Pheromones for Modulating Reproduction in Cattle

Biological Potential of Oestrus-related Substances in Intra- and Inter-species Chemical Communication

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Pheromones for Modulating Reproduction in Cattle. Biological Potential of Oestrus-related Substances in Intra- and Inter-species Chemical Communication

Abstract

Declining dairy cow fertility, largely attributable to increasing milk yields, is of major concern to dairy farmers worldwide. New tools for reproductive management, to replace exogenous hormones, would be of great interest. Chemical communication in cattle has been suggested to cause modifications of the oestrous cycle. Here, reproductive parameters were monitored in ten dairy heifers during five oestrous cycles. They were exposed to distilled water (control) or oestrous urine and vaginal mucus (treatment). Four of them were also subjected to intensive blood sampling to study the treatment effect on the LH pulsatility pattern (LHP) preceding the preovulatory LH surge (LHS).We found that the treatment had significant effects on when the different signs of oestrus occurred and on the intensity of oestrus expression. There was a tendency for treatment to have an effect on LHS characteristics whereas LHP was significantly affected by the treatment. To find a quick bioassay to detect bioactive bovine body fluids, two heifers and two bulls were exposed to different body fluids while their heart rates were registered. Although the exposure caused significant effects, they were not of sufficient magnitude to be useful. Further attempts to find a bioassay to identify oestrus-specific compounds included using the face fly as a biological detector. Female flies were allowed to choose between either oestrous (OU) or luteal urine (LU) and distilled water (control) in a Y-tube olfactometer. Flies were significantly repelled by OU, but not by LU. Gas chromatography coupled with electroantennographic detection (GC-EAD) revealed no behaviourally active, oestrus-specific compounds. Comparison of chromatograms, however, showed that cetyl alcohol was more abundant in OU than in LU. When tested at different doses, the lowest dose of cetyl alcohol was found to attract flies, while the second lowest dose was repellent.

We found effects of exposure to body fluids on cyclicity in heifers, as well as on heart rate in cattle, which supports the existence of bovine pheromones. We also found that the face fly could discriminate between OU and LU, probably based on the amount of cetyl alcohol in the samples. Neither monitoring of heart rate, nor using the face fly as a biological detector, were suitable bioassays for detection of bovine pheromones.

Keywords: Pheromone, cattle, oestrous synchrony, biological detector, face fly

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Dedication

To those who came before and to those who will follow

Ne te lave pas, j'arrive!
Napoléon Bonaparte

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I K. Nordéus, R. Båge, H. Gustafsson, P. Humblot, L. Söderquist (2012). The influence of oestrous substances on cyclicity and oestrous behaviour in dairy heifers. *Acta Veterinaria Scandinavica* 54:26 doi: 10.1186/1751-0147-54-26 (in press).

II K. Nordéus, R. Båge, H. Gustafsson, L. Söderquist (2012). Changes in LH pulsatility profiles in dairy heifers during exposure to oestrous urine and vaginal mucus. *Reproduction in Domestic Animals* doi: 10.1111/j.1439-0531.2012.01997.x.

III K. K. Nordéus, E. Jergil, R. Båge, N. Lundeheim, F. Hultén, L. Söderquist (2012). The effects of pheromones on heart rate in bulls and heifers. *Veterinary Record* doi: 10.1136/vr.100583.

IV K. Nordéus, R. Glinwood, B. Webster, R. Ignell, G. Birgersson, T. Lindblom, R. Båge, L. Söderquist. Using the female face fly (*Musca autumnalis*) as a biological detector to identify oestrus-specific volatile compounds in cow urine. *Manuscript.*

Kristina Nordéus' contribution to the papers included in this thesis was as follows:

I All experimental work in this study, except for extra help during intensive sampling periods, as well as preparation of the data set for statistical analysis and draft of the manuscript, with the exception of blood sample analyses and statistical analysis.

II All experimental work in this study, except for extra help during the intensive sampling periods, as well as preparation of the data set for statistical analysis and draft of the manuscript, with the exception of blood sample analyses as well as statistical analysis and illustrations.

III Part of the experimental work on all animals, preparation of the heifer data set for statistical analysis and draft of the manuscript, with the exception of preparation of the bull data set and statistical analysis.

IV All Y-tube behavioural work, with the exception of the cetyl alcohol dose-response behavioural assay, as well as the collection of oestrous substances and volatiles and part of the work with GC-EAD, not including the identification of compounds. Preparation of the data set for statistical analysis and draft of the manuscript, with the exception of some of the statistical analysis.

Abbreviations

AI	Artificial insemination
AOB	Accessory olfactory bulb
AOS	Accessory olfactory system
BCS	Body condition score
BPM	Beats per minute
CL	Corpus luteum
EAD	Electroantennographic detection
ET	Embryo transfer
GC	Gas chromatography
GC-EAD	Gas chromatography coupled with electroantennographic detection
GC-EPG	Gas chromatography coupled with electropalpogram recording
GC-MS	Gas chromatography coupled with mass spectrometry
GC-SSR	Gas chromatography coupled with single sensillum recording
GnRH	Gonadotrophin releasing hormone
LH	Luteinising hormone
LHP	LH peak
LHS	LH surge
LU	Luteal phase urine
LSM	Least squares means
MHC	Major histocompatibility complex
MOB	Main olfactory bulb
MOE	Main olfactory epithelium
MOS	Main olfactory system
O17β	Oestradiol 17β
OBP	Odour binding protein
OU	Oestrous urine
P_4	Progesterone
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
RIA	Radioimmunoassay

SD	Standard deviation
SEM	Standard error of the mean
TAI	Timed artificial insemination
VNO	Vomeronasal organ

1 Introduction

1.1 Background

During the 20th century, dairy farming underwent great changes, developing into a regular industry. Since the 1950s milk yields have increased vastly, mainly through improved management and extensive breeding programs. In Sweden, just like in most industrialised countries, this development has led to profound changes which are still ongoing. At present 84% of all cows in Sweden are registered in the official milking scheme. During the first decade of this century the number of herds within the scheme decreased from 9 115 to 4 302, while the number of herds with 75 cows or more increased from 670 to 1 097, so the trend is towards fewer but larger farms. In the year 2000, the mean yield of energy corrected milk per cow and year for these cows was 8 612 kg compared to 9 468 kg in 2010 (*Cattle statistics 2011*, 2011). Approximately half of this increase can be ascribed to genetic progress (Pryce, 2001). At the same time decreasing fertility rates is a growing concern to dairy farmers. The final two decades of the last century saw calving rates to first service falling annually by 0.5 and 1 % in the United States (Beam & Butler, 1999; Butler & Smith, 1989) and the United Kingdom (Royal *et al.*, 2000), respectively, and the same trend was also observed in other developed countries (Lucy, 2001). The declining reproductive performance is partly attributable to metabolic stress induced by milk production (Dobson *et al.*, 2007) and partly to the negative genetic correlation that exists between milk yield and fertility (Pryce *et al.*, 2004).

One of the underlying mechanisms of the decreased fertility rates is depressed dairy cow oestrous behaviour, which makes it difficult for farmers to determine the optimal time for artificial insemination (AI) (Dobson *et al.*, 2008). There are methods, such as timed artificial insemination (TAI), to optimise breeding and circumvent the need for time-consuming oestrus detections, but these methods include the administration of several exogenous

hormones and are consequently not used in some European countries, such as Sweden. Hence, there is a need for new reproductive management tools. Being able to manipulate oestrous cycles, enhance oestrous behaviour or accurately determine when a cow is in oestrus with environmentally sustainable, labour-efficient, cheap techniques would be of great interest to farmers and consumers and could possibly be achieved through the use of synthetic pheromones.

1.2 Dairy cow fertility

1.2.1 Costs associated with decreased fertility

The extent of the decline in fertility is significant and brings substantial economic losses to the dairy industry. Several studies have focused on the cost of declining reproductive performance. According to the ”minimum value of fertility” estimated by Boichard (1990) an annual 1 % drop in conception rate per cow leads to a cost of between €1.50 and €3. This span depends on the prevailing conditions, e.g. average fertility level, replacement costs and culling value, which affect the final outcome. Seegers (2006) reviewed studies aimed at estimating fertility costs and found that the effects of a prolonged calving interval ranged from positive, i.e. giving the farmer a profit, to negative, costing up to €5 per day.

1.2.2 Endocrinology of the oestrous cycle

The hypothalamus secretes gonadotrophin-releasing hormone (GnRH), which affects the release of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. A lower GnRH pulse frequency favours FSH and a higher frequency favours LH (Sharma *et al.*, 2012; Bliss *et al.*, 2010).

A schematic overview of changes in the ovaries and in reproductive hormones during the bovine oestrous cycle is presented in Figure 1. FSH surges result in the emergence of follicular waves and follicular growth, whereas the LH surge induces ovulation. Activins, inhibins and follistatins are important regulators of FSH production (Sharma *et al.*, 2012). Other regulators are oestradiol, produced by growing follicles, which has a positive effect on the pulse frequency of the GnRH-secretion, and progesterone, produced by the corpus luteum (CL) , which suppresses pulse frequency (Noakes *et al.*, 2009).

The cohort of emerging follicles typically consists of 5-20 small follicles which are dependent on FSH to maintain their growth. In each follicular wave, the largest healthy follicle will be selected and become dominant. With increasing size, the follicular fluid concentrations of oestradiol and inhibin increase, resulting in a negative feedback on FSH secretion. The dominant

follicle, however, has already acquired LH responsiveness, ensuring its continued growth (Forde *et al.*, 2011).

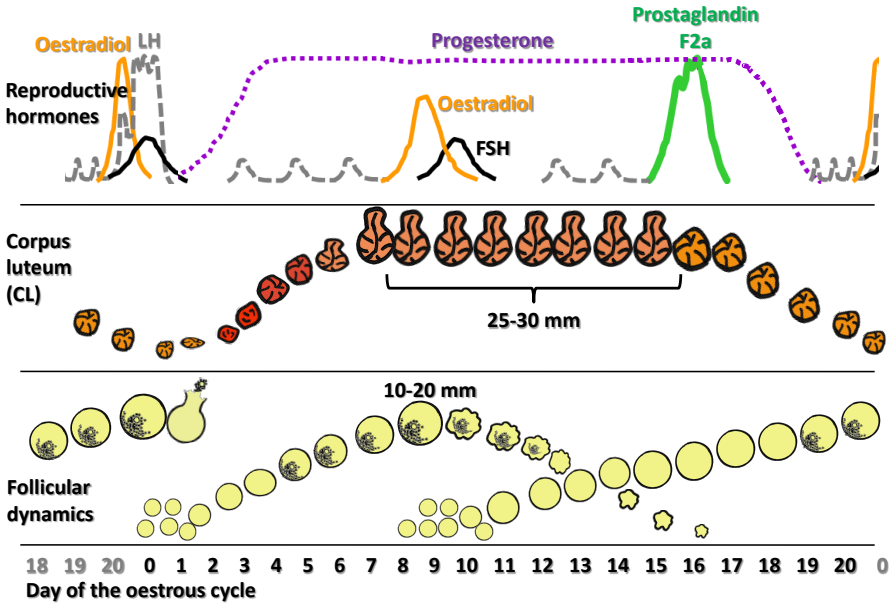

Figure 1. Schematic overview of changes in the ovaries and in reproductive hormones during a normal bovine oestrous cycle with two follicular waves. Illustration by Sudsaijai Petyim, modified by the author.

During the luteal phase, progesterone secreted by the corpus luteum suppresses LH secretion, which prevents the dominant follicle from maturing into an ovulatory follicle (Noakes *et al.*, 2009). Instead, the dominant follicle will go into atresia, which allows FSH to be secreted again and another wave to emerge. When progesterone levels are low, as during the follicular phase, increasing oestradiol levels will cause oestrous behaviour and induce a preovulatory LH surge from the surge center, leading to ovulation.

After ovulation, a corpus luteum (CL) is formed through luteinisation of the ovulatory follicle. The CL produces progesterone, which allows for maintenance of a potential pregnancy. If an embryo fails to secrete enough interferon tau to signal its presence around day 16 of the oestrous cycle, the CL will regress and another follicular phase will occur (Forde *et al.*, 2011).

McCracken *et al.* (1999) proposed a model for the neuroendocrine control of luteolysis in sheep which, given the similarities between the two species, probably also applies to cattle. The decreasing effect of progesterone towards the end of the luteal phase allows a rise in oestradiol concentrations, which stimulate release of oxytocin from the hypothalamic pulse generator. This will

lead to an upregulation of oxytocin receptors in the endometrium causing a release of low levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus. These low levels of $PGF_{2\alpha}$ are enough to trigger a much greater release of oxytocin from the CL, which induces a greater release of $PGF_{2\alpha}$ from the uterus, enough to cause luteolysis.

The functional regression of the CL is rapid, while the structural regression is much slower (Noakes *et al.*, 2009).

1.2.3 Duration of oestrus and oestrus detection

The high concentrations of oestradiol produced by the dominant follicle concomittantly with low levels of progesterone induce behavioural oestrus, during which the animal is receptive to mating, and a surge in LH that allows final maturation and ovulation of the dominant follicle (Forde *et al.*, 2011). Detection of oestrus is a prerequisite for successful artificial insemination and therefore is of utmost importance in the dairy industry, where the vast majority of cows are inseminated. A shorter and weaker display of oestrus is considered to be an important factor contributing to poor fertility rates. Diskin & Sreenan (2000), summarising results from studies on the duration of oestrus in dairy cows and heifers, found that while the duration of oestrus in heifers had remained at approximately 15 h between 1948 and 2000, it had decreased substantially in dairy cows, from 17.8 h in 1948 to 7.1 h in 1998. This, of course, causes poorer oestrus detection rates. In American Holstein herds, studied between 1985 and 1999, oestrus detection rates fell from 50.9 % to 41.5 %. In Sweden, when comparing herd data collected during the 1980s and 1990s to earlier studies, the interval from calving to the first ovulatory oestrus had increased from 47 to 61.3 days, whilst that from calving to commencement of luteal activity remained the same, indicating that the underlying reason for the increased interval between calving and first insemination may be difficulties in detecting oestrus (Petersson *et al.*, 2006).

Although results have been ambiguous (Wiltbank *et al.*, 2006), duration of oestrus and number of standing events seem to be lower in high producing dairy cows than in low producing cows (Lopez *et al.*, 2004). Even though pre-ovulatory follicles were larger in the high producers, the serum concentration of oestradiol was lower than in the low producing animals (Lopez *et al.*, 2004). A possible explanation for the lower level of oestradiol in high producers could be that they have an increased metabolism of steroid hormones, due to increased feed intake and liver blood flow (Wiltbank *et al.*, 2006).

Needless to say, this reduced ability of dairy cows to display standing oestrus makes it even more important to have efficient methods for detection of oestrus. Methods of oestrus detection and their efficiency are reviewed below.

Visual oestrus detection

Even though many different tools have been developed to help farmers determine the optimal time for insemination, the most important tool is still observing the animals visually, both as a group and as individuals. Traditionally the am-pm rule has been used, i.e. if an animal is observed in oestrus am it should be inseminated pm and vice versa (Trimberger, 1948). The only really reliable sign of true oestrus is when the animal stands to be mounted, by a bull or by herd mates. Peralta *et al.* (2005) reported a detection rate of 49 % when visual observation was based primarily on standing to be mounted. However, even when animals are observed at very short intervals, i.e. 2-3 h, the number of oestrous periods during which standing heat is displayed can be quite low, ranging from 37% to 58% (Roelofs *et al.*, 2005; Van Eerdenburg *et al.*, 1996; VanVliet & VanEerdenburg, 1996). Hence, there is a need to include other signs of oestrus in the visual observation.

To circumvent this problem, Van Eerdenburg *et al.* (1996) developed a scoring scale for evaluating oestrous behaviour in free stall herds based on the relative importance of different signs of oestrus (Table 1). Cows were observed 12 times daily for 30 minutes and points were awarded accordingly each time a behaviour was displayed, except for restlessness, for which points could only be awarded once per observation period. When individual cows surpassed 100 points during a period of 24 hours they were considered to be in oestrus. Using this scheme resulted in an oestrus detection rate of 100 %, whereas a more realistic scheme with two observations per day rendered a rate of 74 %. However, when the scoring scale was evaluated by farmers on commercial dairy farms the detection rate was only 47 %, which was lower than during the control period, indicating that this method may be too complicated to use when incorporated into the farmers'daily work.

Table 1. *Oestrus scoring scale by Van Eerdenburg et al. (1996)*

Sign of oestrus	Points
Mucous vaginal discharge	3
Flehmen	3
Restlessness	5
Being mounted but not standing	10
Sniffing the vulva of another cow	10
Chin-resting on another cow	15
Mounting other cows	35
Mounting head side of another cow	45
Standing heat	100

Holman *et al.* (2011) compared different methods of oestrus detection and found that the detection rate of the farm staff, when observing the animals visually for 10 minutes on six different occasions during the day, was 57 %. The visual detection was based on standing to be mounted as well as other behavioural signs of oestrus. According to Firk *et al.* (2002) oestrus detection rates reported for visual observation during the 1990s ranged between 50 and 80 %. Roelofs (2005) found that oestrus detection rates could be vastly improved by increasing the number of observations and by including other signs than stand to be mounted (Table 2).

Table 2. *Detection rates (%) for different oestrous signs and frequencies of 30 minutes-observations by Roelofs (2005)*

	2 times daily	3 times daily	8 times daily
Standing heat	19	30	57
Mounting behaviour	48	61	89
All behavioural signs[a]	77	90	100

[a]At least 50 points scored according to the scoring scale of Van Eerdenburg *et al*. (1996)

Measuring physical activity

Daily physical activity, as measured by pedometers, increases significantly during oestrus (Kiddy, 1977) and can be used for oestrus detection. The oestrus detection rate ranges between 49 % and 100 %, but is commonly over 80 % (Roelofs *et al.*, 2010). Physical activity can also be measured by using transponders attached to neck collars. A detection rate of 37 % has been reported for the neck collars in the Alpro system (Peralta *et al.*, 2005) and a more recent study reported detection rates of 59 % for neck collars and 63 % for pedometers (Holman *et al.*, 2011). However, the pedometer had a much higher number of false positives.

Mount detectors

There are different types of mount detection devices, ranging from simpler methods such as tail paint, scratchcards or KaMar, to more advanced systems such as electronic mount detectors or sterilised teaser bulls that may or may not be fitted with a marking devise. Although the first three methods are cheap and easy to use, the detection rates vary. For tail paint, detection rates ranging from under 50 % to over 85 % have been reported (Roelofs *et al.*, 2010) and, according to a recent study (Holman *et al.*, 2011), detection rates for scratchcards and KaMar were 36 % and 57 %, respectively, with the KaMar renderering more false positives. Peralta *et al.* (2005) reported a detection rate of 48 % for HeatWatch, an electronic mount detector. There are major practical

disadvantages with using a vasectomised bull, such as increased risks for the farm staff as well as for spreading genital infections.

In-line progesterone measurements

Friggens *et al.* (2008) tested a model developed for determining reproductive status from in-line milk progesterone and found that 99 % of oestruses that resulted in a pregnancy could be detected by using this system. Although in-line measurements can be very accurate, the system is very expensive and is consequently not a possible solution for small- and medium-sized herds.

Combining methods

Even if the individual methods of oestrus detection are inadequate, combinations of techniques can increase the rate of oestrus detection. Peralta *et al.* (2005) found that combining visual observation with data from neck collars and an electronic mount detector rendered an oestrus detection rate of 80 %, even though the individual methods each had a detection rate of under 50 %. Holman *et al.* (2011) compared oestrus detection rates for visual observation, pedometers, neck collars, scratchcards and KaMar, and also combinations thereof, and found that only 74 % of all oestrous events were identified using one or more methods. The oestrus detection rate for each individual method was about 60%, except for the scratchcard, which had a much lower rate. Combining the methods did not significantly increase detection rates.

Hormonal synchronisation

Synchronising oestrous cycles in a herd is beneficial to the farmer in many ways. Firstly, it allows the farmer to use labour and housing resources more efficiently, by being able to perform oestrus detections or monitor calvings during concentrated time periods. If TAI is used, it also renders the time-consuming work of oestrus detections superfluous. TAI is a systematic breeding strategy for which many different protocols have been suggested, but the basic ideas is to induce oestrus through a series of hormonal treatments, after which AI is performed at a given time. TAI without oestrus detection has been found to be more efficient than traditional breeding strategies using the am-pm rule since it reduced the median days to conception by 19 days, while maintaining pregnancy rates (Pursley *et al.*, 1997). There is also an additive effect of having more animals in oestrus at the same time. The intensity of oestrous behaviour, measured as the number of mounts, increases with the number of animals simultaneously in oestrus (Helmer & Britt, 1985; Hurnik *et al.*, 1975), facilitating detection of animals in, or approaching, oestrus.

In Sweden, consumers do not favour treating healthy animals with exogenous hormones. As a consequence, the Swedish Dairy Association has adopted a policy against these hormonal treatments, with an exception for embryo transfer.

1.2.4 Future needs

In the Scandinavian countries, recording systems, including fertility and health traits, have existed since the 1960s (Philipsson & Lindhe, 2003) and fertility has been included in the breeding evaluation scheme for several decades. Even though heritabilities for the reproductive traits included are quite low (Janson, 1980), this strategy may explain why the Swedish Red Breed (SRB) does not display the same negative fertility trend as the Swedish Holstein (SH), for which semen from North American bulls has been used extensively (Philipsson & Lindhe, 2003). Today, many countries have included reproductive traits in their breeding programs, new traits with higher heritabilities have been suggested (Petersson *et al.*, 2006), and some breeding companies use genomic breeding values (GEBV) (Hayes *et al.*, 2009) to reduce the extent of progeny testing and shorten generation intervals (Schrooten *et al.*, 2005). Crossbreeding Holstein with more fertile breeds, such as SRB, is another strategy used to improve the poor fertility of pure-bred Holstein cattle (Heins *et al.*, 2006). However, genetic progress concerning fertility is quite slow and not a practical remedy for the current acute situation. When reviewing tools for oestrus detection, Lehrer *et al.* (1992) concluded that there were aids ”more efficient, but not necessarily more accurate than visual observations”. Despite recent developments this conclusion, to some extent, remains. The trend of fewer, bigger herds, decreasing fertility rates and decreasing financial margins for the farmer clashes with the consumers’ demand for ecologically sustainable, locally produced foodstuff, grown under animal-friendly conditions. This underlines the urgent need for alternative, more natural methods to manage reproduction efficiently in cattle.

1.3 Chemical signalling

The term semiochemicals comprises chemical substances used for communication between or within species. These categories are not mutually exclusive (Brown *et al.*, 1970) as signals for intra-species communication can be overheard or mimicked by another species, i.e. inter-species communication.

1.3.1 Inter-species communication

Semiochemicals used interspecifically can be grouped according to who benefits from the communication. Allomones are substances that induce a behavioural or physiological response in the receiver, which is beneficial to the transmitter, e.g. plant volatiles that deter herbivores. Kairomones, on the other hand, are substances for which the response is beneficial to the receiver but not the emitter, e.g. the scent of prey animals being beneficial for the predator (Brown *et al.*, 1970). Finally, synomones are chemical substances that evoke a response in the receiver that is beneficial to both transmitter and receiver, e.g. floral scent that attracts insects that feed on the nectar and transmit pollen to other plants (Nordlund & Lewis, 1976).

1.3.2 Intra-species communication

Pheromones are substances used for communication between individuals of the same species. The word pheromone derives from the Greek words *pherein*, to transfer, and *hormōn*, to excite (Karlson & Luscher, 1959). In other words, pheromones are molecules that carry something exciting. They are grouped, either as releaser or primer pheromones, according to their function. Releaser pheromones induce a direct behavioural effect mediated by the central nervous system, while primer pheromones cause neuroendocrine changes (Wilson & Bossert, 1963). Recently a distinction between pheromones and signature mixtures has been suggested (Wyatt, 2010). By definition, whereas a pheromone is present in all individuals of the same sex and developmental stage, a signature mixture is unique to the individual or a subgroup, can be comprised of different cues for different receivers, and also requires the receiver to learn the signal. In other words, the pheromone signal is anonymous (Hölldobler & Carlin, 1987), while the signature mixture is not (Wyatt, 2010). The specific mixture of major urinary proteins (MUPs) of mice that conveys individual identity is one example of a signature mixture (Wyatt, 2010).

1.3.3 The olfactory system

Most mammals have two olfactory systems, the main olfactory system (MOS) and the accessory olfactory system (AOS). The MOS comprises the main olfactory epithelium (MOE) of the nasal cavity, the main olfactory bulb (MOB), different forebrain nuclei and the neurons that connect these structures. The vomeronasal organ (VNO) and the accessory olfactory bulb (AOB) constitute the accessory olfactory system together with its interconnecting neurons and forebrain nuclei (Figure 2) (Swaney & Keverne, 2009).

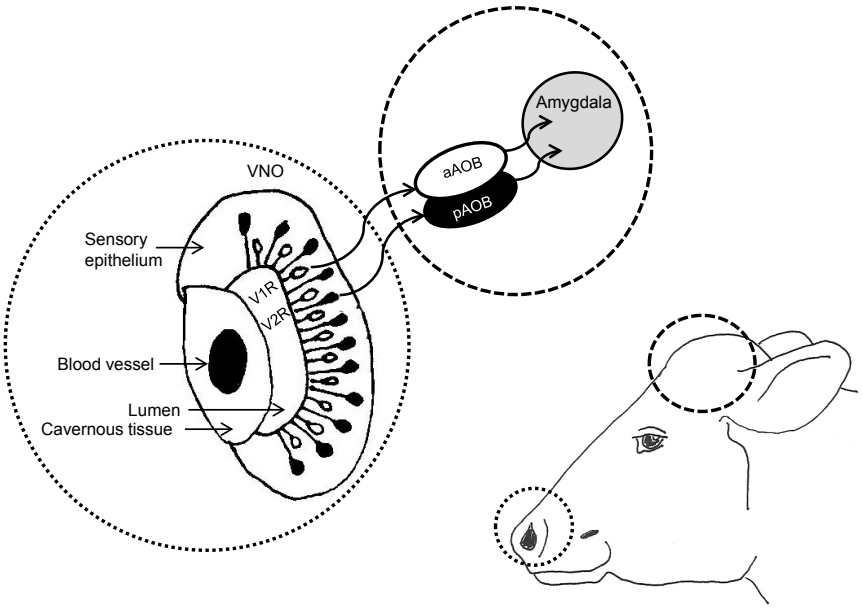

Figure 2. The accessory olfactory system consists of the vomeronasal organ (VNO), the accessory olfactory bulb (AOB), forebrain nuclei and interconnecting neurons. The VNO is located in the nasal septa (dotted line) and the other structures in the brain (dashed line). The sensory epithelium of the VNO contains two distinct receptor types, V1Rs and V2Rs. The information from V1Rs is processed in the anterior AOB (aAOB) while that of V2Rs is processed in the posterior AOB (pAOB). The information then converges in overlapping projections to the bed nucleus of the stria terminalis and the amygdala. Modified by the author after Brennan & Keverne (2004).

The VNO is embedded in the hard palate at the junction with the nasal septum and consists of two fluid-filled parallel ducts that caudally end blindly and rostrally open into the incisive ducts, thus communicating with both the nasal and the oral cavities (Swaney & Keverne, 2009; Doving & Trotier, 1998; Dyce *et al.*, 1996). Since the VNO is encapsulated in bone it is not subjected to the airflow passing over the MOE. Instead a pumping mechanism, achieved through vasoconstriction and vasodilation of surrounding vessels, allows stimuli to access the organ. Flehmen (Figure 3), a behaviour common to both ungulates and carnivores, where the head is lifted, the lips drawn back and the tongue pressed against the palate, is commonly believed to allow the transfer of chemical signals into the VNO (Tirindelli *et al.*, 2009). Indeed it has been shown that when guineapigs were allowed access to conspecific urine containing non-volatile dye, the dye could be found in the VNO, but not the MOE (Wysocki *et al.*, 1980).

Figure 3. Flehmen behaviour displayed by a dairy heifer. Photograph by R. Båge.

The sensory epithelium of the VNO contains two different types of receptors, the V1Rs and the V2Rs. The V1Rs are found in the apical layer of the VNO and also of the MOE: the ligands for this receptor type are small volatile molecules and sulphated steroids. The V2Rs are found in the basal layer of the VNO and bind to peptides, such as MHC (major histocompatibility complex) peptides, major urinary proteins (MUPs) and sulphated steroids (Kaupp, 2010). The number of V1R and V2R genes varies among species. The cow has 40 intact V1R genes and 45 disrupted genes, i.e. pseudogenes, compared to the 187 intact genes of the mouse (Shi & Zhang, 2007). During evolution, ancestors to the cow lost the entire repertoire of V2Rs and the cow now has 0 intact genes and 16 pseudogenes, compared to the 121 intact genes of the mouse (Young & Trask, 2007).

The traditional view of the two olfactory systems has been that the MOS processes ordinary volatiles, while the AOS is responsible for processing non-volatile pheromones (Swaney & Keverne, 2009; Rekwot *et al.*, 2001; Buck, 2000). However, it is now clear that pheromones may be volatile and that both systems can respond to volatiles as well as to non-volatiles (Swaney & Keverne, 2009; Baxi *et al.*, 2006; Brennan & Keverne, 2004). The boar pheromone androstenone and the rabbit nipple-search pheromone are examples of volatile pheromones that are processed by the MOS (Swaney & Keverne, 2009; Baxi *et al.*, 2006).

Lipocalins are proteins that carry volatile pheromones in order to facilitate transport in aqueous media, but they may also act as pheromones by themselves, as has been suggested for aphrodisin, an aphrodisiac found in the vaginal discharge of female golden hamsters (*Mesocricetus auratus*), and for MUPs in male mice urine. The question whether these lipocalins are just carriers of pheromones or if they themselves have a pheromonal function has not, however, been definitely answered (Tirindelli *et al.*, 2009; Brennan & Keverne, 2004). As mentioned before, individual MUP profiles also convey information about identity (Brennan & Kendrick, 2006).

Odour binding proteins (OBPs) are another example of soluble proteins of the lipocalin superfamily found in the mucus layer lining the olfactory epithelium (Mitchell *et al.*, 2011; Pelosi, 1998). They interact with odour molecules and pheromones, but whether they act as mere carriers of hydrophobic molecules or if they are a part of the chemical message has not been established (Mitchell *et al.*, 2011; Pelosi, 1996). Other functions have also been suggested for OBPs, such as scavenging odorants (Vincent *et al.*, 2004) and protecting the airways (Mitchell *et al.*, 2011). 1-octen-3-ol has been found to be the natural ligand of bovine OBP. Since 1-octen-3-ol is an important component of bovine breath and a very potent attractant for many insects, this indicates that one role of bovine OBP may be to clear the breath of 1-octen-3-ol to reduce the attraction of insects (Ramoni *et al.*, 2001).

1.3.4 Mammalian pheromones

Pheromones affect reproduction in many mammals, including domestic animals and human beings. Studies have shown that they may, among other things, induce attraction to the opposite sex and inter-male aggressiveness, accelerate puberty, shorten periods of anoestrus, modify oestrous cycles, elicit mating behaviour in both sexes, block pregnancies and induce the search for, and grasping of, the nipple in offspring (for review see Tirindelli *et al.*, 2009 and Aron, 1979).

1.3.5 Bovine pheromones

Male-to-female communication

Great attention has been paid to the influence of the male on the female in cattle. Izard & Vandenbergh (1982a) found that prepubertal heifers recieving oronasal treatments with bull urine reached puberty earlier than heifers receiving control treatment with water. The urine-treated heifers calved earlier and had a shorter calving season than the control heifers, but pregnancy rates were the same for the two groups.

This biostimulatory effect on ovarian activity has also been demonstrated in zebu cows (Rekwot *et al.*, 2000) and in suckled beef cows (Landaeta-Hernández *et al.*, 2004; Custer *et al.*, 1990; Zalesky *et al.*, 1984), where bull-exposure shortened the postpartum anoestrus. It was further supported by Berardinelli & Joshi (2005b), who found that exposure to bulls, or their excretory products, hastened resumption of luteal activity after calving. However, there have also been reports suggesting no positive effect of bull-exposure on postpartum anoestrus (Larson *et al.*, 1994).

The biostimulatory effect seems to be greater when bulls are exchanged during the course of the experiment, compared to continuous exposure to the

same bulls (Miller & Ungerfeld, 2008). There seems to be an inverse linear relationship between the daily duration of bull exposure and the intervals from either calving or the start of bull exposure to resumption of ovarian activity (Tauck *et al.*, 2010a), with longer daily exposure resulting in a shorter interval. There may also be a more rapid return to cycling activity when cows are exposed later in the postpartum anoestrous period (Berardinelli & Joshi, 2005a). This difference, however, was not seen by Fernandez *et al.* (1993).

The response to male stimuli can be modified by nutritional conditions. Monje *et al.* (1992) found that cows fed above their energy requirements showed a greater response than those fed below their requirements. Stumpf *et al.* (1992), however, demonstrated that the male effect was greater in cows subjected to a low dietary regimen preceding calving than in those subjected to a high dietary regimen. The explanation provided for the discrepancy between this and previous studies was that the exposure to males for cows with lesser body condition score (BCS) may help to overcome the inhibition of LH secretion caused by nutritional deficiencies, making the male effect more substantial than in well-fed cows, whereas for cows in deeper negative energy balance the male effect was not enough to overcome this inhibition.

Contrary to Custer *et al.* (1990), who did not see any effect of bull-exposure on LH characteristics, Tauck *et al.* (2010b) found that the LH pulse frequency tended to be greater in anoestrous suckled beef cows exposed to fence-line contact with bulls. The exposure also resulted in a lower cortisol pulse frequency.

Tauck & Berardinelli (2007) demonstrated that AI pregnancy rates for cows inseminated 12 h after oestrus were greater for suckled beef cows exposed to bulls or their excretory products, than for fence-line contact with bulls, when using a progestin-based protocol for synchronisation of oestrus. TAI pregnancy rates, however, did not differ between bull-exposed cows and unexposed cows. The opposite was seen when using a GnRH-$PGF_{2\alpha}$ protocol (Berardinelli *et al.*, 2007).

Even though the definition of the term ”pheromone” implies intraspecificity, there have been claims that exposure to the boar sex pheromone, androstenone, may have a positive effect on reproduction in cows (Sokolov *et al.*, 1995).

Female-to-male communication

Bull bioassays have been frequently used in attempts to isolate and identify oestrus-specific odors. Many different behavioural parameters assignable to pericopulatory behaviour, such as flehmen, sniffing, licking, erection and mounting, have been evaluated but with varying success.

Recording the preference of bulls, either as total time spent adjacent to heifers or as number of flehmen towards heifers, for either oestrous or dioestrous heifers in a pairwise choice-test, indicated that bulls do not show olfactory preference for heifers in oestrus (Geary *et al.*, 1991). Contrary to these findings, several studies indicate that bulls are able to discriminate between oestrous and non-oestrous odour (Paleologou, 1977; Hart *et al.*, 1946).

Sankar & Archunan (2004) found that the duration of flehmen behaviour displayed by bulls was greater during exposure to oestrous samples of vaginal mucus, saliva, faeces and milk smeared on to the genital region of non-oestrous cows, than during exposure to samples collected during other oestrous cycle stages. Among the substances tested, the response to oestrous vaginal mucus was significantly higher than for the other substances.

When exposing dairy bulls to urine from cows in various stages of cyclicity in a bowl, Houpt *et al.* (1989) found that the number and duration of flehmen, but not sniffing and licking, were parameters suitable to discriminate between oestrous and non-oestrous urine. The flehmen response to vaginal mucus, however, did not differ from that to water. Interestingly enough, only seven of the 15 bulls tested responded to oestrous urine with two flehmen or more, which was the criterion for inclusion in the study. One explanation given for this finding was that sexual experience with females may be important in order for bulls to respond to oestrous cues properly. Indeed, Presicce *et al.* (1993) found that bulls from a stud farm, with no prior contact with female cattle, displayed a stronger pericopulatory behaviour to urine collected from teaser bulls, than to compounds from oestrous blood.

Klemm *et al.* (1987) and Rivard & Klemm (1989) also presented their samples to bulls in a dish. The substances studied were vaginal mucus (Rivard & Klemm, 1989; Klemm *et al.*, 1987), and serum and vulval skin gland secretions (Rivard & Klemm, 1989). They found that all three of these body fluids from cows in oestrus evoked a series of chained behaviours, which could be divided into three cathegories (Rivard & Klemm, 1989). The attraction phase comprised initial responses, such as head orientation toward the sample, sniffing the air, moving toward the sample, sniffing the sample at close range, salivation and urination. It was followed by the detection phase, which included behaviours such as licking, tongue manipulation, hypersalivation, labored breathing, flehmen and vocalisation. During the phase of sexual preparation the bulls displayed penis protrusion, penile secretion, head butting and mounting behaviour. Only eight of 23 oestrous samples induced the complete series of behaviours.

Female-to-female communication

Studies on both dairy cows (Hurnik *et al.*, 1975) and beef cows (Floyd *et al.*, 2009) indicated that oestrous behaviour is intensified and duration of oestrus prolonged with increasing numbers of animals simultaneously in oestrus. Crowding oestrus-cycling cows also hastens postpartum resumption of luteal activity in beef cows (Berardinelli & Joshi, 2005b). In a limited study Hradecky (1989) found that oestrous cows, through the secretion of ”primary attractive odours” induce the production of ”secondary attractive odours” in pen-mates, which attract the interest of the bull, but to a lesser extent than the former. When exposing cycling dairy heifers to vaginal mucus and urine from oestrous cows following treatment with $PGF_{2\alpha}$, Izard & Vandenbergh (1982b) found that the degree of oestrous synchrony was higher than in control animals exposed to water. Nishimura *et al.*(1991) reported that dioestrous heifers smeared with their own vaginal discharge from previous cycles were mounted more than control animals treated with water. This effect, however, did not manifest when animals were smeared with oestrous vaginal discharge from another animal, indicating that the vaginal mucus may contain individual-specific volatile cues.

Chemistry

Ramesh Kumar *et al.* (2000) analysed urine from cows in different stages of cyclicity and found that 1-iodo undecane and di-n-propyl phthalate were unique to the oestrous samples. Further evidence that 1-iodo undecane is oestrus-specific was provided by Sankar & Archunan (2008). When they compared volatile profiles from faeces of cows in different stages of the reproductive cycle, they found three compounds, acetic acid, proprionic acid and 1-iodo undecane, that were specific for the oestrous phase. As a bioassay, they smeared compounds on to the genital region of non-oestrous cows. Bulls were allowed to sniff the cows for 30 minutes, with pre-copulatory behaviours and copulation being recorded. They found that the mixture of the three compounds resulted in significantly longer duration of flehmen behaviour and increased number of mounts than the individual compounds and control, indicating that these substances may be involved in the induction of mating behaviours.

The dispersion of oestrus-specific compounds in the bovine body has been demonstrated previously in swabs from the vulva and fluids from the vagina, urine, milk and blood (Rivard & Klemm, 1989; Kiddy *et al.*, 1984). Weidong *et al.* (1997) investigated the presence of oestrus-specific volatiles in milk, but did not find any compounds that were qualitatively different between stages. They did, however, find that there were significant quantitative differences and

concluded that cycle stage can be determined by analysing 15 compounds in milk. Klemm *et al.* (1994) found that blood acetaldehyde levels decreased rapidly just before, or at onset of, oestrus, and suggested that oestrus and ovulation could potentially be predicted by monitoring levels of acetaldehyde in milk, saliva, sweat or breath. Acetaldehyde was also found to be oestrus-specific in bovine vaginal secretions (Ma *et al.*, 1995). Increasing amounts were found followed by a drop in quantities 0-3 days before oestrus. Acetaldehyde has, however, been tested in a bull bioassay previously (Presicce *et al.*, 1993) and was not behaviourally active when tested as a singular component. Five oestrus-specific compounds, trimethylamine, acetic acid, phenol 4-propyl, pentanoic acid and proprionic acid, were identified in saliva (Sankar *et al.*, 2007). Results from the bioassay indicated that trimethylamine may be involved in attracting the bull to the oestrous cow.

At the end of the 90s, an attempt to develop an electronic nose to detect changes in perineal odours during oestrus was made (Lane & Wathes, 1998). The odour was quantified in terms of changes in sensor resistance by using twelve sensors consisting of polymer material. Values peaked on the day before oestrus and this pattern correlated strongly with oestradiol concentrations. The authors concluded that identification of oestrus-specific volatiles was required to improve the specificity of the system.

Several studies have focused on vaginal secretions, which are profound during oestrus. Preti (1984) patented a method for detecting bovine oestrus based on the quantification of methyl heptanol in vaginal secretion. Hradecky (1986) found that the concentration of free fatty acids in oestrous vaginal discharge increased gradually before oestrus and decreased rapidly thereafter. The concentration of free fatty acids in urine, but not in vaginal discharge, was affected by the ruminal concentration. Klemm *et al.* (1987) found nine oestrus-specific compounds in samples that had tested positively in a bull behavioural assay. These compounds included two ketones, four amines, one alcohol, one diol and one ether.

According to a study by Kiddy & Mitchell (1981), in which dogs were trained to detect oestrous samples of bovine vaginal fluids, oestrous odour emerges from day 3 before oestrus, peaks on the day of oestrus and disappears the day after. Similarly, trained rats show behavioural response towards samples collected 2 days before to 2 days after oestrus (Dehnhard *et al.*, 1991).

1.3.6 Modifications of the reproductive cycle

Exposure to conspecifics or their excreta has been shown to cause modifications of reproductive cycles in female mice (Whitten, 1956; Vanderlee & Boot, 1955), rats (McClintock, 1978), sheep (Radford & Watson, 1957) and

humans (McClintock, 1971). When female rats are housed together, inter-oestrus intervals decrease, an effect known as enhancement (McClintock, 1983). Conversely, when female mice are housed together, longer inter-oestrus intervals, or suppression, may occur (McClintock, 1983; Vanderlee & Boot, 1955). Exposing female mice to males or their excreta, on the other hand, may result in shorter cycles (Whitten, 1956).

Oestrous synchrony

Oestrous synchrony has been widely debated over the years (Schank, 2004; Schank, 2001), but there are studies to suggest that inter-female chemical communication may cause synchrony in rats (McClintock, 1978) and in humans (Stern & McClintock, 1998; McClintock, 1971).

Farmers occasionally provide anecdotal evidence of oestrous synchrony in cattle. However, since cows in general are either in postpartum or lactational anoestrus or are pregnant, and the cyclicity of heifers is often much less closely monitored, opportunities to observe such an effect scientifically in the field are scarce. Not much experimental work has been done either, but, as previously mentioned, Izard & Vandenbergh (1982b) found that ovulations in heifers exposed to oestrous cervical mucus and urine following treatment with $PGF_{2\alpha}$ were more synchronous than in unexposed animals. This effect was mainly caused by the vaginal mucus. However, although there are studies to suggest that bovine inter-female pheromones actually exist and can cause synchronisation of oestrous cycles or enhancement of oestrous behaviour, proof is still lacking.

In 1979 Aron suggested three different mechanisms by which manipulation of female cyclicity can occur. One explanation could be that the the follicular growth rate is altered. Another possible explanation is that a preovulatory LH surge is induced. Finally, the neuroendocrine structures controlling the corpus luteum may be affected.

Follicular growth (via FSH) and final maturation of the dominant follicle (via LH) as well as the LH surge are affected by the secretion of GnRH (Forde *et al.*, 2011), so effects on GnRH secretion can result in both a more rapid maturation of the follicle and an induction of the preovulatory LH surge.

Effects on LH pulse frequency (Shinohara *et al.*, 2001) as well as on the timing of the preovulatory LH surge (Stern & McClintock, 1998), have been demonstrated in women exposed to female axillary secretions. Moreover, introduction of a ram into a group of anoestrous ewes induces pulsatile secretion of LH within minutes after introduction and a preovulatory LH surge can be detected within 36 h (Signoret, 1991).

According to McCracken *et al.* (1999), the first step in luteolysis is the loss in progesterone action due to the downregulation of its receptors, which then allows higher levels of oestradiol to be secreted. Hence, an effect of pheromones directed specifically at the neuroendocrine structures controlling the luteal phase would have to include modulation of the progesterone receptors in the hypothalamus. Beyond this, luteolysis can be affected by modulations of GnRH secretion.

In conclusion, the three aforementioned mechanisms are, in reality, three aspects of the same mechanism, i.e. the manipulation of GnRH secretion, which can manifest in different effects.

1.3.7 Identification of pheromones

The first pheromone to be identified was the sex pheromone of the silk moth, *Bombyx mori* and this was done by Butenandt *et al.* (1959). Since then, chemical communication in both insects and vertebrates has been thoroughly investigated and a vast number of pheromones have been identified.

The first step in identifying a pheromone is to observe a behavioural or physiological effect that is mediated through chemical communication, and to find a bioassay that works for the species that can be used to test this effect under experimental conditions.

The next step is to collect the chemical signals of the substance used to mediate this effect and identify bioactive compounds. There are many different methods to collect chemical signals from bioactive substances, depending on the nature of the substance, or to identify bioactive compounds, depending on the organism.

Dynamic headspace analysis (air entrainment) is commonly used to collect volatiles emanating from biological samples. A stream of purified air or nitrogen is passed over a sample and then through an adsorbent polymer where volatiles are trapped. The volatiles can then be extracted by eluting the polymer with a solvent or can be desorbed thermally.

Gas chromatography (GC) is an extensively-used technique in the field of chemical communication. The gas chromatograph consists of an injector, a capillary column with a mobile and a stationary phase, which is housed in an oven, and a detector. The sample is injected on to the column, where the volatiles are transported by the carrier gas (typically hydrogen) which constitutes the mobile phase. As the oven heats up, separation of compounds is achieved depending on the boiling points of the different compounds and their interaction with the stationary phase, as well as on the temperature program and gas flow used. The retention time of a compound is the time taken for that compound to pass through the column and reach the detector. Compounds are

detected using a flame ionisation detector (FID) and a chromatogram is plotted using specialist software (Figure 4a). The magnitude of the FID response is proportional to the number of carbon atoms reaching the detector, allowing for quantification based on peak area on the chromatogram (Flanagan, 1993).

For identification, gas chromatography coupled with mass spectrometry (GC-MS) is commonly used (Wyatt, 2003). In the mass spectrometer, molecules are ionised, usually by electron impact. The molecules are bombarded with electrons, which causes ionisation and fragmentation of the molecules. The ions can then be filtered through magnetic and/or electrostatic fields and identified according to their mass to charge ratio (*m/z*). The mass spectrum of a compound consists of the molecular ion and its fragment ions and is specific for that particular compound (Figure 4b). Unknown substances can be identified by comparing the mass spectrum to reference libraries of mass spectral data (Andresen & Wise, 1986).

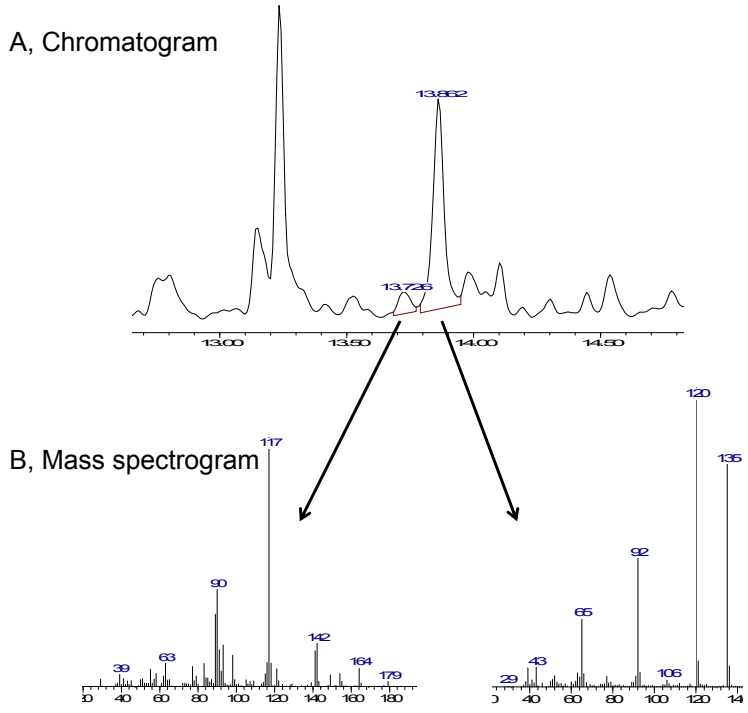

Figure 4. A, Part of a chromatogram of bovine urine. The compounds elute at different retention times. The amount of each compound in the sample is indicated by the area of the peaks. B, The most prominent ion fractions of two mass spectra for two of the peaks in the chromatogram. Illustration by the author.

Identifications are then confirmed by comparing the retention time of the compound to be identified with that of an authentic standard.

When identification has been made, the final step is to confirm identification by testing synthetic analogues to the compounds in bioassays. If the compounds are not commercially available, the analogues have to be synthesised for the purpose. If the same effect is achieved as with the original substance, the identification of the pheromone has been verified.

The effect may be mediated by the presence or absence of one or more specific compounds, i.e. a qualitative difference, or by specific quantities or relative concentrations of compounds, i.e. a quantitative difference.

Bioassays

Despite the fact that there has been a number of studies aimed at finding bovine pheromones by identifying oestrus-specific molecules in oestrous body fluids, the results have been diverse and the number of compounds suggested to be bovine pheromones is probably greater than the number of papers published on this subject. A possible explanation for this is that there are one or more oestrus-specific compounds present at very low concentrations in body fluids from all oestrous cows, but that conventional techniques are not sensitive enough to detect them. It is also possible that unsuitable bioassays were used in the experiments, resulting in false positives. Finding a quick and reliable bioassay in mammals can be quite difficult. Bioassays suitable for identification of primer pheromones are generally more difficult to find than bioassays for releaser pheromones, given that the endocrinological response may be much slower and quantifiable only with invasive techniques, such as blood sampling or biopsy.

The field of insect chemical ecology is more extensively explored than that of mammalian chemical ecology (Figure 5). Approximately 80 % of all pheromone research has been done on insects, compared to 8 % on mammals (Symonds & Elgar, 2008). Given the vast knowledge in the field of insect chemical ecology, it is important to look there for techniques that may be suitable for use also in mammals.

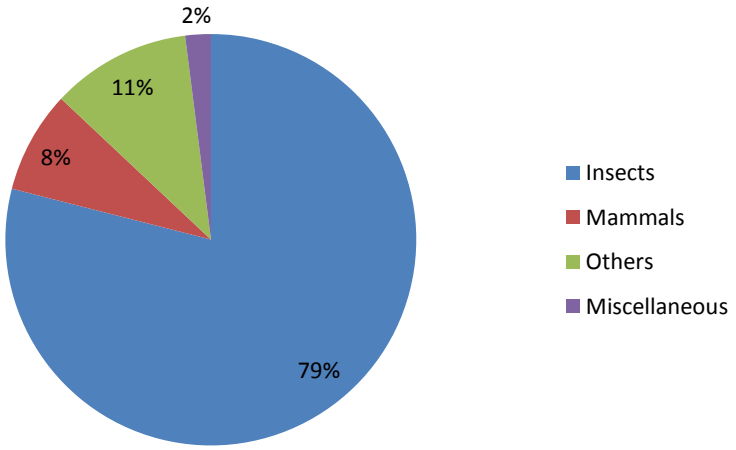

Figure 5. Pheromone research effort on different taxa. Illustration by the author, modified after Symonds & Elgar (2008). Others = taxa other than insects and mammals; Miscellaneous = unspecified from all taxa.

Apart from this, other advantages with using insects are that they often display easily quantifiable distinct behaviours, such as anemotactic movement towards an odour-sorce, and that they can be kept and used for experiments in large numbers.

Another aspect of using insects as biological detectors is that their olfactory system is extremely sensitive. It was demonstrated that a cardiac response could be triggered in the moth *Spodoptera littoralis* by less than six molecules of sex pheromone or other important olfactory cues hitting the antennae (Angioy *et al.*, 2003). This extreme sensitivity was explained as a preparation for behavioural responses at higher concentrations.

Using other animals than the studied species as biological detectors has been attempted previously. Rats (Dehnhard & Claus, 1988; Ladewig & Hart, 1981) mice (Sankar & Archunan, 2005) and dogs (Fischer-Tenhagen *et al.*, 2011; Kiddy *et al.*, 1978) have been trained successfully to identify samples from cows in oestrous. There are, however, many techniques available in insect chemical ecology, such as gas chromatography coupled with electroantennographic detection (GC-EAD) or single sensillum recording (GC-SSR), that are not possible to use on mammals, given practical and animal welfare considerations. Thus it is sometimes possible to use insects as biological detectors.

1.4 Insects as biological detectors

1.4.1 Chemical communication between insects and mammals

Hematophagous Diptera are known to use visual and olfactory cues to locate their hosts. Besides specific host volatiles, many Diptera species also respond to carbon dioxide and octenol. The host-specific odours are of greater importance for nocturnal dipterans, which use them to discriminate hosts, than for diurnal species (Gibson & Torr, 1999). Olfactory cues used in the host-seeking behaviour of tsetse flies (*Glossina* spp.) have been extensively investigated. They use both general cues, such as octenol and acetone (Paynter & Brady, 1993), and host-specific cues, such as a mixture of phenolic compounds found in cattle urine (Bursell *et al.*, 1988; Vale *et al.*, 1988).

It is known that some haematophagous Diptera display preference for certain individuals of a certain host species (Rebollar-Téllez, 2005; Jensen *et al.*, 2004; Acree *et al.*, 1968). Varying levels of attractant, as well as repellent, volatile compounds emanating from hosts have been found to influence the attractiveness to insects (Logan *et al.*, 2008; Birkett *et al.*, 2004; Acree *et al.*, 1968).

In humans, the effect of several demographic parameters, such as age, body size, skin colour, blood type, disease status and pregnancy, on attractiveness to blood-sucking insects has been demonstrated (Kelly, 2001). It has also been suggested that the attractiveness of women to mosquitoes may vary throughout the menstrual cycle. Roessler (1963) found that the maximum attractiveness of female mosquitoes (*Aedes aegypti*) to cycling women occurred on days 13, 18 and 23 after the onset of menses, which corresponds well to the peaks in oestrogen secretion during the menstrual cycle. The mosquitoes were also attracted to fractions of blood and urine containing oestrogens. There was a peak that could not be related to oestrogen secretion, but it was related to the secretion of other attractive steroids. Gilbert *et al.* (1966) found a similar, but not identical, pattern which followed cyclic endometrial changes, but the differences between stages were not statistically significant. The maximum attractiveness of cycling women was about equal to that of non-cyclic women. There are, however, confounding factors that make it difficult to investigate the influence of reproductive stage on attractiveness to blood-sucking insects. Lindsay *et al.* (2000) found that pregnant women produced more exhaled breath and had greater heat dissipation, resulting in more volatiles being given off than from non-pregnant women. It has also been shown that the regulation of body temperature is affected by the menstrual cycle, with higher temperature and a higher threshold for sweating during the luteal phase (Kolka & Stephenson, 1989), which could affect the release of volatiles.

1.4.2 Identification of semiochemicals in insects

Gas chromatography coupled with electroantennographic detection (GC-EAD) is a technique that has been widely used to identify semiochemicals in dipterans (e.g. Logan *et al.*, 2008; Birkett *et al.*, 2004; Bursell *et al.*, 1988). In GC-EAD, the sample is injected on to the column of a GC, but part of the effluvium from the GC passes over the insect antenna instead of going to the FID. The insect is mounted and, by using micromanipulators, a small electrode is put in contact with the antenna and a ground electrode inserted into the insect's brain. Alternatively, only an excised antenna can be used. Compounds, shown as peaks in the chromatogram, for which the insect has receptors, will trigger an electrophysiological response. This signal is amplified and recorded alongside the gas chromatogram, resulting in a GC-EAD recording (Figure 6). Compounds which elicit electrophysiological responses are then identified using GC-MS and comparison of retention times with authentic standards. (Wyatt, 2003; Hummel & Miller, 1984).

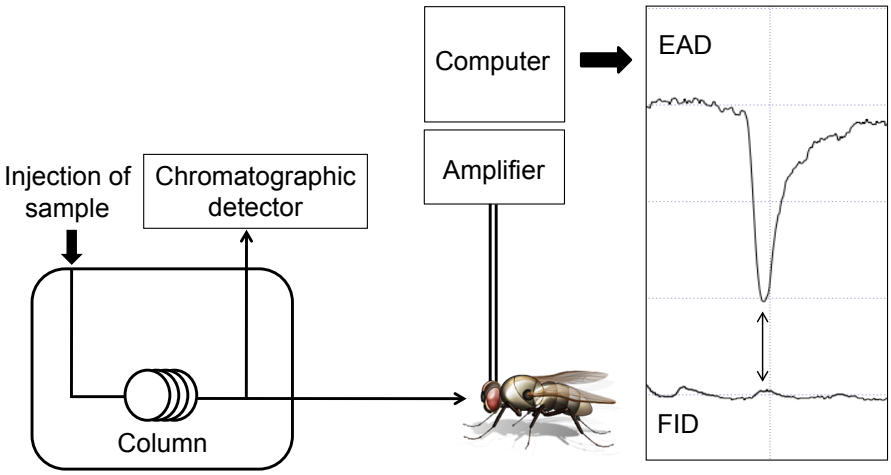

Figure 6. GC-EAD. The sample is injected on to the GC column, which separates the compounds. The effluvium is partly passed over the detector and partly over the insect's antenna. One electrode is put in contact with the antenna and a ground electrode is inserted into the head of the insect. The signal is amplified and recorded in a computer, resulting in the GC-EAD recording seen to the left. The arrow indicates a peak in the chromatogram (FID), which elicits an electrophysiological response in the insect (EAD). Illustration by the author.

Whereas GC-EAD records the combined response of many neurons GC-SSR only records the electrophysiological response of a single olfactory cell. This is a very useful technique when only few neurons respond to stimuli, making it difficult to detect the responses with GC-EAD (Jackson & Linskens, 2002; Hummel & Miller, 1984).

1.4.3 Using insects as biological detectors

Insects have been used as biological detectors previously. It is possible to teach insects to respond to certain olfactory stimuli. Parasitoid wasps (*Microplitis croceipes*) can learn to detect odours by associative learning. A portable volatile odour detector, the "Wasp Hound", has been developed based on these wasps, which may be used for monitoring food stuff for fungal growth associated with production of toxins (Rains *et al.*, 2006). The wasps have also been trained to detect indole and skatole, compounds associated with boar taint, and this might be used as a biosensor for boar taint (Wäckers *et al.*, 2011). Also the honey bee (*Apis mellifera*) is able to learn olfactory stimuli and display conditioned extension of the proboscis in response to these stimuli, with success rates between 70 and 90 % (De Jong & Pham-Delegue, 1991).

It is also possible to take advantage of the natural response of insects to certain odours, either to attractants or repellents. One example of this is a biosensor for plant damage based on antennae from the Colorado potato beetle (*Leptinotarsa decemlineata*), developed by Schütz (Schütz *et al.*, 2000; Schütz, 1996).

1.4.4 The face fly

The face fly, *Musca autumnalis* de Geer (Diptera: Muscidae), is endemic to temperate latitudes of Europe, central Asia and Northern Africa. Since the 1950s, it has also spread over large parts of North America (Krafsur & Moon, 1997). It is the most prominent cattle-visiting fly on pasture in Sweden, with some local and seasonal variation (Chirico, 1994). In addition to being a nuisance to grazing cattle, the face fly has been implicated as a biological vector of *Parafilaria bovicola*, a parasite of bovine connective tissue (Bech-Nielsen *et al.*, 1982), and *Thelazia* parasites, as well as a mechanical vector of *Moraxella bovis*, the causative agent of bovine keratoconjunctivitis or pink eye (Krafsur & Moon, 1997). It feeds on proteinaceous secretions, such as tear fluid, saliva and nasal discharge, but is also a facultative blood-sucker and can use its sucking proboscis to suck blood from wounds, e.g. bites from haematophagous insects. Nectar and dung fluid are also part of the diet (Hammer, 1941), and sugar is a prerequisite for survival (Turner Jr & Hair, 1967). Approximately 90 % of flies found on cattle or in droppings are female (Teskey, 1969) and the females depend on bovine secretions and dung to procure the proteins necessary for egg development (Van Geem & Broce, 1985; Chaudhury & Ball, 1973). Females display cyclic feeding responses to blood and dung, which can be ascribed to the nutritional requirements during the cycle of egg development and oviposition (Miller & Treece, 1968). Eggs are deposited in fresh bovine faeces and the face fly is among the first insects

to reach a fecal dropping (Teskey, 1969). The face fly population during one year may consist of 3-12 generations, depending on location. The flies overwinter as unmated adults that resume the breeding season in the spring (Krafsur & Moon, 1997).

The face fly as a biological detector

Although not numerous, some studies have focused on the olfaction of the face fly. Chaudhury *et al.* (1972) used a Y-tube olfactometer to demonstrate the existence of a female sex pheromone, and hypothesised that the pheromones attracted males to females and elicited copulatory behaviour. The attraction of face flies to bovine faeces has also been investigated using a Y-tube olfactometer (Bay & Pitts, 1976). Both males and females were attracted to the faeces, but whereas males were equally attracted on each of the seven days following emergence, the females were uninterested during the first four days and showed peak attraction on day seven. The number of attracted females correlated to the number of females carrying mature ova. Raman & Gerhardt (1997) developed an insect odourmeter, based on gravid female face flies, for measuring odours emanating from bovine manure. Even though the system was highly dependent on controlled conditions with regard to the odour exposure history of the insects and their reproductive status, they underlined the usefulness of the device, given that the response could be correlated with human odour panel responses.

For another dipteran, *Haematobia irritans,* it has been demonstrated that the fly load in a herd is determined by the presence or absence of individuals that are fly-susceptible or fly-resistant (Jensen *et al.*, 2004). This difference is partly due to differences in volatile semiochemicals emanating from those individuals (Oyarzun *et al.*, 2009; Birkett *et al.*, 2004). In the same study, Birkett *et al.* (2004) also investigated the response of the face fly to bovine volatiles, by using GC-EAD and behavioural assays in a wind-tunnel. The face flies responded electrophysiologically to 16 compounds collected from cattle head space and aged urine. In the behavioural assay three compounds, 1-octen-3-ol, 6-methyl-5-heptene and 3-octanol, attracted flies, while naphthalene and linalool were repellent at certain concentrations. When two of the compounds were tested in the field, results were contradictory. However, these findings confirm that face flies respond to volatiles released from cattle, both electrophysiologically and behaviourally.

The number of face flies on a specific individual is believed to vary over time. Teskey (1969) found that some mature cattle during certain periods of one to four weeks carried more flies than other individuals in the herd. At times

this increase in fly load could be ascribed to smears of faeces, but at other instances no such explanation could be found.

Since the fly depends on bovine body secretions, and bovine pheromones are thought to be dispersed in different body fluids (Rivard & Klemm, 1989; Kiddy *et al.*, 1984), it is possible that the fly has receptors to discriminate between oestrous and luteal phase secretions, provided that the fluctuations in reproductive hormones change food quality for the fly. This might explain the variation in fly load reported by Teskey (1969). If this holds true, the face fly could be used as a biological detector to detect oestrus-specific molecules.

2 Aims

The overall aim of this thesis was to investigate whether bovine inter-female pheromones exist and, if so, whether they can be identified and used to modulate reproduction in cattle. The specific aims were to:

- Explore the potential of female oestrous secretions for olfactory modulation of reproductive functions, such as cycle length, oestrous behaviour, secretion of reproductive hormones and follicular dynamics.

- Find a bioassay that can be used to distinguish quickly between active and inactive scent.

- Isolate and identify oestrus-specific bioactive compounds in oestrous secretions.

3 Methodological Considerations

A general presentation of the methods used in the thesis is provided here, while more detailed descriptions are provided in papers I-IV. Given the diverse nature of this thesis, papers I-II and III-IV are presented separately. All experiments on cattle described below have been approved by the Uppsala regional ethical committee.

3.1 The effect of oestrous substances on cyclicity (papers I-II)

3.1.1 Experimental animals

Ten heifers of the Swedish Red Breed (SRB) were used to study the effect of oestrous substances on cyclicity (paper I). Of these, four were used for the study on LH pulsatility pattern following exposure to oestrous substances (paper II). The animals were kept isolated from each other and from other animals in separate rooms throughout the experiments and changing of clothes was mandatory before entering each room. The animals were kept in tie stalls and fed hay *ad libitum* with limited amounts of concentrate. The age of the heifers at the onset of the experiments ranged from 14.9 to 16.9 months and the ovaries of all animals were examined with transrectal ultrasound at the start to verify that sexual maturity had been attained.

3.1.2 Donor animals

The donor animals were cows and heifers of the SRB and the Swedish Holstein Breed (SH) kept in tie stalls. The experimental animals mentioned above were not used as donors. Oestrus was induced through intramuscular administration of 2 ml (0.5 mg) of $PGF_{2\alpha}$ (Cloprostenol sodium, Estrumat® vet., Intervet International B.V., Boxmeer, Netherlands). Collection of oestrous substances was initiated when signs of oestrus were observed and a large follicle was present in the ovaries. The vaginal mucus was collected using handmade cotton tampons manufactured for the purpose and urine was collected after

stimulation of the perineum. The substances were frozen and stored at -80°C until needed. Donor animals were monitored by visual oestrus detection and ultrasonographic examination of the ovaries (Agroscan ALR 757, ECM, Angoulème, France; 7.5 MHz linear rectal probe). Only samples collected during the last 48 h leading up to ovulation were used.

3.1.3 Experimental design and exposure to oestrous substances

In both studies (paper I and II) each animal was its own control. The study on LH pulsatility pattern (paper II) was carried out directly following the cyclicity study (paper I) on the last group of animals. A schematic presentation of the experimental design is presented in figure 7.

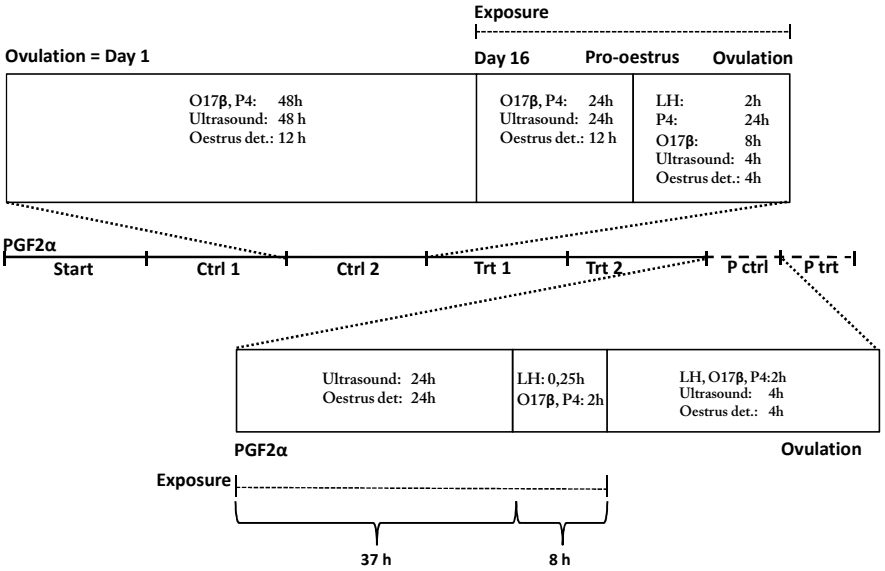

Figure 7. Experimental design. A schematic view of the five cycles constituting the cyclicity study (paper I) and the additional two oestrous periods constituting the pulsatility study on the last group of four animals (paper II). One control period in each of the studies, representative of all other cycles, is shown in detail with sampling intervals given in hours (above the time line for paper I and below for paper II). Reproductive hormones sampled were progesterone (P4), oestradiol (O17β) and LH. In addition ultrasonographic examinations of the uterus and ovaries (ultrasound) as well as visual oestrus detection (oestrus det.) were performed. Start = start cycle; Ctrl = control cycle in paper I; Trt = treatment cycle in paper I; P ctrl = control cycle in paper II; P trt = treatment cycle in paper II. Illustration by the author.

Both studies started with the induction of oestrus through intramuscular administration of $PGF_{2\alpha}$ (as described above), which in the pulsatility study was preceded by intramuscular administration of 5 ml (21 μg) of GnRH

(Buserelin acetate, Receptal® vet., Intervet International B.V., Boxmeer, Netherlands).

In the cyclicity study (paper I) the animals were monitored for five consecutive oestrous cycles (one start cycle followed by two control cycles and finally two treatment cycles). In the pulsatility study (paper II) the animals were monitored during parts of two oestrous cycles. Monitoring included blood sampling, ultrasonographic examinations of the uterus and ovaries (using the same equipment as described above) and visual oestrus detection.

From day 16 of the oestrous cycle in paper I and from the administration of $PGF_{2\alpha}$ in paper II, the animals were exposed to either the control (water) or the test (oestrous urine and vaginal mucus) substances. The substances were presented to the animals on tampons placed in a modified nose ring (Figure 8), originally intended to prevent unwanted suckling (Cattle Weaner Müller, Albert Kerbl GmbH, Buchbach, Germany), and replaced with fresh ones every 12 h.

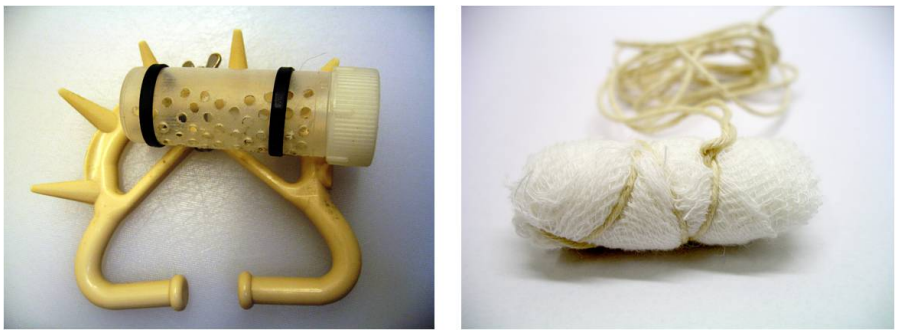

Figure 8. Modified nose ring and tampon. Photograph by the author.

3.1.4 Reproductive hormones

For paper I, blood was sampled every second day during day 1-15 of the oestrous cycle and from day 16 daily for determination of oestradiol and progesterone concentrations. From the onset of pro-oestrus until ovulation, blood was sampled at two-hourly intervals. Of these samples, those taken every 24 h were analysed for progesterone, those taken every 8 h for oestradiol and those taken between 34 and 14 h before ovulation were analysed to pinpoint the timing of the preovulatory LH surge. For paper II, blood was sampled every 15 min during a period of 8 h, starting 37 h after administration of $PGF_{2\alpha}$. During this time, and until ovulation occurred, blood samples taken every two h were analysed for oestradiol and progesterone. Samples taken between 34 and 14 h before ovulation were also analysed to determine the timing of the preovulatory LH surge.

To quantify the total production of reproductive hormones, two different measurements were used in papers I and II. In paper I, the sum of the values from all measurements during a sampling period were calculated (= sum of concentrations). In paper II, the total production of reproductive hormones was calculated as area under the curve (AUC), using the trapezoidal rule.

Plasma concentrations of LH and oestradiol (Double Antibody Estradiol, KE2D1, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) were determined using double antibody radioimmunoassays (RIA), while a direct RIA without extraction was used to determine progesterone concentrations (Coat-A-Count, Siemens Healthcare Diagnostics Inc., Tarrytown, NY. USA).

3.1.5 Reproductive parameters

In paper I, ultrasonographic examinations were carried out every second day from day 1-15 and from day 16 once daily. In both papers I and II, ultrasonographic examinations were carried out once daily until the onset of pro-oestrus and thereafter at four-hourly intervals until ovulation. Each examination was recorded as a digital file, with a selection of these recordings being used for retrospective analysis of follicular dynamics. The time of ovulation was calculated as the midpoint between the last ultrasound examination when the ovulatory follicle was seen and the first examination when it was no longer seen. The temporal relationships between ovulation and several physiological events were investigated (ovulatory interval, interval from standing oestrus to ovulation and between the LH peak and ovulation). Other temporal relationships were also investigated (e.g. LH peak interval, interval between LH peak and standing oestrus, as well as between maximal oestradiol concentration an standing oestrus and, for paper II, also the timing of certain events in relation to the window of intensive blood sampling).

3.1.6 Oestrus detection and scoring scale

Visual oestrus detection was performed twice daily until the onset of pro-oestrus in paper I and once daily in paper II. It was performed at four-hourly intervals from the onset of pro-oestrus until ovulation had occurred. To evaluate duration of oestrus and the strength of display as objectively as possible, the animals were observed in a standardised manner with five signs of oestrus being evaluated and scored (body position at the beginning of the observation, degree of restlessness, occurrence of lordosis, presence of vaginal discharge and appearance of the external genitalia). The scoring of each parameter is presented in table 3. To compare the effect of the type of exposure on expression of oestrus in paper I, data were divided into three different time

periods (data collected 0-14, 14-26 or 26-38 hours before ovulation), based on the mean onset and end of standing oestrus.

Table 3. *Scoring scale for oestrus detection*

Sign of oestrus	Score
Position	
Standing	3
Getting up	1
Lying	0
Restlessness	
Strong	3
Average	1
Calm	0
Lordosis	
At eye contact	15
At approach	12
At touch	10
None	0
Vaginal discharge	
Clear	5
Cloudy	2
Bloody or none	0
External genitalia	
Red and swollen	3
Red or swollen	1
Normal	0

3.1.7 Statistical analyses

In paper I, the data were analysed using SAS software (ver. 9, SAS Inst. Inc.,Cary, USA). Cyclicity parameters and hormonal data were analysed using the GLM procedure, while the PROC MIXED was used to analyse the scoring scale data.

In paper II, the characteristics of the LH pulsatility were determined using the ”Pulsar” algorithm (”Munro”, Zaristow Software, Haddington, UK) and the data were analysed using a marginalised estimating equations linear regression model. Cyclicity parameters for treatment and control were compared using a paired *t*-test.

3.2 Identification of bioactive compounds (paper III-IV)

3.2.1 Experimental animals

Two SRB heifers (additional to those mentioned above) and two SRB bulls were used in the experiment described in paper III. The heifers were approximately 15 months old at the start of the experiment and were cyclic. They were kept isolated from each other and from other animals for the duration of the experiment. The bulls were 17 and 19 months, respectively, and displayed normal libido. They had been separated from females since the age of six months although they were not isolated from other animals.

3.2.2 Insects

The face fly, *Musca autumnalis*, was used as a biological detector to identify bovine oestrus-specific volatiles in paper IV. The flies originated from a laboratory-reared colony kept at the Department of Entomology, University of Minnesota, St. Paul, USA. They were imported as pupae to Sweden where they were hatched in cages and fed *ad libitum* sugar and water. Only female flies (775 in total) at the age of 4-5 days were used in the behavioural assays. For GC-EAD, three-to-five day old females were used.

3.2.3 Substances

Oestrous urine and vaginal mucus were collected in the same manner as for papers I and II. In the heifer experiment (paper III) all oestruses except one were spontaneous. Urine from the luteal phase and bull urine (collected from one charolais bull and the two experimental bulls) were collected either during spontaneous or manually induced urination. The test substances were stored at -20°C until used. An insect behavioural assay was attempted for urine and vaginal mucus, but was only successful for urine. The following description of insect experiments concerns urine only.

3.2.4 Bovine behavioural assay

Heart rate was monitored using non-invasive heart rate monitors (Models S610 and S610i, Polar Electro Oy, Kempele, Finland), which recorded the heart rate every five seconds. For test substance administration, the heifers were fitted with plastic, non-invasive nose rings, similar to those described above, to which a metal plate was attached (Figure 9), whereas the bulls' own metal nose rings were used. The substances were presented to the animals on cotton tampons (described above) placed in plastic cassettes containing a magnet, which attached to the metal of the plate or the nose ring and allowed the cassettes to be exchanged swiftly. Each exposure lasted 10 minutes and was

followed by a 10-minute recovery period, during which a cassette containing a clean tampon was attached to the nose ring. The sequence of samples was bull urine, water, oestrous urine and vaginal mucus for heifers and bull urine, luteal urine, water, oestrous urine and vaginal mucus for bulls. After the first sequence was completed, it was repeated in reverse order. Experiments on heifers were carried out during three consecutive periods of oestrus and two luteal phases, and on bulls on three different days.

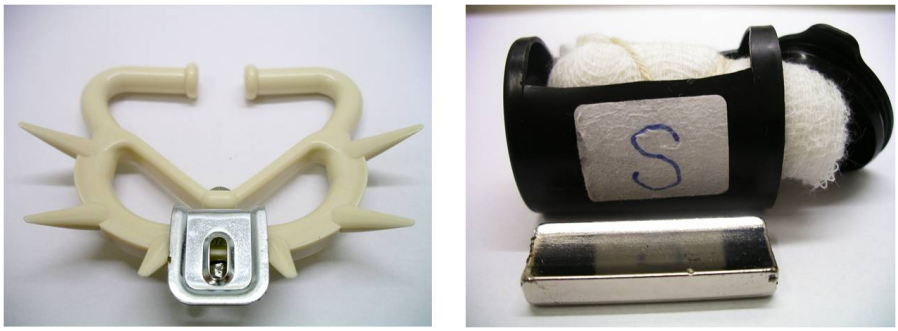

Figure 9. Modified nose ring, magnet, cassette and tampon. Photograph by the author.

3.2.5 Insect behavioural assay

A glass Y-tube olfactometer was used for the behavioural assay (Figure 10). Air was pulled over the test substances and through the arms of the tube into the stem where the flies could enter the tube. The flies were allowed to choose between the two arms and choices were recorded at the branching of the tube. In the urine behavioural assay, the flies were given the choice between oestrous or luteal phase urine as treatment and distilled water as control. In the cetyl alcohol dose-response behavioural assay the flies were given the choice between one of five doses of cetyl alcohol (1-hexadecanol) diluted in ether (100 ng, 10 ng, 1 ng, 0.1 ng and 0.01 ng) and pure ether.

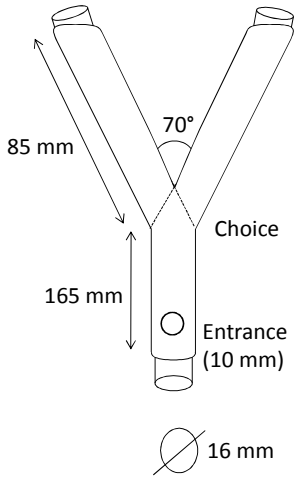

Figure 10. Y-tube olfactometer. Illustration by the author.

3.2.6 Volatile collection

Freshly thawed aliquots of the urine samples used for the behavioural assay were placed in glass flasks in a water bath at 38°C. Head space volatiles were collected for 4.5 h, by blowing nitrogen over the samples and through glass Porapak Q adsorbent tubes (Alltech Associates, Carnforth, Lancashire, UK; 50 mg Porapak in 5 mm glass tube). The volatiles were eluted with freshly redistilled pentane and the extracts were stored at -20°C until used.

3.2.7 GC-EAD

GC-EAD was performed on mounted intact flies using the extracts of headspace volatiles collected as described above. For this purpose, samples were injected on a J & W scientific HP5 US8775725H column, housed in a Hewlett-Packard 6890 GC equipped with a Gerstel Crosspiece splitless injector and a FID. Electrophysiological responses were recorded using a 10 x amplifying Syntech Universal probe connected to a Syntech IDAC-2 (Ochenfels Syntech GmbH, Kirchzarten, Germany). EAD traces were aligned in time using the retention time of prominent FID chromatogram peaks, after which averages of antennal responses were calculated. These responses were then verified by examining individual traces.

3.2.8 Chemical analysis

The extracts from the headspace collection of urinary volatiles were injected on to a HP5 column housed in a Hewlett-Packard 6890N GC equipped with a cold

on-column injector and a flame ionisation detector (FID). For identification of prominent peaks in the chromatograms, the samples were also analysed using GC-MS. For this purpose, a column, similar to that above, housed in an Agilent 7890A GC that was directly coupled to a mass spectrometer (Agilent 5975C inert MSD with triple-axis detector) was used. Identification of compounds eliciting an electrophysiological response was attempted by GC-MS analysis. The samples were injected on an Agilent 6890N/5975B inert GC-MS equipped with a column similar to that used for GC-EAD.

3.2.9 Statistical analyses

Data from the bovine behavioural assay were analysed using SAS software (ver. 9). For each animal and category of exposure, the average heart rate and standard deviation was calculated and then analysed using analysis of variance (PROC GLM).

Choice differences between oestrous and luteal urine in the face fly behavioural assay were analysed by a logistic regression and a log likelihood test using SAS software (ver. 9.2). Choices between treatment and control for each of the doses in the cetyl alcohol dose-response behavioural assay, were analysed by a χ^2 test using Minitab software (ver 16, Minitab Ltd., Coventry, UK). The same software was used to analyse differences in amounts of candidate compounds in the chromatograms from individual cows by Student's *t*-test.

4 Results and general discussion

4.1 Effects of oestrous substances on cyclicity (papers I-II)

4.1.1 Reproductive parameters

Overall, there were no significant differences between the treatment and control cycles on the reproductive parameters investigated in either study. In paper I, the mean cycle length (measured as LH peak interval) was 20.2 ± 1.5 (mean ± SD) days for control cycles and 20.4 ± 1.1 days for treatment cycles. The mean duration of standing oestrus was 12.2 h (range 0-20.4 h) and 10.9 h (range 0-20.0 h) for control and treatment cycles, respectively.

Despite the lack of difference in cycle length, we did find a significant effect of cycle number within type of exposure on both ovulatory interval (P = 0.038) and LH peak interval (P = 0.027), and a tendency for an interaction between cycle number within type of exposure and type of exposure for both parameters (P = 0.068 and 0.073 respectively). The cycle length (measured as LH peak interval) differed significantly between the two control cycles (P = 0.042), but not between the two treatment cycles. This observation could be a result of a modulating effect of the treatment.

4.1.2 Reproductive hormones

Luteinising hormone

In paper I, we saw a tendency for a treatment effect on both the sum of concentrations of LH during the surge (P = 0.063) and on the maximum concentration of the LH surge (P = 0.073), with higher values for the control cycles. A possible explanation for this finding could be that there is a suppressive effect of the treatment on LH secretion.

In paper II, we found significant differences between the control and treatment cycles in LH pulsatility pattern during the intensive blood sampling.

The mean LH concentration at the nadir was 2.04 ± 0.18 ng/ml (LSM ± SEM) for the treatment cycle and 1.79 ± 0.16 ng/ml for the control cycle, and this difference was significant ($P < 0.001$). Conversely, the mean peak amplitude was significantly lower ($P = 0.001$) during the treatment cycle (0.87 ± 0.09 ng/ml) than during the control cycle (1.03 ± 0.09 ng/ml). The mean peak LH concentrations did not differ significantly between treatment (2.92 ± 0.17 ng/ml) and control (2.83 ± 0.13 ng/ml) cycles. There were no significant differences between control and treatment for any of the parameters reflecting the total release of LH during the intensive sampling period, i.e. mean number of the peaks (7.0 and 7.3 for treatment and control, respectively) and mean area of the peaks (25.59 ± 3.02 and 27.60 ± 4.42 ng × ml^{-1} × min for treatment and control cycle, respectively).

There is evidence to suggest that the pre-luteolytic period in cows is characterised by greater pulse amplitudes and lower nadir concentrations than the luteolytic period, but without any difference in peak concentrations between the two periods (Ginther *et al.*, 2011). One possible explanation for the difference seen in LH pulsatility profiles between control and treatment in the present study could be that the luteolysis occurred later in animals receiving the treatment than in those receiving the control.

It has been shown that LH pulsatility patterns during the pre-ovulatory LH surge and early luteal phase resemble each other, with high frequency (20-30 pulses/24 h) and low amplitude (Δ0.3-1.8 ng), whereas, conversely, the midluteal phase is characterised by high amplitude and low frequency (Rahe *et al.*, 1980). This pattern may be caused by changes in concentrations of steroidal hormones. It is known that exogenous progesterone suppresses LH release, whereas exogenous oestradiol has an enhancing effect on mean concentration of LH, by increasing LH pulse amplitude (Rathbone *et al.*, 2001).

Acute psychosocial stress (Breen *et al.*, 2007; Dobson *et al.*, 1999) and negative energy balance (Butler & Smith, 1989) negatively affect LH secretion in ruminants. In the present study, the animals, housing, management and experimental design were the same for all four experimental cycles, with the exception of the type of exposure, and thus are not likely to have influenced the results.

Steroidal hormones

We found no significant influence of the treatment either on progesterone or on oestradiol concentrations.

In paper I we found a significant effect of cycle number within type of exposure on maximum oestradiol concentration ($P = 0.049$), the sum of

oestradiol concentrations during the 48 h sampling ($P = 0.009$) and the sum of oestradiol concentrations during the 8 h sampling ($P = 0.020$). The maximum concentration of oestradiol for the two control cycles was quite similar, whereas it differed more between the treatment cycles. An interesting finding was that the cycle pattern for the sum of oestradiol concentrations during days 1-16 seemed to be an inversion of the pattern seen during the last 24 h before ovulation.

At the beginning of the intensive sampling period in paper II, all animals had progesterone levels ≤ 0.9 ng/ml, regardless of type of exposure. Given the fact that the end of the luteolytic period has been defined as the time when the progesterone concentration decreases to 1 ng/ml (Ginther *et al.*, 2010), these animals must all be considered to be post-luteolytic. There were no differences in total production of progesterone and oestradiol between control and treatment cycles.

4.1.3 Scoring scale for oestrus detection (paper I)

The time period (data collected 0-14, 14-26 or 26-38 hours before ovulation), had a highly significant effect on the score for all variables ($P < 0.001$) except for the appearance of the external genitalia ($P = 0.025$). This result was to be expected given that the time periods corresponded to pro-oestrus, oestrus and post-oestrus, respectively.

We found a significant effect of treatment on the score for the appearance of the external genitalia ($P = 0.004$), with higher scores during control than treatment cycles. The effect of cycle within type of exposure was also significant ($P = 0.007$), with increased scores for the second control cycle and, conversely, decreased scores for the second treatment cycle.

Similarly, the score for the variable ”discharge” was also higher during the second control cycle and lower during the second treatment cycle, and this effect of cycle within type of exposure was significant ($P = 0.026$).

For the variable ”restlessness” there was a significant interaction between type of exposure and time period ($P = 0.011$), with maximum scores occurring 26-38 h before ovulation during treatment cycles, i.e. before the onset of oestrus, and for the control cycles 14-26 h before ovulation, i.e. during oestrus.

There was also a tendency for an interaction between type of exposure and time period for the variable ”position” ($P = 0.076$), which resembled the pattern seen for the variable ”restlessness”.

Other than the effect of time period, there were no significant effects on the score for lordosis or on the total score.

Even though we found no differences between control and treatment cycles with regard to duration and intensity of the expression of oestrus (measured as the total score), we did see other differences in the expression of oestrus. These differences may have been the effects of endocrinological changes induced by the treatment.

4.1.4 General discussion

A comparison between some cyclicity parameters from the control cycles in the paper I and data from previous studies on SRB heifers is given in Table 4 (Bage *et al.*, 2002; Gustafsson *et al.*, 1986). The data from the present study correspond well to previous results, indicating that the animals studied were in good reproductive health. This is further supported by the fact that 9 out of 10 animals became pregnant after the first AI or ET. The duration of standing oestrus is somewhat shorter than in previous studies. This could be explained by the fact that the animals were kept in isolation in the present study, but not during the other two studies. It has been shown that the duration of oestrus increases with the number of animals simultaneously in oestrus (Floyd *et al.*, 2009; Hurnik *et al.*, 1975).

Table 4. *Comparison of some reproductive parameters from three studies on SRB heifers*

Parameter	Paper I	Gustafsson *et al.* 1986	Båge *et al.* 2002
	Mean ± SD	Mean ± SEM	Mean ± SD
Cycle length (days)	20.1 ± 1.5	-	20.0 ± 1.6
Duration of standing oestrus (h)	12.2 ± 5.3	23.8 ± 2.0	15.2 ± 5.4
Onset of standing oestrus to LH peak (h)[a]	2.0 ± 4.9	4.8 ± 1.5	0.4 ±1.0
Onset of standing oestrus to ovulation (h)	26.9 ± 4.8	-	24.1 ± 6.3
LH peak to ovulation (h)	24.9 ± 1.9	-	25.7 ± 4.4
Size of ovulatory follicle (mm)	13.1 ± 1.4	-	14.2 ± 1.2

[a]Standing oestrus defined as the time period when the animal displays lordosis

We believe that the differences in LH secretion in papers I-II, as well as the difference seen in expression of oestrus in paper I, are effects of the exposure to oestrous urine and vaginal discharge.

The fact that progesterone levels were below luteolytic levels at the beginning of the intensive sampling in all animals during both cycles, tells us that the difference in pulsatility patterns was not due to treatment animals being pre-luteolytic and control animals luteolytic. There is a possibility, however, that the timing of luteolysis differed between the groups. Unfortunately, the post-luteolytic LH pulsatility pattern has not been as

extensively investigated as the pre-luteolytic and luteolytic patterns, which prevents us from drawing any conclusion in this regard.

We did not see any differences in the concentrations of progesterone and oestradiol in papers I and II, which would have been expected given the differences in LH secretion between control and treatment cycles.

It is still possible that the differences in LH secretion and oestrus expression are caused by endocrinological changes induced by the treatment, even if no such differences were detected during the present studies. The studies in papers I and II are unique, in that the heifers were kept in conventional barns while at the same time being isolated from other animals. These conditions entailed a limitation on the number of animals in the studies, which in turn may have influenced the result, especially in view of the large variation seen in several of the variables investigated. The small number of animals may have obscured potential differences, as suggested by the maximum oestradiol concentrations being quite similar between the control cycles while differing more between treatment cycles in paper I.

The cycle effect seen for the variables ”genitalia” and ”discharge” might have been caused by the lengthy isolation. Randomising the types of exposure would have made the study less susceptible to such factors. However, the studies in papers I and II were designed to minimise the risk of a treatment effect spilling over into the control cycles, given the lack of information regarding the properties of bovine pheromones. Another objective with the design was to reduce the potential risk of contamination when handling the substances simultaneously.

We believe that the findings presented above support, to some extent, the existence of bovine inter-female pheromones as suggested by anecdotal evidence from farmers and previous studies (Nishimura *et al.*, 1991; Izard & Vandenbergh, 1982b). Contrary to our hypothesis, we found no differences in cycle length or oestrus duration between the control and treatment cycles in paper I. The fact that the LH peak interval differed significantly between the two control cycles, but not between the two treatment cycles, may indicate that there is an effect of the treatment on cycle length, although it was not manifested clearly under the prevailing conditions of the experiment.

Variation in cycle length may be one explanation for this observation. The length of a normal bovine oestrous cycle in Sweden may vary between 18 and 24 days. When designing the study in paper I, we assumed the cycle length to be quite constant within the same unexposed animal. This assumption is not supported by the data from the present study. It is also possible that the time span of two oestrous cycles was not long enough for the effect to be fully manifested.

Furthermore, knowledge about the nature of a potential bovine pheromone is very limited. The bioactivity of the samples may be lost if they are handled inappropriately. Based on studies of oestrous secretions from different ruminants (Rasmussen, 2001; Klemm *et al.*, 1987), we believe the risk for this to be slight, given the experimental protocol in papers I and II. At present, we do not know whether we are looking for one or several olfactory cues and, if more than one, how these compounds may interact. In humans and rodents (Stern & McClintock, 1998; Schank & McClintock, 1992; McClintock, 1984), a coupled oscillator model has been developed to explain oestrous synchrony, with one enhancing pheromone released before the LH surge and another, suppressing, pheromone released afterwards. We collected substances before, during and after the LH surge, which may have masked a potential pheromonal effect. It is also possible that other biostimuli than the olfactory cues, such as increased vocalisation or mounting, are needed to produce the full effect. The expression of such additional biostimuli was very limited, due to the fact that the animals were kept isolated. Another aspect of isolation is that the effect may be mediated by a signature mixture, rather than by pheromones. A signature mixture is a mix of compounds that is individual-specific and that has to be learnt by the receiver (Wyatt, 2010). Nishimura *et al.* (1991) found that heifers smeared with their own oestrous discharge during dioestrus were mounted by their herd mates, while heifers who received discharge from another individual were not. This releaser effect is likely to be mediated by a signature mixture, i.e. an individual is only mounted when the cue from the discharge matches the cue of that individual. In the present study, where the animals were exposed only to the substances, the lack of an individual signal may have influenced the result, e.g. it is possible that only a dominant cow can control the cycle length of her herd mates. Individual differences in secretion and receptivity may also have influenced the result, especially given the limited number of animals in the present study.

We believe that further studies on a larger number of animals would be of great interest, especially given the possible sources of bias, such as the large variation in variables investigated and possible individual effects on secretion and receptivity to pheromones. Investigating the effects of exposure on LH secretion should be prioritised, and there is a need to map the post-luteolytic LH pulsatility pattern. Although the follicular phase was the main focus of the present study, the end of the luteal phase, especially at the time of the $PGF_{2\alpha}$ release, is also of interest.

4.2 Identification of bioactive compounds (paper III-IV)

4.2.1 Bovine bioassay

Heifers

In general, we did not see any significant differences in heart rate during exposure to the different substances. Exposure to bull urine, however, elicited a significantly higher ($P < 0.01$) heart rate than that registered during the recovery periods. The average heart rate was significantly higher during the first minute of exposure than during the second minute ($P < 0.01$) and also during the following minutes ($P < 0.001$). The influence of animal on the heart rate was also significant ($P < 0.001$).

Oestrous cycle stage influenced the heart rate in several ways. We found that the average heart rate during oestrus was 75.7 beats per minute (bpm) whereas it was 66.3 bpm during dioestrus, and this difference was highly significant ($P < 0.001$). Furthermore, we found a significant interaction between cycle stage and type of substance. For the exposure that occurred during dioestrus, heart rates were significantly higher when the animals were exposed to oestrous urine ($P < 0.05$), vaginal mucus ($P < 0.001$) and bull urine ($P < 0.001$) than during the recovery periods. We did not see this effect during oestrus.

We believe that the observed effect of oestrous stage on average heart rate is an interesting finding, which merits further investigation. Since standing oestrus occurred mainly during night-time, there was a bias in the experimental design, with exposure during oestrus and dioestrus occurring at night-time and during the day, respectively, which may have influenced the result. The great effect of stage of oestrus on average heart rate, observed in the present study, is not supported in the literature (Lewis & Newman, 1984) and may be a consequence of the experimental design, given that the exposure during oestrus occurred at night-time, while the dioestrus experiments were carried out during the day. Koelsch (1992) demonstrated that heart rate in cattle is lower in the morning and higher at night, which may explain the pattern seen in the present study.

Bulls

We found no significant differences in heart rate in bulls when individual substances were compared. We did see, however, that the average heart rate was significantly higher ($P < 0.05$) during the first minute of exposure to vaginal mucus compared to the first minute of exposure to all other substances, when they were analysed together as one variable.

Conversely to the heifers, the average heart rate in bulls during the first minute of exposure did not differ from the following minutes. However, as in the heifers, there was a significant effect ($P < 0.001$) of animal on the heart rate.

General discussion

We found changes in average heart rate during exposure to potential sources of pheromones in both bulls and heifers. These changes were greater in bulls, but the effect may not be sufficiently great to enable heart rate monitoring to be used as a bioassay to measure bioactivity in body fluids.

There are several factors that may have influenced the cardiac response to exposure. It is possible that other olfactory, visual or auditory stimuli could have influenced heart rates, thereby increasing the variation in heart rate and making it hard to detect changes induced by the exposure.

The animal factor may also be important in the present study, especially considering the limited number of animals used. For instance, the sexual inexperience of the animals may have affected the results, as it is known that response to sexual chemical cues may depend on sexual experiences (Keverne, 2002).

As discussed above, individual differences in secretion of, and receptivity to, olfactory cues may also be important. Again, the limited number of animals, i.e. both donors and recipients, makes this study susceptible to such variations.

Even if the findings presented above are very interesting, no definitive conclusions can be drawn from these results of the effect of pheromones on heart rate. It is possible that the great complexity of mammalian physiology makes heart rate an unsuitable biodetector, but we firmly believe that further studies on a larger number of animals are desirable. Moreover, it would be useful to investigate the influence of sexual experience on response in this context.

4.2.2 Chemical analysis and insect bioassay

Urine behavioural assay

The urine behavioural assay revealed that female face flies were repelled by urine from cows in oestrus. Only 19 flies chose treatment compared to 39 flies choosing the control. This difference was not seen during exposure to urine from the luteal phase, with 28 flies choosing treatment and 25 choosing control. When the odds ratio was calculated for the choice difference between the two treatments, we found that the flies were 2.3 times more likely to choose the control arm when given the choice between control and oestrous urine as

when they were exposed to urine from the luteal phase, and this difference was significant ($P = 0.03$). The proportion of flies entering the olfactometer was 40-45 % during exposure to urine and water. The proportion of flies entering the empty olfactometer was numerically higher (65%). This may be an effect of the increased humidity from the aqueous samples. Of the flies entering the olfactometer, 88 % made a choice when exposed to oestrous urine and 83 % during the luteal exposure.

The proportion of flies entering the Y-tube was higher for urine from one specific cow (51 %) than for urine from the other cows (35-41 %) and this difference was significant ($P = 0.03$). However, the choice difference between treatment and control for oestrous and luteal urine was not affected by the animal factor, i.e. the identity of the animal did not influence the choice pattern.

The finding that female face flies were repelled by oestrous urine may be explained by the hormonal fluctuations in the blood during the bovine oestrous cycle. It is possible that secretions from an animal in oestrus are less suitable as a food source for face flies, than those secreted during other stages of the oestrous cycle and that the flies use oestrus-specific olfactory cues, e.g. from urine, to discriminate between suitable and unsuitable food sources. However, oestrus is characterised by high concentrations of oestradiol in blood and in other haematophageous insects, i.e. the anthropophilic mosquito *Aedes aegypti*, oestrogens have proven to be attractive rather than repellent (Bos & Laarman, 1975; Roessler, 1961).

Other than hormonal fluctuations, the peri-oestrous period is also characterised by behavioural changes, such as increased restlessness and attempts of pro-estrous animals to mount herd mates in oestrus. These changes may make it difficult for flies to settle on, and feed from, peri-oestrous cows. To avoid these individual animals in oestrus, or even a herd with a high proportion of oestrous animals, the flies might use chemical cues to identify suitable hosts in this regard. It is known from studies on biting insects that relatively small differences in host suitability can lead to great differences in effective host choice (Kelly, 2001).

GC-EAD

Using GC-EAD, we found eight peaks associated with six electrophysiological responses in female face flies during exposure to both oestrous and luteal extracts. For two of the six responses recorded, two compounds co-eluted at the time of the response. Three of the compounds were identified as dimethyl trisulfide, indole and skatole, whereas the remaining five compounds were tentatively identified as 2,2,5,5-tetramethyl-3-cyclopenten-1-one, 2,3,4,5-

tetramethyl-2-cyclopenten-1-one, 2-methyl-2-nonen-4-one, 1-(2-aminophenyl)-ethanone (co-eluting with indole) and 2-(ethylthio)-phenol (co-eluting with skatole). However, since these compounds were not commercially available they would have had to be synthesised, which was not possible within the timeframe of the present study and given the available resources. Pure samples of the three compounds that had been identified, dimethyl trisulfide, indole and skatole, were verified as electrophysiologically active. The quantified amounts of each compound in oestrous and luteal extracts, respectively, are given in Table 5. None of the compounds were found in significantly greater amounts in either oestrous or luteal extracts.

Table 5. *Quantified amounts (ng/µl) of compounds eliciting electrophysiological response in M. autumnalis (compounds with the same number are co-eluting)*

Compound	Oestrus (Mean ± SEM)	Luteal phase (Mean ± SEM)
1. Dimethyl trisulfide	0.22 ± 0.02	0.13 ±0.03
2. 2,2,5,5-tetramethyl-3-cyclopenten-1-one	0.20 ± 0.04	0.16 ± 0.002
3. 2,3,4,5-tetramethyl-2-cyclopenten-1-one	0.15 ± 0.06	0.10 ± 0.002
4. 2-methyl-2-nonen-4-one	3.24 ± 0.83	4.41 ± 0.75
5. Indole	0.22 ± 0.15	0.11 ± 0.007
5. 1-(2-aminophenyl)-ethanone	0.97 ± 0.26	0.89 ± 0.02
6. Skatole	0.29 ± 0.12	0.13 ± 0.02
6. 2-(ethylthio)-phenol	0.26 ± 0.07	0.16 ± 0.06

Chemical analysis

When the chromatograms for oestrous and luteal extracts were compared, we found one compound that was present in approximately 3.5 times greater abundance during oestrus than during luteal phase. The compound was identified as cetyl alcohol (also known as 1-hexadecanol) and the amount in each individual extract was quantified. The oestrous extracts contained significantly more cetyl alcohol ($P = 0.02$), with a mean (± SEM) of 1.99 ± 0.31 ng/µl, while the luteal extracts contained an average of only 0.60 ± 0.19 ng/µl. This difference was consistent for all four oestrous and three luteal extracts that were analysed.

Cetyl alcohol dose-response behavioural assay

Only the two lowest doses of cetyl alcohol, 0.01 and 0.1 ng, elicited a significant behavioural response during the dose response test. The number of flies choosing the arm with the treatment at the lowest dose was 32, compared to 8 choosing the control. This difference was highly significant ($P = 0.0001$).

For the second lowest dose, 17 flies chose the treatment whereas 32 chose the control: this difference was significant ($P = 0.03$).

These findings correspond well with the data from the urine bioassay, where flies seemed to be repelled by oestrous urine, which contained greater amounts of cetyl alcohol, but not by urine from the luteal phase, which contained lower amounts of cetyl alcohol. Furthermore, it is quite surprising that this behavioural switch occurred between two relatively similar doses (only a tenfold increase). However, this is also consistent with data from the urine bioassay, given that the average amount of cetyl alcohol in oestrous urine was only 3.5 times greater than in urine from the luteal phase.

We believe that female face flies may use cetyl alcohol, as part of a blend or on its own, to discriminate between suitable and less suitable hosts, i.e. animals in oestrus.

General discussion

As far as we know, this is the first time that cetyl alcohol concentrations have been reported to vary during the bovine oestrous cycle.

GC-EAD did not reveal cetyl alcohol as an electrophysiologically active compound, which is surprising, given the behavioural response in the dose response test. However, it is known that insects may respond to compounds at concentrations far below the detection limit of conventional techniques used in chemical analysis, i.e. only a few molecules may be needed to elicit a response (Angioy *et al.*, 2003). If only a few olfactory receptors are activated when an animal is exposed to a chemical cue, the resulting change in potential across the antenna may be of insufficient magnitude to be detected using GC-EAD. Furthermore, in the present study the recording electrode was placed at the apical part of the antenna, but different parts of the antenna, or even other olfactory organs such as the palp, may be involved in the detection of olfactory cues. It is possible that an electrophysiological response to cetyl alcohol would have been detected by using the more sensitive GC-SSR technique or using GC coupled with electropalpogram (GC-EPG) recordings.

In the present study we wanted to identify compounds that could be used to manipulate reproduction in cows. Even though no oestrus-specific molecules were found, we did see that cetyl alcohol was present in significantly greater amounts during oestrus, than during the luteal phase. The question of whether or not the four cows in the present study are representative of the cow population, i.e. whether the difference in concentration of cetyl alcohol between oestrus and the luteal phase may be seen in all cows, remains unanswered at present. To address this issue, oestrous and luteal volatile profiles from a larger number of cows would need to be investigated.

It would also be useful to study the dynamics of cetyl alcohol secretion. If the shift in concentration is rapid and occurs simultaneously with oestrus, cetyl alcohol could possibly be analysed using a cow-side test, i.e. similar to human pregnancy tests.

Even though differences in urinary cetyl alchol concentrations between oestrus and the luteal phase are quantitative rather than qualitative, cetyl alcohol may act as a bovine pheromone, either on its own or as a part of an oestrus-specific blend.

It has previously been shown that cetyl alcohol is a natural ligand of aphrodisin (Briand *et al.*, 2004). As mentioned above, aphrodisin is found in the vaginal secretions of golden hamsters and is a male aphrodisiac, on its own or in combination with its ligands (Tirindelli *et al.*, 2009; Brennan & Keverne, 2004). Given this indication that cetyl alcohol may be a mammalian pheromone, it would be of great interest to test the effect of cetyl alcohol at different doses on dairy cow reproduction.

We did not find any additional oestrus-specific compounds when using the face fly as a biological detector. Even so, we still believe that the technique should be tested on other mammalian and insect species. The face fly was able to discriminate between oestrous and luteal urine and this supports earlier evidence that haematophagous insects can detect and respond to mammalian cycle-specific olfactory cues.

The focus of the present study was mainly the cow, but even so we present findings that are of interest from an entomological point of view. The ability of the face fly to discriminate between stages of the bovine oestrous cycle is, to our knowledge, a novel finding, but also one of very few reports on this phenomenon in general.

Furthermore, host-specific olfactory cues may be used to control pest insects. It has been shown that individual heifers may be more or less attractive to the horn fly (Jensen *et al.*, 2004) and that the difference in attractiveness to cattle flies in general, can be ascribed, to some extent, to individual differences in volatile profiles (Birkett *et al.*, 2004). This has also been shown for humans, where the individual differences in attractiveness to *Aedes aegypti* were attributable to certain compounds, either acting as repellents or masking attractants (Logan *et al.*, 2008). If behaviourally active cues are identified, they may be put to use as alternatives to pesticides in controlling insect populations. Attractants can be used in lure-and-kill strategies (El-Sayed *et al.*, 2009) or for mass-trapping (El-Sayed *et al.*, 2006), whereby the insect is attracted to a certain location where it is exposed to an insecticide or trapped. Repellents, on the other hand, may be used as they are or together with an attractant in a push-

and-pull strategy, where the insect is repelled from one location and attracted to another (Hassanali *et al.*, 2008).

5 Conclusions

- Exposing heifers to vaginal mucus and urine collected during oestrus, from day 16 of the oestrous cycle to ovulation, had the following effects on reproductive parameters:
    - LH pulsatility pattern preceding the LH surge was altered
    - Secretion of LH during the surge seemed to be suppressed
    - The temporal pattern, i.e. when the different signs occur, and the intensity of oestrus expression were altered
    - Consecutive oestrous cycles were more consistent in length

- Heart rate, in both bulls and heifers, was affected by exposure to potential sources of pheromones, such as urine and vaginal mucus
    - This effect may not be of sufficient magnitude to use as a bioassay to test for bioactivity in samples

- Female face flies (*Musca autumnalis*) were repelled by oestrous urine from cows, but not by urine collected during the luteal phase

- The discrimination between substances collected during oestrus and luteal phase seemed to be based on the amount of cetyl alcohol in the sample and the behavioural switch occurred with small differences in concentration

- The attempt to use the face fly as a biological detector was not successful, but may prove valuable for other species

- There is a possibility that cetyl alcohol could be useful in the field of bovine reproduction

- These results do, to some extent, support the existence of a bovine inter-female pheromone

6 Future perspectives

Studies should be repeated on a larger number of animals

In general, the results from studies I-III are believed to be of great interest, but the limited number of animals may have prevented the effects of the exposure to be fully manifested. Further studies on a larger number of animals are needed, especially given the large variation in variables investigated and possible individual effects on secretion and receptivity to pheromones.

Using randomised design

Given the large variation seen in several of the parameters investigated here, it is important to use a randomised design for future studies.

LH secretion should be prioritised

Studies on the effect of exposure on LH secretion should be prioritised. However, to find the underlying mechanism behind the differences seen in LH pulsatility patterns, postluteolytic LH pulsatility pattern in unexposed animals needs to be thoroughly investigated.

Studying the luteal phase

Furthermore, to obtain a broader picture, attention should also be paid to the end of the luteal phase, especially around the time of $PGF_{2\alpha}$ release.

Investigating the effect of sexual experience

Further studies on the change of heart rate during exposure to putative sources of pheromones should include a greater number of animals and also sexually experienced individuals.

Scanning volatile profiles in cows

The volatile profiles of a larger number of animals need to be scanned for cetyl alcohol, to determine whether the significant difference seen between oestrus

and the luteal phase in the present study is seen in all cows. At the same time, the dynamics of the changes in cetyl alcohol concentrations throughout the bovine oestrous cycle should be investigated.

The effect of cetyl alcohol on cows

Studies measuring the effect of exposing heifers to different doses of cetyl alcohol, to see whether the compound is bioactive also in cattle, would be very interesting.

Further use of insects as biological detectors

Although the technique of using insects as biodetectors to identify mammalian olfactory cues did not provide any additional findings in the present studies, it ought to be tested on other mammalian and insect species, especially given the fact that the face fly can detect and respond to urine from different cycle stages. Furthermore, it would be interesting to investigate whether the fly load on individual heifers in the field varies with their oestrous cycles.

7 Populärvetenskaplig sammanfattning

7.1 Bakgrund och syfte

Under den senare delen av 1900-talet har förbättrad skötsel och omfattande avelsprogram bidragit till en markant ökning av den mängd mjölk som produceras per ko och år. Under det första decenniet av 2000-talet ökade årsproduktionen för svenska kor med nästan ett ton per djur, från 8,6 ton per år till strax under 9,5 ton. Under samma period minskade antalet besättningar i Sverige medan antalet kor per besättning ökade. Större besättningar kan innebära att mindre tid läggs på varje djur, vilket t.ex. kan leda till bristande brunstkontroll. Brunstkontrollen görs för att se när djuren är parningsvilliga, vilket är en förutsättning för att kunna välja lämplig tidpunkt för insemination i förhållande till ägglossningen och få djuren dräktiga.

Det finns en negativ genetisk koppling mellan mjölkproduktion och fruktsamhet hos mjölkkor, vilket betyder att en ensidig avel för ökad mjölkproduktion givit en negativ genetisk trend för fruktsamhetsegenskaperna. Mjölkbonden måste idag kompensera för detta med förbättrade skötselåtgärder. En komponent i den negativa fruktsamhetstrenden är att brunsten, den tid då djuren visar sig parningsvilliga, blivit kortare och svagare hos korna. Detta försvårar brunstkontrollen och leder till att kor går ”tomma” (dvs. inte blir dräktiga) längre än nödvändigt, vilket i sin tur medför en ekonomisk förlust för djurägaren.

I många länder används idag olika hormonprogram för att synkronisera djurens brunstcykler, dvs. få flera djur att bli brunstiga samtidigt. I Sverige har branschen tagit ett policybeslut som innebär att en sådan rutinmässig användning av hormoner till friska mjölkande kor skall undvikas. Alternativa metoder för att synkronisera brunster, förstärka kornas brunstbeteende eller på ett objektivt sätt kunna mäta vilka djur som är brunstiga vore därför särskilt värdefulla att utveckla.

Feromoner är doftämnen som används för kemisk kommunikation mellan individer av samma art och som kan framkalla beteendemässiga eller hormonella förändringar hos mottagaren. Ett exempel på en tillämpning av detta finns redan idag tillgängligt inom grisnäringen i form av en spray på burk. Produkten är baserad på ett feromon, androstenon, som utsöndras av galten och som framkallar ståreflex, vilket är ett tecken på brunst, hos suggor och gyltor. Ett flertal studier har visat att kemisk kommunikation mellan nötkreatur förekommer men dessa studier har huvudsakligen fokuserat på tjurens förmåga att känna igen och svara på brunstdoft samt tjurars påverkan på de honliga könsfunktionerna. Studier på människa och råtta tyder dock på att hondjur omedvetet kan synkronisera sina sexualcykler och att detta tycks styras via kemisk signalering.

Det övergripande syftet med projektet var att undersöka om kor och kvigor påverkar varandras brunstcykler med hjälp av doftämnen i urin och brunstslem och i så fall försöka identifiera de biologiskt aktiva molekylerna i dessa substanser eller, om detta inte var möjligt, försöka identifiera brunstspecifika molekyler (ämnen som bara hittas under brunsten) i allmänhet.

7.2 Resultat

För att undersöka om exponering för brunsturin och brunstslem påverkar könsfuntionerna hos kvigor genomförde vi två studier. I den första studien undersökte vi effekten av exponering på hela brunstcykeln hos 10 kvigor. Djuren fick bära modifierade nosringar försedda med kassetter, i vilka tamponger innehållande de aktuella substanserna kunde placeras. Exponeringen började på dag 16 i brunstcykeln (dag 1 = ägglossning) och tampongerna med urin och slem byttes var 12:e timme fram till ägglossning. Under tiden övervakades djuren med täta blodprovstagningar, brunstpassningar och ultraljudsundersökningar för att följa utvecklingen av äggblåsor i äggstockarna. I den andra studien fokuserade vi på en avgränsad period av brunstcykeln och undersökte den stötvisa frisättningen av luteiniserande hormon (LH) som styr ägglossningen.

Vi kunde inte se någon skillnad i brunstcykelns längd mellan kontroll (vatten) och behandling (urin och vaginalslem från brunstiga kor) men de två kontrollcyklerna skiljde sig mer åt än de två behandlade cyklerna, vilket skulle kunna vara en effekt av behandlingen. Vidare fann vi att frisättningen av LH påverkades av exponeringen. Vi såg också att brunstbeteendet skiljde sig åt tidsmässigt, dvs. att de olika brunstsymptomen nådde maxvärden vid olika tidpunkter för kontroll respektive behandling. Våra fynd tyder på att det kan

finnas någon form av kemisk kommunikation mellan honliga nötkreatur som påverkar frisättningen av hormoner och djurens brunstbeteende.

Vi undersökte även möjligheten att utveckla en snabbmetod för att testa om olika substanser är bioaktiva eller inte, dvs. huruvida substanserna kan framkalla en fysiologisk reaktion hos det exponerade djuret. Vi exponerade två kvigor och två tjurar för tjururin, brunsturin, brunstslem, icke-brunsturin (endast tjurar) och vatten (som kontroll) samtidigt som vi registrerade hjärtfrekvensen var femte sekund. Resultaten tyder på att hjärtfrekvensen påverkas vid exponering för potentiellt bioaktiva substanser men att effekten troligtvis inte är tillräckligt stark för att denna metod skall kunna användas rent praktiskt som ett snabbtest.

Syftet med den sista studien var att undersöka om ansiktsflugan (*Musca autumnalis*) kan användas som en s.k. biodetektor (ett levande mätinstrument) för att hitta brunstspecifika molekyler i kourin. Ansiktsflugan är den vanligast förekommande ko-nära flugan på beten i södra Sverige och honorna är beroende av protein från de betande djurens kroppsvätskor för att kunna producera ägg. Studier på myggor och fästingar tyder på att de kan skilja mellan olika stadier av sexualcykeln hos honliga värddjur men det är inte känt om detsamma gäller för ansiktsflugan.

För att undersöka om honliga ansiktsflugor kunde skilja mellan urin från brunstiga och icke-brunstiga kor studerades deras beteende vid exponering för substanserna i en s.k. olfaktometer. Olfaktometern utgjordes av ett Y-format glasrör där flugorna kunde göra vägval då olika dofter presenterades i rörets skänklar. De fick välja mellan antingen brunsturin och vatten eller urin från icke-brunstiga kor och vatten. Vi fann att flugorna kunde skilja mellan de båda typerna av kourin och att brunsturin tycktes vara frånstötande.

För att undersöka vilka ämnen i urinen som orsakar detta använde vi oss av en gaskromatograf kopplad till en elektrofysiologisk detektor (GC-EAD). Gaskromatografen förångar doftextrakt och separerar de olika ämnena i extraktet beroende på deras kokpunkt och struktur. Efter genomgången analys får man ett kromatogram, där varje ämne syns som en egen topp. Den elektrofysiologiska detektorn utgjordes av flugan själv och ett system som känner av elektriska impulser i flugans antenn. När de två systemen är sammankopplade kan toppar i kromatogrammet kopplas till elektrofysiologiska svar i antennen, dvs. man kan se vilka ämnen som flugorna är känsliga för. Denna metod registrerade sex olika svar, varav inget var brunst-specifikt.

När kromatogrammen för urin från brunstiga och icke-brunstiga kor jämfördes sågs en topp som var betydligt större i brunsturinen. Detta ämne identifierades som cetylalkohol.

Flugornas beteende vid exponering för olika doser av cetylalkohol studerades också i olfaktometern. Vi fann då stora skillnader i beteende mellan två ganska likartade doser. Vid den lägsta dosen valde flugorna behandlingen (cetylalkohol i lösningsmedel) framför kontrollen (lösningmedel) medan det omvända sågs vid en endast 10 gånger högre dos. Detta stämmer väl med våra fynd från urinförsöken, där brunsturin (som innehåller mer cetylalkohol) hade en frånstötande effekt medan urin från icke-brunstiga kor (som innehåller mindre cetylalkohol) inte var frånstötande.

7.3 Slutsatser

Det faktum att vi fann förändringar i frisättningen av hormoner och i brunstbeteende hos djuren vid exponering för urin och slem från brunstiga kor tyder på att honliga nötkreatur kan kommunicera med kemiska signaler och att dessa kan påverka könsfunktionerna hos mottagardjuret.

Vi fann även effekter av exponering för potentiellt bioaktiva substanser på hjärtfrekvensen hos både tjurar och kvigor. Det är dock oklart om denna effekt är stark nog för att registrering av hjärtfrekvensen skall kunna användas för undersökning av substansers bioaktivitet.

Så vitt vi vet, är detta första gången någon har visat att ansiktsflugan kan skilja mellan urin från brunstiga och icke-brunstiga kor. Det är även en av få studier som överhuvudtaget ger stöd för hypotesen att insekter kan känna av sexualcykelstadium hos ett värddjur. Våra försök att använda ansiktsflugan som en biologisk detektor gav inte önskat resultat, eftersom den kemiska signalen flugorna använde sig av för att skilja mellan urin från brunstiga och icke-brunstiga kor inte kunde detekteras med GC-EAD. Det är dock möjligt att metoden kan vara användbar för andra arter av däggdjur och insekter, där topparna av de bioaktiva ämnena inte är lika påtagliga. Om variationerna i urinens innehåll av cetylalkohol i olika faser av brunstcykeln är desamma för kor i allmänhet återstår att undersöka, liksom om exponering för cetylalkohol har någon effekt på kor.

Sammanfattningsvis anser vi att våra resultat, som visar på en påverkan av kvigors könsfunktioner efter exponering för brunsturin och brunstslem, är lovande. En fortsatt forskning omfattande ett större antal djur skulle kunna bidra till att hitta nya hjälpmedel som kan användas i mjölkkobesättningar för att styra reproduktionen hos kor utan användning av hormoner.

Figure 11. Oestrus research. Illustration by L. Rydén in Land Lantbruk nr 17 2008. Reproduced with permission from the artist.

–I see you have taped her tail? –Yes, I didn't want her to brush off your research!!

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