicals might also be useful as sexual stimulants for increasing the libido of unmotivated males. These techniques, based on a better and more natural utilisation of animal behaviour within their environment may contribute to lower and/or suppress the use of exogenous compounds and may then be more acceptable by the consumer. Moreover, as they will facilitate farmer's work and reduce manpower needs related to heat detection, these bio techniques should greatly contribute to the development of sustainable production systems integrating the new tools for multiple character genetic selection.

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