

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

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### 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 oestrus in dairy cows makes it difficult to determine optimal time for artificial insemination (Dobson et al. 2008). Many tools to aid oestrus 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, Kiddy 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 (Angioy 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 PGF<sub>2α</sub> (Cloprostenol sodium, Estrumat® 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 placed on ice after collection and transferred to a freezer at  $-20^{\circ}\text{C}$  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  $22\text{--}25^{\circ}\text{C}$  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  $-20^{\circ}\text{C}$  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  $180^{\circ}$  to account for any directional bias.

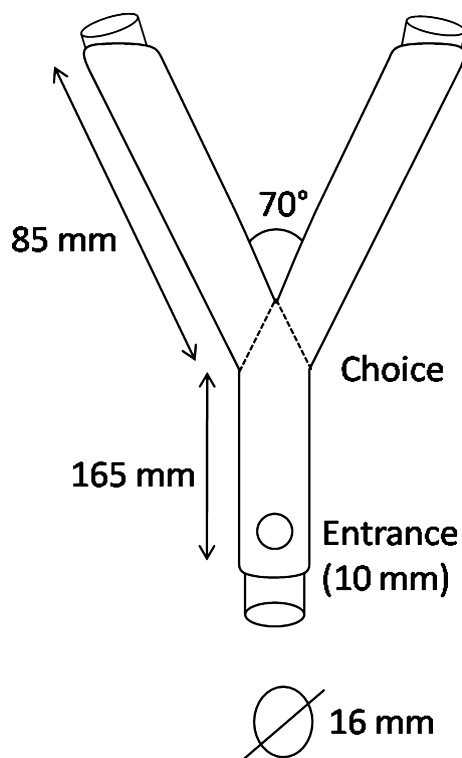


Fig. 1. Y-tube olfactometer

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  $150^{\circ}\text{C}$ .

### 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  $\mu\text{l}$  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  $38^{\circ}\text{C}$  (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  $\mu\text{l}$  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/ $\mu\text{l}$  (concentrations verified by GC-MS). Prior to experiments, 1  $\mu\text{l}$  of the test 1-hexadecanol solution and 1  $\mu\text{l}$  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 × 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  $\chi^2$  test. The  $\chi^2$  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

Table 2. Response of female *Musca autumnalis* to odour of urine from cows in oestrus or luteal phase in a Y-tube olfactometer bioassay. Control treatment was distilled water. Absolute numbers (and %) of flies tested, number of entries into olfactometer and choices. The choice difference between oestrous and luteal phase urine was calculated as odds ratio and the point estimate was 2.3 (p = 0.03), i.e. flies were 2.3 times more likely to choose the control arm when exposed to oestrous urine, than when exposed to luteal urine. Separate tests compared two empty olfactometer arms and two arms containing filter paper moistened with distilled water

	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., i.e. 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  $3.2 \pm 0.4$  (mean  $\pm$  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

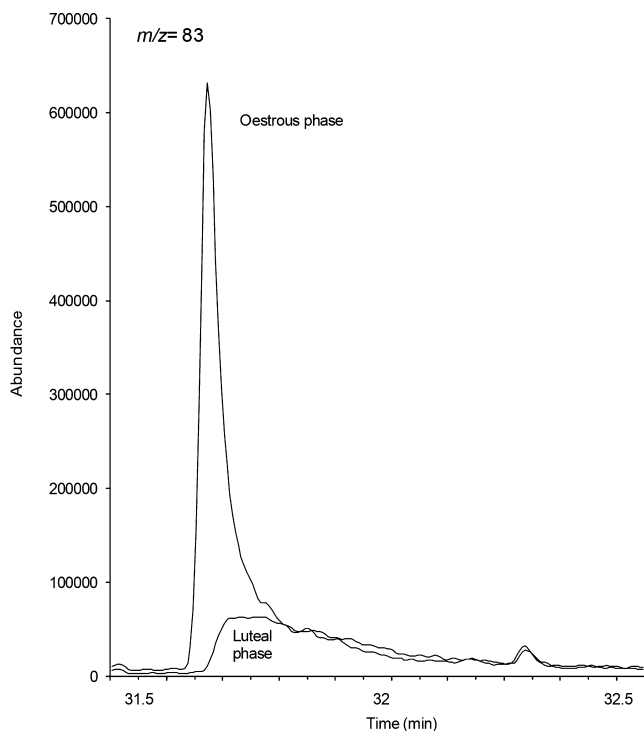


Fig. 2. Representative GC-MS trace of ion  $m/z = 83$  from the headspace of urine from an individual cow during luteal and oestrous phases showing the amount of 1-hexadecanol (cetyl alcohol) present in the sample

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 ( $\pm$ SEM) in the oestrous extracts was  $5.49 \pm 0.89$  ng/ $\mu$ l and in the luteal extracts  $1.86 \pm 0.36$  ng/ $\mu$ l, and this difference was statistically significant (ANOVA;  $F_{1,6} = 18.4$ ;  $p = 0.005$ ).

### 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 ( $p = 0.0001$ ;  $\chi^2 = 14.50$ ; 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) ( $p = 0.03$ ;  $\chi^2 = 4.59$ ; 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.

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 oestrus-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 quantitatively 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 oestradiol 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 oestradiol 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 oestrus 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 oestrus-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).

#### Acknowledgements

The authors thank U. Emanuelson for help with the statistical analysis, H. Muñoz and E. Qvarfordt for valuable assistance in the lab, J. Chirico for advice and equipment for fly rearing and R. Moon for providing pupae. K. Nordéus was funded by the Swedish Research Council Formas and the Faculty of Veterinary Medicine and Animal Science, SLU. L. Söderquist and R. Båge were funded by the Faculty of Veterinary Medicine and Animal Science, SLU. R. Glinwood was funded by Mistra through the PlantComMistra program. The collection of bovine urine was approved by the Uppsala regional ethical committee (approval no. C160/6).

#### 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 Nordéus carried out most of the practical work and draft of the manuscript. Ben Webster, Lennart Söderquist, Renée Båge and Robert Glinwood contributed to draft of the manuscript and the revision thereof.

#### References

- Angioy AM, Desogus A, Barbarossa IT, Anderson P, Hansson BS, 2003: Extreme sensitivity in an olfactory system. *Chem Senses* **28**, 279–284.
- Birkett MA, Agelopoulos N, Jensen KMV, Jespersen JB, Pickett JA, Prijs HJ, Thomas G, Trapman JJ, Wadhams LJ, Woodcock CM, 2004: The role of volatile semiochemicals in mediating host location and selection by nuisance and dis-

- ease-transmitting cattle flies. *Med Vet Entomol* **18**, 313–322.
- Bos H, Laarman J, 1975: Guinea pig, lysine, cadaverine and estradiol as attractants for the malaria mosquito *Anopheles stephensi*. *Entomol Exp Appl* **18**, 161–172.
- Briand L, Blon F, Trotier D, Pernollet JC, 2004: Natural ligands of hamster aphrodisin. *Chem Senses* **29**, 425–430.
- Carroll J, 2000: Responses of adult *Ixodes scapularis* (Acari: Ixodidae) to urine produced by white-tailed deer of various reproductive conditions. *J Med Entomol* **37**, 472–475.
- Dehnhard M, Claus R, Pfeiffer S, Schopper D, 1991: Variation in estrus-related odors in the cow and its dependency on the ovary. *Theriogenology* **35**, 645–652.
- Dobson H, Walker SL, Morris MJ, Routly JE, Smith RF, 2008: Why is it getting more difficult to successfully artificially inseminate dairy cows? *Animal* **2**, 1104–1111.
- Forde N, Beltman ME, Lonergan P, Diskin M, Roche JF, Crowe MA, 2011: Oestrous cycles in *Bos taurus* cattle. *Anim Reprod Sci* **124**, 163–169.
- Gibson G, Torr S, 1999: Visual and olfactory responses of haematophagous Diptera to host stimuli. *Med Vet Entomol* **13**, 2–23.
- Gilbert IH, Gouck HK, Smith N, 1966: Attractiveness of men and women to *Aedes aegypti* and relative protection time obtained with DEET. *Fla Entomol* **49**, 53–66.
- Hagemeyer P, 2010: Pheromones in social mole-rats and implications for the study of mammalian chemical communication. Universität Duisburg-Essen, Fakultät für Biologie und Geografie. [http://duepublico.uni-duisburg-essen.de/servlets/DocumentServlet/Document-24753/Diss\\_Hagemeyer.pdf](http://duepublico.uni-duisburg-essen.de/servlets/DocumentServlet/Document-24753/Diss_Hagemeyer.pdf). (accessed 2 August 2014).
- Hammer O, 1941: Biological and ecological investigations on flies associated with pasturing cattle and their excrement. *Videnskabelige meddelelser fra dansk naturhistorisk forening* **105**, 141–393.
- Haupt KA, Rivera W, Glickstein L, 1989: The flehmen response of bulls and cows. *Theriogenology* **32**, 343–350.
- Karlson P, Luscher M, 1959: Pheromones – a new term for a class of biologically active substances. *Nature* **183**, 55–56.
- Kelly DW, 2001: Why are some people bitten more than others? *Trends Parasitol* **17**, 578–581.
- Kiddy CA, 1977: Variation in physical activity as an indication of estrus in dairy cows. *J Dairy Sci* **60**, 235–243.
- Kiddy CA, Mitchell DS, 1981: Estrus-related odors in cows: time of occurrence. *J Dairy Sci* **64**, 267–271.
- Klemm WR, Hawkins GN, Delossantos E, 1987: Identification of compounds in bovine cervicovaginal mucus extracts that evoke male sexual-behaviour. *Chem Senses* **12**, 77–87.
- Ma W, Clement B, Klemm W, 1995: Cyclic changes in volatile constituents of bovine vaginal secretions. *J Chem Ecol* **21**, 1895–1906.
- Pérez Pavón JL, del Nogal Sánchez M, Pinto CG, Fernández Laespada ME, Cordero BM, Peña AG, 2006: Strategies for qualitative and quantitative analyses with mass spectrometry-based electronic noses. *Trends Analyt Chem* **25**, 257–266.
- Preti G, 1984: Method for detecting bovine estrus by determining methyl heptanol concentrations in vaginal secretions. United States Patent.
- Pryce JE, Royal MD, Garnsworthy PC, Mao IL, 2004: Fertility in the high-producing dairy cow. *Livest Prod Sci* **86**, 125–135.
- Ramesh Kumar K, Archunan G, Jeyaraman R, Narasimhan S, 2000: Chemical characterization of bovine urine with special reference to oestrus. *Vet Res Commun* **24**, 445–454.
- Rivard G, Klemm W, 1989: Two body fluids containing bovine estrous pheromone(s). *Chem Senses* **14**, 273–279.
- Roessler HP, 1961: Versuche zur geruchlichen Anlockung weiblicher Stechmücken (*Aedes aegypti* L., Culicidae). *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* **44**, 184–231.
- Roessler P, 1963: The attractiveness of steroids and amino acids to female *Aedes aegypti*. 50th Annual Meeting of the New Jersey Mosquito Extermination Association. pp.250–255.
- Royal MD, Darwash AO, Flint APE, Webb R, Woolliams JA, Lamming GE, 2000: Declining fertility in dairy cattle: changes in traditional and endocrine parameters of fertility. *Anim Sci* **70**, 487–501.
- Sankar R, Archunan G, 2004: Flehmen response in bull: role of vaginal mucus and other body fluids of bovine with special reference to estrus. *Behav Process* **67**, 81–86.
- Schott M, Wehrenfennig C, Gasch T, Vilcinskas A, 2013: Insect antenna-based biosensors for in situ detection of volatiles. *Adv Biochem Eng Biotechnol* **136**, 101–122.
- Tirindelli R, Dibattista M, Pifferi S, Menini A, 2009: From pheromones to behavior. *Physiol Rev* **89**, 921–956.
- Van Eerdenburg F, Loeffler HSH, vanVliet JH, 1996: Detection of oestrus in dairy cows: a new approach to an old problem. *Vet Q* **18**, 52–54.
- Van Geem T, Broce AB, 1985: Significance of cattle discharges and secretions as protein sources for ovarian development in the face fly (Diptera: Muscidae). *Environ Entomol* **14**, 60–64.
- Zhang J-X, Rao X-P, Sun L, Zhao C-H, Qin X-W, 2007: Putative chemical signals about sex, individuality, and genetic background in the preputial gland and urine of the house mouse (*Mus musculus*). *Chem Senses* **32**, 293–303.

Submitted: 24 Apr 2014; Accepted: 11 Jul 2014

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