Cycle-Characteristic Odour of Cow Urine Can Be Detected by the Female Face Fly (Musca autumnalis)

K Nordens1, B Webster2, L Söderquist1, R Bäg1 and R Gilwood3

1Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden; 2Department of Entomology, University of California, Riverside, CA, USA; 3Department of Crop Production Ecology, Swedish University of Agricultural Sciences, Uppsala, Sweden

Contents
Due to declining dairy cow fertility rates, there is great interest in developing tools for oestrus detection. Compounds in the volatile profile of oestrous cows are suggested as oestrus-specific, but consistent results have not been presented. Certain haematophagous arthropods can discriminate stages of the mammalian reproductive cycle based on host volatiles. This study investigated whether the face fly, Musca autumnalis de Geer (Diptera: Muscidae), can discriminate between urine from cows in oestrus and urine collected during the luteal phase. Individual flies were tested in a two-choice behavioural assay with choice between odour of oestrous or luteal urine and water (control). Flies chose the control arm significantly more when exposed to oestrous urine than when exposed to luteal urine. Analysis of volatiles showed that 1-hexadecanol (cetyl alcohol) was released in greater amounts from oestrous urine than from urine collected during the luteal phase. In a dose response assay, flies were significantly attracted by 0.01 ng of 1-hexadecanol but significantly repelled by 0.1 ng, a pattern consistent with fly responses to urine. In conclusion, M. autumnalis can discriminate between oestrous and luteal urine, and this may be mediated by differences in 1-hexadecanol concentration.

Introduction
Declining fertility rates in dairy cows (Royal et al. 2000), largely attributable to increasing milk yields and the unfavourable genetic correlation between yield and fertility (Pryce et al. 2004), are a growing concern for dairy farmers. Depressed expression of oestrous in dairy cows makes it difficult to determine optimal time for artificial insemination (Dobson et al. 2008). Many tools to aid oestrous detection have been developed, but a cheap and accurate method to determine when an animal is in oestrus is still needed.

Pheromones are compounds used for communication between individuals of the same species (Karlson and Luscher 1959). Pheromones affect several aspects of reproduction in mammals and one area of special interest in bovine reproduction has been their role in oestrus detection. Bulls can discriminate between oestrous and non-oestrous stages in a wide range of body fluids, such as urine, vaginal mucus, vulval skin gland secretions, saliva, faeces, milk and serum (Klemm et al. 1987; Houpt et al. 1989; Rivard and Klemm 1989; Sankar and Archunan 2004). Further, dogs and rats have been successfully trained to discriminate samples from different stages of the bovine oestrous cycle. Using dogs, Kidd and Mitchell (1981) found that oestrous odour in vaginal fluids emerges from day three before oestrus, reaches a peak on the day of oestrus and disappears within 1 day. A similar pattern was seen when testing urine in a rat bioassay (Dehnhard et al. 1991). However, attempts to identify oestrus-specific molecules in urine and vaginal mucus have given variable results, with several different compounds reported, e.g. 1-iodo undecane and di-n-propyl phthalate (Ramesh Kumar et al. 2000) acetaldehyde (Ma et al. 1995) and methyl heptanol (Preti 1984).

Insect olfactory systems are extremely sensitive, sometimes capable of responding to a few molecules of certain compounds (Angiyo et al. 2003). Insects often display distinct, quantifiable behaviours when exposed to olfactory cues and large numbers can easily be reared and used for experiments. This could open the possibility to develop biosensors for oestrus detection based on insect antennal response (Schott et al. 2013). These may be based on olfactory receptor neurons, excised antennae or intact, live insects. Specialized cells in the antennae generate electrical signals on detecting a specific volatile compound. This signal is then amplified and analysed electronically outside of the biological preparation.

Many haematophagous arthropods use host-characteristic volatile cues to find their animal or human hosts (Gibson and Torr 1999), and some appear to change their behaviour according to the reproductive status of the host (Roessler 1963; Gilbert et al. 1966; Carroll 2000).

The face fly, Musca autumnalis, is a cattle-visiting fly that is dependent on bovine proteinaceous secretions (Van Geem and Broce 1985) and is considered a facultative bloodsucker (Hammer 1941). The face fly has been shown to respond to bovine volatiles in behavioural assays (Birkett et al. 2004), so it is possible that it also uses volatiles from bovine secretions to locate its host.

The aim of this study was to investigate whether the face fly can discriminate between bovine luteal and oestrous phase urine using volatile chemical cues, and to identify oestrus-specific volatiles with potential for future use in management of cattle reproduction, for example by developing biosensors for oestrus detection.

Material and Methods
Urine collection and preparation
Urine was collected from four cows from the experimental herd at the Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, during two periods of oestrus and once during luteal phase. The animals were cyclic, non-
lactating cows of the Swedish Red (n = 2) and Swedish Holstein (n = 2) breeds, between 4.5 and 5.5 years of age. They were kept in tie stalls and fed hay *ad libitum*.

Oestrus was induced through two intramuscular injections of 2 ml PGF₂α (Cloprostenol sodium, Estrumate® vet., Intervet International B.V., Boxmeer, Netherlands) administered 11 days apart. Collection of oestrous urine once daily started when symptoms of oestrus were observed and a follicle larger than 12 mm was present in the ovaries. During collection, cows were monitored through visual oestrus detection and ultrasonographic examination of the ovaries twice daily until ovulation had occurred. Urine was collected, either at spontaneous urination or after stimulation of the perineum, in a glass container and divided into aliquots that were placed on ice after collection and transferred to a freezer at −20°C until further use. After ovulation, one oestrous urine sample from each cow was chosen, based on oestrous behaviour and on the time of collection in relation to ovulation (the sample closest to a time window of 24–12 h before estimated time of ovulation based on two consecutive ultrasound examinations), and only aliquots of this sample were used in subsequent experiments.

**Insects**

*Musca autumnalis* pupae were obtained from a laboratory-reared colony at the Department of Entomology, University of Minnesota, St. Paul, MN, USA and stored in a refrigerator until use, for a maximum period of 1 week. To hatch, pupae were put in cages at 22–25°C in a refrigerator until use, for a maximum period of 1 week. To hatch, pupae were put in cages at 22–25°C with 16:8 h light:dark. Adult flies were fed *ad libitum* on water and sugar cubes. Four- to five-day-old female flies were used in the behavioural experiments. Before all experiments flies were sedated by placing them in a freezer at −20°C for 1–2 min, and then sexed and placed individually into plastic vials.

**Behavioural assays**

A glass Y-tube olfactometer placed horizontally on a bench (Fig. 1) was used for the behavioural assays of odour choice. Two plastic tubes were attached to the branches of the Y-tube with silicone stoppers. The test substances were applied to pieces of filter paper, placed in the plastic tubes. Air was pulled through these and of the stem of the Y-tube at 3.5 l/min. A plastic vial containing a fly was attached to an entrance hole in the Y-tube, located 125 mm from the intersection, and flies were allowed to climb upwards into the Y-tube. If a fly had not entered the Y-tube within 3 min, a ‘no entry’ was recorded. When flies had entered the Y-tube, they were allowed another 3 min to choose between the two arms. The choice was recorded at the intersection of the Y-tube.

Experiments were carried out in a randomised block design. In each block, every treatment was run once in random order. For every treatment, five flies were used per block. Two treatments were carried out consecutively using two separate Y-tubes. Each fly was only used once. Between each trial, the Y-tube was rotated through 180° to account for any directional bias.

Y-tubes were cleaned before every treatment and after five flies had been tested with the same treatment by rinsing with acetone and distilled water and oven drying at 150°C.

**Response of flies to oestrous and luteal urine**

Flies were given a choice between either oestrous urine and distilled water or between luteal urine and distilled water. Fifty μl of urine or distilled water were applied and volatiles allowed to disperse for 30 s before a vial containing a fly was attached to the Y-tube. Fluids were kept in a water bath at 38°C (bovine body temperature) until application. In addition to choice tests between luteal phase and oestrous urine samples from each of the four cows and distilled water, separate tests compared two empty olfactometer arms and two arms containing filter paper with 50 μl of distilled water (double control). In total, there were 10 treatments (Table 1).

**Dose response of flies to 1-hexadecanol**

Five solutions of 1-hexadecanol (≥ 99% pure; Sigma-Aldrich Inc., St. Louis, MO, USA) were prepared in redistilled diethyl ether at concentrations of 100, 10, 1, 0.1 and 0.01 ng/μl (concentrations verified by GC-MS). Prior to experiments, 1 μl of the test 1-hexadecanol solution and 1 μl diethyl ether were applied to filter papers that were left outside the Y-tube for 1 min to allow the ether to evaporate. The papers were then placed in the plastic tubes and volatiles allowed to disperse for 30 s before a vial containing a fly was attached to the Y-tube. To determine whether the presence of ether affected the number of insects entering...
Table 1. Treatment comparisons in a Y-tube olfactometer bioassay of female *Musca autumnalis* response to urine from cows in oestrous and luteal cycles

| Treatment               | Arm 1          | Arm 2          |
|-------------------------|----------------|----------------|
| 1                       | Empty          | Empty          |
| 2                       | Distilled water| Distilled water|
| 3                       | Cow A luteal urine| Distilled water|
| 4                       | Cow A oestrous urine| Distilled water|
| 5                       | Cow B luteal urine| Distilled water|
| 6                       | Cow B oestrous urine| Distilled water|
| 7                       | Cow C luteal urine| Distilled water|
| 8                       | Cow C oestrous urine| Distilled water|
| 9                       | Cow D luteal urine| Distilled water|
| 10                      | Cow D oestrous urine| Distilled water|

the Y-tube, the proportion of flies entering when an ether double control was applied was also tested.

**Comparison of headspace of oestrous and luteal urine**

Three ml of each urine sample was thawed and placed in a double-necked 25-ml glass flask. Each flask was placed in a water bath at 38°C and nitrogen was introduced at a flow rate of 0.4 l/min through a Teflon tube in one of the necks. In the other neck, a glass tube (5 mm diameter) containing the molecular adsorbent Porapak Q (50 mg) (Supelco, Bellefonte, PA, USA) was inserted and the headspace drawn through at a rate of 0.3 l/min. The difference in flow rate created a positive pressure ensuring that air did not enter from outside the system. Volatiles were collected for 3 h, after which there was a 30 min pause, during which the equipment and the Porapak were dried to minimize condensation. The inside of the glass collection vessel was wicked with a clean, neutral laboratory paper wipe, and the Porapak tube was dried by passing through a gentle stream of nitrogen for 3 min. Volatiles were then collected for another 1.5 h. After this time, the Porapak tubes were eluted with 750 µl redistilled pentane and extracts stored in capped microvials at −20°C.

Volatiles were collected from aliquots of the four oestrous and four luteal phase urine samples used in the behavioural assay and from a control (distilled water). Three collections were made for each sample on three separate days. The three extracts were then combined to form one oestrous and one luteal phase extract for each cow. Extracted headspace samples were spiked with an internal standard (3,4-dimethoxybenzaldehyde to give 1.4 ng/µl in concentrated samples) and then concentrated to 10% of their original volume under a flow of nitrogen.

Volatiles were analysed by coupled gas chromatography-mass spectrometry. A 1 µl aliquot of each sample was injected onto an Agilent 7890A GC coupled to an Agilent 5975C mass spectrometer. The GC was fitted with an HP5 column (30 x 0.25 mm i.d., 0.25 µm film thickness, J & W scientific, Folsom, CA, USA) and oven temperature was maintained at 30°C for 1 min, then programmed at 5°C/min to 150°C, held for 0.1 min, then 10°C/min to 250°C. The carrier gas was helium and ionization was by electron impact at 70 eV.

GC traces from control and urine samples were compared to identify compounds of biological origin. Traces from oestrous aliquots were compared with the corresponding luteal traces to identify qualitative or quantitative differences between cycle stages. Compounds consistently present in greater amounts (minimum 2× greater peak area) in either luteal or oestrous extracts were noted. Only one compound fulfilled this criterion. This was identified as 1-hexadecanol by comparison of the mass spectrum with a commercially available library (NIST 08 MS Library, National Institute of Standards and Technology, Gaithersburg, MD, USA) and by comparing the mass spectrum and retention time with an authentic standard. The amount of 1-hexadecanol in each sample was calculated by selecting a characteristic, abundant ion (m/z = 83) and quantifying on a four-point response curve constructed using an authentic 1-hexadecanol standard (>99% pure; Sigma-Aldrich Inc.). Ion counts were first normalized against the internal standard to correct for any small differences in sample concentration or injection volume.

**Statistical analysis**

To compare differences in choice between the two treatments (oestrous and luteal urine), a logistic regression and a log likelihood test were carried out, using SAS software (ver. 9.2, SAS Inst. Inc., Cary, NC, USA). The statistical model included the fixed effect of type of urine and the random effect of cow, as well as the interaction between cow and type of urine. p-Values <0.05 were considered significant. Comparison of the mean amount of 1-hexadecanol in oestrous and luteal extracts was made using ANOVA on square root transformed data. The choices between each dose and the control in the 1-hexadecanol dose response test were compared using a ch² test. The ch² test was carried out using Minitab software (version 16, Minitab Ltd, Coventry, UK).

**Results**

**Response of flies to oestrous and luteal urine**

Results from the behavioural assay for oestrous and luteal urine are presented in Table 2. The choice

| Treatment               | Oestrous urine | Luteal urine | Distilled water | Empty arm |
|-------------------------|----------------|--------------|-----------------|-----------|
| Total no. flies tested  | 160            | 160          | 40              | 40        |
| No. entries into olfactometer | 66 (41)    | 64 (40)      | 18 (45)         | 26 (65)   |
| Total no. making a choice | 58 (88)  | 53 (83)      | –               | –         |
| No. choosing treatment | 19             | 28           | –               | –         |
| No. choosing control   | 39             | 25           | –               | –         |
difference between oestrous and luteal phase urine was calculated as odds ratio and the point estimate was 2.3 (p = 0.03) with a confidence interval at the 95% level ranging from 1.1 to 5.1. The flies were 2.3 times more likely to choose the control arm when exposed to oestrous urine, than when exposed to luteal urine. Preferences for either urine sample were not affected by which cow the urine came from. There was no statistically significant difference in number of entries between luteal phase and oestrous urine (p = 0.82).

Urine from one cow elicited significantly more entries (51%) than the other cows (35–41%) (p = 0.03), but the response pattern was similar to that of urine from the other cows for flies that entered the olfactometer. There was no significant interaction between cow and type of urine (p > 0.05).

**Comparison of headspace of oestrous and luteal urine**

Fifty-two compounds were found in the headspace from urine samples but not in the headspace from the control and were considered as being of biological origin. The majority of these compounds gave poor matches with the commercially available mass spectral library. Comparison of the chromatograms revealed little consistent variation in individual compounds between oestrous and luteal samples. However, one compound was present in consistently greater amounts in oestrous samples and was on average 3.2 ± 0.4 (mean ± SEM; range 1.8–3.8) times more abundant in oestrous than in luteal extracts. This compound was identified as 1-hexadecanol. Figure 2 shows a representative GC-MS ion trace from an individual cow during luteal and oestrous phases. The amount of 1-hexadecanol in each individual extract was quantified. The mean amount (±SEM) in the oestrous extracts was 5.49 ± 0.89 ng/μl and in the luteal extracts 1.86 ± 0.36 ng/μl, and this difference was statistically significant (ANOVA; F1,6 = 18.4; p = 0.005).

**Dose response of flies to 1-hexadecanol**

At the lowest 1-hexadecanol dose (0.01 ng), a significantly higher number of flies chose the arm containing the treatment over the control arm (p = 0.0001; \( \chi^2 = 14.50; \text{d.f.} = 1 \)) (Table 3). In contrast, a significantly higher number of flies chose the control over the treatment when exposed to the second lowest dose of 1-hexadecanol (0.1 ng) (p = 0.03; \( \chi^2 = 4.59; \text{d.f.} = 1 \)). For the higher doses, no significant preference for either arm was observed.

**Discussion**

*Musca autumnalis* distinguished between the odour of luteal and oestrous urine from cows, its principal host. Levels of 1-hexadecanol were higher in oestrous than in luteal urine, and this compound elicited a behavioural response in flies similar to that observed for oestrous urine. While it is difficult to compare the exact concentration of 1-hexadecanol flies were exposed to in the olfactometer assays with amounts collected from the different urine samples, this switch in behaviour may explain the significantly higher number of flies choosing the control arm when exposed to oestrous urine, which contained a higher amount of 1-hexadecanol compared with urine from the luteal phase.

Concentrations of 1-hexadecanol have not previously been reported to vary during the bovine oestrous cycle. Possible explanations for this discrepancy in results between studies may be that several compounds are involved in the signalling of oestrus and that the methods used for collecting and analysing substances vary greatly. There may also be differences between individuals of the same breed, between different breeds and also between animals in different environments.

Interestingly, 1-hexadecanol has previously been identified as a natural ligand of aphrodisin (Briand et al. 2004). Aphrodisin is a lipocalycin protein found in vaginal discharge of golden hamsters (*Mesocricetus auratus*) that induces mating behaviour in males. It is not known whether aphrodisin itself has pheromonal properties or whether it carries smaller, volatile pheromones (Tirindelli et al. 2009). The fact that 1-hexadecanol is a natural ligand of aphrodisin suggests that it could be a mammalian pheromone, which is further supported by other studies (Zhang et al. 2007; Hagemeyer 2010).

Interestingly, more flies entered the olfactometer when both arms were empty compared to those containing water. It is surprising that flies would show an aversion to high humidity. An alternative explanation is flies may be more active in low humidity as the greater risk of dehydration increases need to find a suitable feeding site, thus increasing their propensity to fly.
Bovine Oestrous Urine Odour Detected by Face Fly

Table 3. Response of female *Musca autumnalis* to different doses of 1-hexadecanol and ether control in a Y-tube olfactometer bioassay. Absolute numbers (and %) of flies tested, number of entries into olfactometer and choices. The number of choices between each dose and the control were compared using a $\chi^2$ test

| 1-Hexadecanol dose | Control Ether |
|---------------------|--------------|
| 100 ng              | 10 ng        | 1 ng        | 0.1 ng      | 0.01 ng     |
| Total no. flies tested | 70           | 70          | 70          | 75          | 70          | 20          |
| No. entries into olfactometer | 52 (74)      | 55 (79)     | 60 (86)     | 57 (76)     | 52 (74)     | 11 (55)     |
| Total no. making a choice | 47 (67)     | 41 (59)     | 47 (67)     | 49 (65)     | 40 (57)     | 9 (45)      |
| No. choosing treatment | 21           | 16          | 23          | 17          | 32          | 5           |
| No. choosing control | 26           | 25          | 24          | 32          | 8           | 4           |
| $p$ control vs treatment | NS         | NS          | NS         | 0.03        | 0.0001      | NS          |

Analysing urine 1-hexadecanol content could potentially be used as a cow-side test to determine oestrus. Both qualitative and quantitative differences in compound composition can be detected using electronic noses (Pérez Pavón et al. 2006). Further experiments are needed to determine whether oestrous-specific increase in concentration is common for all cows and to investigate the oestrous cycle dynamics of urine 1-hexadecanol concentrations. Although 1-hexadecanol varied qualitatively rather than qualitatively, it could potentially function as a pheromone on its own or in the context of the volatile blend. It would be interesting to investigate the effect on cyclicity parameters of exposing cows to 1-hexadecanol at different doses.

It is interesting that fly behaviour switched from preference of treated arm to preference of control arm across only a tenfold increase in 1-hexadecanol concentration, which is unusual in insects. As urine samples showed relatively little difference in their chemical composition, this could allow flies to modify their behaviour in response to subtle differences in urine odour.

Female face flies could potentially use 1-hexadecanol to determine whether a cow is in oestrus. One explanation for reduced preference of oestrous urine may be that bovine blood and other secretions are less suitable food sources for female flies during oestrus than during the luteal phase. The perioestrous period is characterized by great fluctuations of the steroidal hormones, with a fast decrease in blood progesterone after onset of luteolysis and a sharp oestriodial peak, emanating from the dominant follicle (Forde et al. 2011). However, previous studies show that anthropophilic mosquitoes are attracted to, not repelled by, oestrogens (Roessler 1961; Bos and Laarman 1975) and that the differences in women’s attractiveness during the menstrual cycle are correlated with changes in oestriodial secretions (Roessler 1963).

An alternative explanation may be the behavioural changes that occur in cows and heifers during the perioestrous period that could make settling and feeding more difficult. Before and during oestrus, animals become restless, spending less time lying down and more time interacting with herd mates including being mounted (Kiddy 1977; Van Eerdenburg et al. 1996). This implies that an individual cow in oestrus, or a herd with a large proportion of oestrous cows, may provide a risky environment for flies seeking to feed. Studies on biting insects have shown that relatively small differences in host suitability can strongly influence host choice (Kelly 2001).

This study shows that female face flies show a reduced preference for bovine oestrous compared with luteal urine and that can be at least partly explained by different concentrations of 1-hexadecanol. Further experiments are needed to determine whether this oestrous-associated difference in concentration is typical for cows in general, and to determine the dynamics of 1-hexadecanol concentrations in urine during the oestrous cycle. This could open the possibility to develop biosensors for oestrus detection based on insect antennal responses (Schott et al. 2013).

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Conflict of interest

None of the authors have any conflict of interest to declare.

Author contributions

All five authors participated equally in designing the study and in discussing data analysis and the results. Kristina Nordeus carried out most of the practical work and draft of the manuscript. Ben Webster, Lennart Söderquist, Renée Bäge and Robert Glinwood contributed to draft of the manuscript and the revision thereof.

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Author’s address (for correspondence): Robert Glinton, Department of Crop Production Ecology, PO Box 7043, SE-750 07 Uppsala, Sweden. E-mail: robert.glinton@slu.se