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The relationship between milk oestradiol concentrations and oestrus activity in lactating Holstein Friesian cows

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Short title: Milk oestradiol concentrations during oestrus

Abstract.

Context. Detecting oestrus to facilitate the optimal timing of artificial insemination is key to optimal reproductive performance in dairy cows.

Aims. The aims of the present study were to investigate the relationship between activity and milk oestradiol concentrations during oestrus.

Methods. Accelerometers (IceQubes, IceRobotics Ltd., UK) were used to monitor the activity (the number of steps per day) of 37 lactating Holstein Friesian cows during the peri-oestrus period. Daily milk samples were analysed for oestradiol and progesterone concentrations.

Key Results. An increase in activity sufficient to generate an oestrus alert (behavioural oestrus) from an activity sensor (GEA Rescounter II) was detected in 29 of 37 (78%) cows. Milk oestradiol increased from 2.0 ± 0.5 pg/mL 4 days before oestrus to 8.3 ± 1.1 pg/mL on the day of behavioural oestrus, then fell to 2.6 ± 0.6 pg/mL by three days afterwards. Similarly, activity also increased and peaked on the day of oestrus. However, in 8 of 37 cows (22%), although milk oestradiol concentrations increased following the fall in progesterone there was no significant increase in activity (silent oestrus). Milk oestradiol concentrations were positively correlated (r=0.37; p = 0.03) with activity on the day of oestrus. Cows that subsequently became pregnant took a similar number of steps (2806 ± 282.3 vs 2850 ± 372.5 steps; PD+ vs PD- cows, respectively) but had higher oestradiol concentrations (11.2 ± 2.06 vs. 5.1 ± 0.51 pg/ml; PD+ vs PD- cows, respectively) on the day of oestrus than cows that did not become pregnant.

Conclusions. Increases in oestradiol concentrations above a threshold of approximately 10 pg/ml are associated with increased activity, and higher oestral oestradiol concentrations are associated with a higher rate of pregnancy following insemination.

Implications. This study demonstrates the significance of oestradiol concentration to the fertility of dairy cows. Thus, strategies to enhance oestradiol secretion may enhance fertility. Further, milk oestradiol measures may be developed as an aid to oestrus detection in lactating dairy cows, although further studies are required to determine whether on-farm assessment of milk oestradiol concentration can be developed to add to the repertoire of biomarkers of oestrus in lactating cattle.

Keywords: Dairy cow; Oestrus; Activity; Oestradiol

Introduction

Oestrus is the event in the cows' reproductive cycle that precedes ovulation. In dairy production systems where artificial insemination is used, accurate oestrus detection is one of the keys to reproductive success (Walsh *et al.* 2011). Oestrus detection rates are dictated by both observation of oestrus behaviours and the extent to which the cow expresses oestrus (Van Vliet and van Eerdenburg 1996). Previous studies show that there is considerable variation in the extent to which dairy cows express oestrus (Zebari *et al*. 2018), with less than 50% of oestrus events being detected (Palmer *et al.* 2010), resulting in considerable economic loss to the dairy industry because of reduced reproductive performance (Inchaisri *et al*. 2010).

Oestrous activity is stimulated by oestradiol (Dobson 1978; Mondal *et al.* 2006). During the follicular phase of the cow's oestrous cycle, the developing ovulatory follicle secretes oestradiol in increasing concentrations (Kaneko *et al.* 1991), following the fall in progesterone concentrations at the end of the previous luteal phase (Domènech *et al.* 2011). Both the high concentrations of oestradiol during oestrus and the preceding period of high progesterone are considered to be important in inducing oestrous behaviour through their influence on the hypothalamus (Allrich 1994).

The variation in oestrous behaviour that exists between cows is considered by some to reflect variation in circulating oestradiol concentrations. In support of this hypothesis, measurements of plasma oestradiol have been used to demonstrate a positive correlation between maximum oestradiol concentrations and both oestrous intensity score (Lyimo *et al.* 2000; Roelofs *et al.* 2004) and the duration of oestrus (Lopez *et al.* 2004). However, other studies have found a weak correlation (Madureira *et al.* 2015) or no relationship between blood oestradiol and oestrus activity when cows or heifers were observed for their oestrus activity during spontaneous (Coe and Allrich 1989; Glencross *et al.* 1981) or synchronised oestrus (Walton *et al.* 1987). In addition, the treatment of ovariectomised cows with oestradiol benzoate has resulted in varying concentrations of circulating oestradiol but no significant relationship with the strength of oestrous expression (Cook *et al.* 1986). These observations are attributed to oestradiol concentrations passing a threshold sufficient to induce the ovulatory surge of luteinising hormone and to the abundance of other factors that influence oestrus expression (Allrich 1994).

Oestradiol is measurable in milk (Pape-Zambito *et al*. 2007; Gyawu and Pope 1983). It is thought to transfer passively from plasma to milk, so milk concentrations of oestradiol are positively related to plasma concentrations (Glencross and Abeywardene 1983) and follow the same profile during oestrous cycles (Abeyawardene *et al*. 1984; Meisterling and Dailey 1987; Walker *et al.* 2010). To date, studies suggest that there is no correlation between oestrus intensity scores and milk oestradiol (Walker *et al.* 2008). However, there is a paucity of published information on the relationship between oestrus activity as measured by activity sensors and the concentrations of oestradiol in the milk of dairy cows. Therefore, the objective of the present study was to investigate the relationship between concentrations of oestradiol in milk and activity during oestrus. In addition, the relationship between both milk oestradiol, oestrus activity and the likelihood of pregnancy following artificial insemination in lactating Holstein Friesian cows undergoing spontaneous oestrous cycles was explored.

Materials and methods

The experiment was undertaken between January and March 2018 at the dairy unit of Harper Adams University, Newport, Shropshire, TF10 8NB, UK, in partial fulfilment of the PhD of HZ (Zebari, 2019). The Harper Adams University Research Ethics Committee approved the study protocol.

Experimental animals, housing and management

Non-pregnant, multiparous (parity 3.1 ± 0.29; mean ± sem) Holstein Friesian cows (n=37; 69.6 ± 3.55 days post-partum) were used for the study. The cows selected for the study had been observed in oestrus earlier in the post-partum period and were recruited approximately 7 days before their next expected oestrus. The average body condition score (BCS; Scale 1-5 AHDB no date) and locomotion score (LS; Scale 1-5 Chapinal *et al*. 2009) of the selected cows were 2.75 (2.5 - 3) (median (quartile 1-quartile 3)) and 2.0 (1-3), respectively. The cows were milked twice a day from approximately 05:00 and 16:30 through a 40-point rotary parlour (Westfalia, GEA Milking System, Germany). The milk yields of the cows were recorded at each milking. Average milk yield at the start of the study period was 35.2 ± 1.07 kg/day and the mean milk fat concentration of the samples collected was 36.4 ± 1.14 g/l.

Cows were housed within a group of approximately 200 early to mid-lactation cows, in a cubicle yard (stocking density was maintained at approximately 105 cubicles per 100 cows; cubicles 2.7x1.2 m). The cubicles were bedded with 3 cm rubber mattresses covered with sawdust and lime. The sawdust and lime were replenished three times per week. Grooved concrete passageways were scraped by automatic scrapers 4 to 5 times per day. The cows were fed a total mixed ration (dry matter (DM) 42.5%, metabolizable energy 12.2 MJ per kg DM, crude protein 16.5% of DM, neutral detergent fibre 36.4% of DM) *ad libitum* which was provided daily at approximately 08:30. Water was also provided *ad libitum* from water troughs at the end of each passageway.

Oestrous activity

Oestrous activity of the cows was monitored using GEA Rescounter ll pedometers (GEA Farm Technologies, Düsseldorf, Germany) attached to right front leg according to the manufacturer recommendations. The data generated by these sensors were downloaded at each milking to the GEA herd management software DairyPlan® (GEA Farm Technologies, Düsseldorf, Germany), according to standard practice on this dairy unit. This software compares the activity of a cow with its average activity over the preceding ten days. An oestrous alert was generated when the activity recorded over three consecutive 2-h periods, was at least two standard deviations above the average for the preceding ten days.

In addition, the activity of each cow was monitored using IceQube accelerometers (IceRobotics Ltd., Edinburgh, UK) attached to their back left leg using a Velcro hook and loop strap. IceQubes use 3-axis accelerometers to determine the number of steps taken per day, as well as time spent lying down per day, summarised in 15-minute blocks (Dolecheck *et al*. 2015). These data were also downloaded twice a day as the cows entered the parlour for milking and from these data oestrus was defined when activity increased to >80% above the mean activity for the preceding three days, followed by a decrease to <80% over the following two days (López-Gatius *et al*. 2008). The interval between the first increase in activity above the threshold and the fall below this threshold was considered as the duration of oestrus (López-Gatius *et al*. 2008).

Artificial insemination and pregnancy diagnosis

All of the cows which showed an increase in activity sufficient to generate an oestrus alert (see below) were artificially inseminated (n=29) approximately 12 hours after the oestrus alert. All artificial inseminations were performed by one trained dairy herd technician by transcervical fixation, using frozen-thawed semen (non-sexed) from one of six Holstein Friesian bulls selected independently of the present study. Cows that did not return to oestrus within 30 days of insemination (n = 27; 93%) were submitted for pregnancy diagnosis by the herd veterinarian between 30 and 40 days post-insemination using transrectal ultrasonography (Easi Scan-3, BCF Technology, UK) and cows were designated as either pregnant (PD+) or non-pregnant (PD-). Cows that had returned to oestrus between 18 and 30 days after AI were considered to be non-pregnant (n = 2; 7%). Overall, of the 29 cows that were artificially inseminated at oestrus, n = 15 (52%) were diagnosed PD+ and n = 14 (48%) were diagnosed PD-.

Milk samples

Composite whole milk samples were collected from each cow, daily at the afternoon milking from 7 days before the expected day of oestrus (to accommodate short oestrous cycles) until 4 days after the day that oestrus was detected by the GEA Rescounter ll (see below). The milk samples were stored frozen (-20°C) in two aliquots until assay for oestradiol and progesterone concentrations. The milk samples were analysed for the period from 4 days before to 3 days after the day of oestrus. The day of oestrus was defined as described below.

Milk oestradiol concentrations

Oestradiol concentrations were determined in daily milk samples collected from 4 days before to 3 days after oestrus, using a commercial enzyme-linked immunosorbent assay (ELISA; Oestradiol Ultrasensitive; ALPCO, Salem, NH 03079, US; assay sensitivity <1.399 pg/mL). The samples were analysed in duplicate (100 µL / duplicate) according to the method described by ALPCO with slight modification; an additional standard (1.5 pg/mL) was prepared by mixing equal volumes of 0 pg/mL and 3 pg/mL standards. Before assay the milk samples were brought to room temperature (22°C) and mixed well to ensure homogeneity. The intra- and inter- assay coefficients of variation were 4.3% and 8.1%, respectively.

Milk progesterone assay

Progesterone concentrations were determined in milk samples collected daily from 4 days before to 3 days after oestrus, using a commercial ELISA (Ridgeway Science Ltd., Gloucestershire, UK; assay sensitivity 0.15 ng/mL). The milk samples were brought to room temperature (22°C), mixed well to ensure homogeneity and assayed in duplicate (10 µL / duplicate). The intra- and inter- assay coefficients of variation were 9.5% and 12.6%, respectively.

Definitions of behavioural and silent oestrus

It was considered that a cow could be in oestrus when milk progesterone concentrations fell to <3 ng/mL for at least two days (follicular phase), before a period when progesterone rose to >5 ng/mL for at least 5 days (luteal phase) (Isobe et al. 2004). Each oestrus identified from the cow's progesterone profile was then classified as a *behavioural* or *silent* oestrus based on the GEA Rescounters activity output. A cow was defined to be in *behavioural oestrus* when her activity increased sufficiently to generate an oestrous alert. A cow was considered to be in *silent oestrus* when there were no changes in cow activity sufficient to generate an activity alert from the GEA Rescounter during a period of low milk progesterone concentrations (<3 ng/ml). The day of *silent oestrus* was taken as the day of highest milk oestradiol concentration while progesterone concentrations were low.

Data-set construction

During the study, 37 cows were detected in their second or third oestrus post-partum based on their milk progesterone profile. The five parameters analysed from four days before to three days after oestrus were the total number of steps taken by cows on each day (from the IceQubes), activity (GEA Rescounter II in arbitrary units; AU), the duration of oestrus (hours), milk oestradiol concentrations (pg/mL) and milk progesterone concentrations (ng/mL).

Statistical analysis

Statistical analyses were performed using the Genstat statistical software package (Genstat 19th edition, 19.1.0.21390, VSN International Ltd, UK). All of the data sets analysed were normally distributed (Shapiro-Wilk W test). The data (number of steps recorded by the IceQube, oestrous activity measured by the GEA Rescounter II, milk oestradiol, milk progesterone and milk yield) were analysed using a repeated measures ANOVA to compare between groups (*behavioural* and *silent oestrus*) from four days before to three days after oestrus and the group x day interaction. A factorial one-way ANOVA was used to compare parameters on the day of oestrus of cows in *behavioural* or *silent* oestrus and cows diagnosed *PD+* or *PD-*. Paired t-tests were used to compare between the days before oestrus, the day of oestrus and the days after oestrus.

Simple regression analysis was used to explore the relationships between the number of steps taken (response variable) and explanatory variables: milk oestradiol and progesterone concentrations on the day of oestrus, milk yield, days in milk, locomotion score, body condition score and parity, Y = a + bX + e, where Y = dependent variable, X = independent variable, b = regression coefficient, a = intercept, e = residual (error). The relationship between pregnancy outcome (response variable; pregnant = 1, non-pregnant = 0) and the explanatory variables including milk oestradiol concentrations, the number of steps and parity were compared by logistic regression analysis, Logit ($\hat{p}$) = $b0 + b_1X_1, +b_2X_2 +\ldots b_mX_m$ where b0 = constant, b_i = regression coefficients, X_i = independent variables. Additionally, the influence of other explanatory variables (progesterone on day 0 and day +3, milk yield, bulls, LS, BCS and days postpartum) on this relationship were assessed using forward stepwise logistic regression. This involved assessing the change in deviance on adding each of the selected variables in turn to a model including the constant term alone. Regression analysis was used to determine the relationship between the duration of behavioural oestrus and milk oestradiol concentration (pg/mL). Differences were reported as significant at $P \leq 0.05$.

Results

Effects of oestrus on activity during the perioestrus period

Of the 37 oestrus events detected by milk progesterone profile, 29 (78%) were behavioural oestruses. The duration of behavioural oestrus recorded by the GEA Rescounter II pedometers and the IceQube accelerometers were 13.4 ± 0.65 h and 12.9 ± 0.72 h, respectively. Activity measured by Rescounters was higher on the day of behavioural oestrus (1149.8 ± 303.61 AU) compared to the day of presumed silent oestrus (406.8 ± 41.66 AU). In cows that were determined in behavioural oestrus, activity recorded by the GEA Rescounter II had increased ($p < 0.001$) between day -1 and the day of oestrus (day 0) and had returned to basal levels by day +1. There was an effect of day and an oestrous expression x time interaction ($p < 0.001$) for steps per day recorded by the IceQubes (Table 1). In cows which were deemed in behavioural oestrus, the number of steps per day recorded by the IceQubes were not significantly different from days -4 to day -2 ($p = 0.434$). The number of steps per day increased ($p < 0.01$) from 1000.2 ± 55.54 on day -2 to 1180.7 ± 97.84 on day -1 and further increased ($p < 0.001$) to 2827.4 ± 227.50 steps on the day of behavioural oestrus (day 0). From day 0 to day +1 there was a fall ($p < 0.001$) in the step count to 1236.9 ± 122.84. By day +2 activity had fallen ($p = 0.004$) further to basal levels in the cows defined as having a silent oestrus and the number of steps taken per day was not significantly different from day -4 to day -1 ($p=0.496$). Steps per day increased ($p = 0.04$) from day -1 to day 0 (1140.0 ± 157.09 steps) and they were at basal levels by day +2 (see Table 1).

Milk oestradiol concentrations are shown in Fig. 1a. In cows that showed behavioural oestrus, milk oestradiol concentrations increased ($p = 0.001$) from 2 days before oestrus (1.9 ± 0.53 pg/ml), to 3.3 ± 0.75 pg/ml on day -1 and increased further ($p <0.001$) to 8.3 ± 1.22 pg/ml on the day of oestrus. Following oestrus, oestradiol concentrations fell ($p < 0.001$) to 5.0 ± 0.94 pg/mL on day +1 and 2.0 ± 0.73 pg/mL on day +2 and had fallen ($p = 0.037$) further to basal concentrations (1.1 ± 0.39 pg/ml) by 3 days after oestrus. The concentrations of oestradiol in milk were lower ($p = 0.018$) on the day of oestrus in cows which had silent oestrus compared to cows which showed behavioural oestrus. During silent oestrus, oestradiol concentrations were only significantly elevated on the day of presumed oestrus, increasing ($p <0.001$) from 1.0 ± 0.25 pg/mL on day -1 to

2.4 ± 0.29 pg/mL on day 0, then falling to 1.3 ± 0.19 pg/mL by day +1 ($p < 0.001$) after oestrus.

Milk progesterone concentrations (see Fig. 1b) fell from four days before oestrus to the day of oestrus in both cows that showed behavioural oestrus ($p < 0.001$) and those that had silent oestrus ($p = 0.02$). Subsequently, progesterone concentrations increased from the day of oestrus to one day after in both groups of cows ($p < 0.01$). By day 3, milk progesterone concentrations in cows that showed behavioural oestrus (15.6 ± 1.27 ng/mL) were higher ($p < 0.001$) than in those of cows that had a silent oestrus (5.8 ± 0.93 ng/mL).

Effects of oestrus on milk yield during the peri-oestrus period

The milk yields of cows that had a behavioural or a silent oestrus are shown in Table 1. Milk yields were reduced ($p < 0.001$) on the day of behavioural oestrus (31.4 ± 1.43 kg/d) compared to four days before (34.8 ± 1.51 kg/d) and three days after (34.3 ± 1.54 kg/d) oestrus. On the day of silent oestrus, milk yields (36.6 ± 2.04 kg/d) were similar ($p = 0.444$) in comparison to four days before (37.3 ± 2.34 kg/d) and three days after (35.5 ± 3.05 kg/d).

There was a weak, positive correlation ($r(37) = 0.39$, $p = 0.017$) between the activity of cows on the day of oestrus (as measured by the number of steps recorded by the IceQubes) and milk yield. This relationship is described by the following equation: IceQube steps = 4655 – 67.4(MY) + 1218; MY = milk yield kg/d).

The relationships among oestrous activity, milk hormone concentrations and cow factors

There was a weak, positive correlation between the concentrations of oestradiol in milk and the number of steps taken on the day of oestrus (Fig. 2). This relationship is described by the following equation: IceQube steps = 1215.7 + 263.5(E2) - 6.8($E2^2$) +1208.9; (r(37) = 0.37; p = 0.03; E2 = oestradiol). However, the relationship between milk oestradiol concentrations and the duration of behavioural oestrus was not significant (p = 0.495). There was no correlation (r(37) = -0.64; p = 0.472) between the concentrations of progesterone in milk and the number of steps taken on the day of behavioural oestrus. Neither the relationship between milk oestradiol and total milk yield (p = 0.549) or milk fat (p = 0.237) on the day of oestrus were significant.

The relationship between milk oestradiol and pregnancy outcome

Of 29 behavioural oestrus events which were followed by artificial insemination, 15 (52%) resulted in pregnancy (PD+). While the milk concentrations of oestradiol were higher (P = 0.009) on the day of oestrus in PD+ (11.2 ± 2.06 pg/ml) compared to PD- (5.1 ± 0.51 pg/ml) cows, oestrus was shorter (P = 0.022) in PD+ cows (11.4 ± 0.9 h) compared to PD- (14.6 ± 1.0 h) cows. However, there was no significant difference in the magnitude of oestrus activity, measured by either the IceQubes (2806 ± 282.3 vs 2850 ± 372.5 steps; PD+ vs PD-, respectively; p = 0.925) or Rescounters (1595 ± 570.2 vs 672 ± 48.3 PD+ vs PD- respectively; p = 0.131) on the day of insemination in the cows that became pregnant compared with those that did not. Milk progesterone concentrations were not significantly different (p = 0.138) on the day of insemination in PD+ (1.6±0.56 ng/ml) compared to PD- (0.7±0.11 ng/ml) cows. Milk progesterone concentrations increased after oestrus, so that by 3 days after oestrus, they were 17.8 ± 1.74 ng/ml in milk of PD+ cows and 13.2 ± 1.68 ng/ml in milk of PD- cows (p = 0.07). There was no difference (p = 0.767) in milk yield on the day of insemination in PD+ (31.8 ± 2.12 kg/day) and PD- (30.9 ± 1.97 kg/day) cows.

Stepwise logistic regression analysis indicated that on the day of behavioural oestrus, oestradiol concentrations were positively ($P < 0.001$) associated with the pregnancy outcome following AI. However, the pregnancy outcome following AI was negatively ($p = 0.01$) associated with parity. The pregnancy outcome following AI was not significantly associated with milk progesterone concentrations on the day of oestrus ($p = 0.097$) or on day +3 ($p = 0.060$). Milk yield ($p = 0.756$), oestrus activity ($p = 0.922$), locomotion score ($p = 0.390$), body condition score ($p = 0.178$) and days in milk ($p = 0.455$) were also not significantly associated with pregnancy outcome. When parity, milk progesterone concentrations on the day of oestrus (d0) and milk progesterone concentrations three days after oestrus (day +3) were added to the model that already included oestradiol and a constant, these factors reduced the residual deviance further.

The regression equation for this model is:

$$\text{Logit}(p) = -2.35 + 0.267(E2) - 0.307(\text{Parity}) + 0.079(P4\ d{+}3) + 0.158(P4\ d0); \quad (1)$$

$p = 0.008$; E2 = oestradiol; P4 = progesterone; d+3 = 3 days after oestrus; d0 = day of oestrus

Discussion

From the 37 oestrous events identified from milk progesterone profiles during this study, 29 were associated with behavioural oestrus as detected by both the IceQube and GEA Rescounter II activity sensors. This falls within the range of 51 to 87% oestrus detection previously reported in studies using pedometers (Roelofs *et al*. 2005a) or neck-mounted activity sensors (Fricke *et al*. 2014). It is also in line with the findings of a previous study of the herd used in the present study (Zebari *et al*. 2018), in which 65.5% of cows showed behavioural oestrus.

The peak of oestrus activity and the oestrus duration recorded by the IceQube accelerometers and GEA Rescounters were co-incident and similar to previous studies on the herd of dairy cows used in the present study (Zebari *et al*. 2018) and another published study (Roelofs *et al*. 2005b). Other published studies of cows wearing activity sensors report longer durations of oestrus (e.g. 14.3 h (Silper *et al*. 2015), 16.1 h (Valenza *et al*. 2012)). This may be due to heifers being used in one of these studies (Silper *et al*. 2015) and the fact that there were more cows in oestrus at the same time following oestrous synchronisation in the other study (Valenza *et al*. 2012).

In the present study, cows which showed a spontaneous, behavioural oestrus had a 4.4-fold increase in milk oestradiol concentrations, from basal concentrations 4 days before oestrus to a peak on the day of oestrous activity. Thus, the milk oestradiol profiles were similar to those reported for plasma oestradiol (Abeyawardene *et al*. 1984; Glencross and Pope 1981). However, the magnitude of the increase was about half that measured in plasma and while milk oestradiol concentrations were increased for only one day before behavioural oestrus in the present study, they are generally reported to be elevated for 2 to 3 days before oestrus in plasma (Glencross and Pope 1981). Further, the milk oestradiol profiles reported here were different from published plasma oestradiol profiles in that milk oestradiol concentrations remained elevated on the day after oestrus, while in plasma they are generally expected to have returned to basal concentrations by this time (Abeyawardene *et al*. 1984; Glencross and Pope 1981). Elevated oestradiol concentrations have previously been reported in milk on the day following behavioural oestrus (Monk *et al*. 1975), perhaps reflecting the difference in clearance of oestradiol from the blood stream compared with the udder.

In contrast to the cows which showed behavioural oestrus, those with silent oestrus had milk oestradiol concentrations that were only elevated on the day of presumed oestrus (the day when activity was highest during the low progesterone, follicular phase) and had returned to basal concentrations by the day after presumed oestrus. This finding is not surprising given the role of oestradiol, derived from the pre-ovulatory follicle, in stimulating oestrous behaviour (Boer *et al*. 2010). Overall, there was a positive relationship between oestrus activity (step count per day) and milk oestradiol concentrations. Previously published studies presented contradictory findings in this regard. In studies where oestradiol was measured in plasma, some studies also report a positive relationship between oestradiol concentrations and pedometer activity (for example, Madureira *et al*. 2015) or between plasma oestradiol and oestrus intensity scores (Lyimo *et al*. 2000; Roelofs *et al*. 2004; Aungier *et al*. 2015). Others (Cook *et al*. 1986; Pape-Zambito *et al*. 2007; Glencross 1987; Ribeiro *et al*. 2012) found no positive relationship, suggesting that cows will express oestrus once plasma oestradiol concentrations increase above a threshold (Coe and Allrich 1989). This suggestion fits with the quadratic equation found to best explain the relationship between milk oestradiol and step count in the present study, whereby there was no further stimulation of oestrus activity once milk oestradiol reached about 10 pg/ml.

In the present study, oestradiol concentrations, measured in whole milk, were higher (mean peak oestradiol 8.3 pg/ml) than those previously reported (Walker *et al*. 2010; Gyawu and Pope 1990). However, the oestradiol concentrations in these two published studies were measured in defatted milk (4.5 and 5.9 pg/mL, respectively). Given that oestradiol is a steroid and lipophilic, this difference can be explained, at least in part, by the partitioning of this hormone to the fat fraction of the milk. In support of this, another study (Meisterling and Dailey 1987) reported that 52% of the oestradiol content in milk is distributed in the lipid fraction and previous studies have shown a positive relationship between milk oestradiol and milk fat content (Glencross and Abeywardene 1983). However, it is also worthy of note that there was no relationship between the milk oestradiol and fat concentrations measured in the present study. In support of this, no difference between defatted (5.3 pg/ml) and whole milk (5.4 pg/ml) oestradiol concentrations has also been reported where a solid-phase radioimmunoassay was used to analyse milk samples without an extraction step (Lopez *et al*. 2002) perhaps reflecting that oestradiol is less lipophilic than other steroids (Pape-Zambito *et al*. 2007). Differences in oestradiol concentrations reported in the present study and those published previously may also be attributed to the milk sampling and processing regime and to differences in the analytical techniques employed, including the inclusion of extraction steps before hormone assay (e.g. Walker *et al*. 2010; Walker *et al*. 2008; Pape-Zambito *et al*. 2008) and differences in the nature of the assay employed (Lopez *et al*. 2002; Walker *et al*. 2010; Walker *et al*. 2008; Gyawu and Pope 1990).

The milk yield of cows used in the present study was reduced by nearly 10% on the day of behavioural oestrus compared to the 4 days before and 3 days after oestrus. Previous studies have reported similar reductions in milk production on the day of behavioural oestrus compared to non-oestrus days (Gwazdauskas *et al*. 1983). Lower milk yields on the day of oestrus may be a consequence of reduced dry matter intake during oestrus (Zebari *et al*. 2018) and reduced rumination time (Reith and Hoy 2012) since cows spend more time performing oestrus-related behaviours. Also, reduced milk yields may be a consequence of the negative effects of oestradiol on mammary gland milk synthesis (Tong *et al*. 2018) and oestrus on milk let-down.

Oestradiol concentrations were higher in the milk of cows that became pregnant following AI compared to those that were diagnosed as non-pregnant. As far as we are aware, this is the first study to report a positive relationship between milk oestradiol and the likelihood of pregnancy following AI in dairy cows undergoing spontaneous oestrous cycles. This finding is not surprising since oestradiol is involved with regulating the environment within the female reproductive tract, making it more suitable for the migration of the spermatozoa and oocyte and the developing embryo (Cruppe 2001) and agrees with other studies (López-Gatius *et al*. 2005; Rabeiro *et al*. 2012) which report higher plasma oestradiol in cows that subsequently became pregnant following artificial insemination.

Despite having differences in the maximum milk oestradiol concentrations on the day of oestrus, oestrus activity, as measured by the IceQubes, was similar in cows that became pregnant and those that did not. This contradicts other studies (Madureira *et al*. 2015; López-Gatius *et al*. 2005; Garcia *et al*. 2011) which show positive relationships between oestrous intensity and pregnancy outcomes and is unexpected as a more intense oestrus would be expected to make a more accurately timed AI possible, which may lead to better pregnancy rates (Cruppe 2001). Further, with regards to the duration of oestrus, oestrus activity was, on average, 3 hours longer in cows that did not become pregnant following AI in the present study. Longer oestrus periods have been reported (Båge *et al*. 2002) in heifers that return to oestrus following insemination, attributed to a delay in the surge of luteinising hormone, responsible for ovulation. Thus, the longer oestrus duration of cows that failed to conceive may have made it difficult to identify the optimal time for AI relative to ovulation (Bloch *et al*. 2006). Where the AM:PM rule is used, AI is timed relative to the onset of oestrus (Foote 1979). However, ovulation is reported as occurring approximately 19h after the end of oestrus (Roelofs *et al*. 2005a), so AI may be too early in cows with extended oestrus periods.

With regards to the success of AI in the present study, milk progesterone concentrations on the third day after oestrus was significant in the logistic regression model of factors associated with pregnancy outcome. These findings are consistent with the findings of a study by Mann *et al*. (2006) which showed that higher concentrations of progesterone during the early luteal phase resulted in increased dairy cow pregnancy rates. The positive relationship between pregnancy rate and progesterone concentrations following ovulation are thought to reflect the effects of progesterone on the uterine environment that lead to enhanced embryo development (Mann and Lamming 2006), increasing the likelihood of the maternal recognition of pregnancy.

Finally, cow parity was significant in the model of factors influencing pregnancy outcome in the present study, which agrees with findings that younger cows have significantly higher conception rates than older cows (Pursley *et al*. 1998; Chebel *et al*. 2004; Balendran *et al*. 2008; Opsomer *et al*. 2000). Older cows generally have higher milk yields than younger cows, which would increase the energy demands for milk synthesis, therefore negatively affecting their energy status (Butler and Smith 1989) and so their fertility. Also, the uterine environment may be altered with increasing parity (Opsomer *et al*. 2000).

Conclusions

In conclusion, the findings of the present study demonstrate positive associations between milk oestradiol concentrations and both oestrus expression and the likelihood of pregnancy following artificial insemination. With regards to oestrus expression, results support the idea of a threshold concentration of oestradiol to stimulate oestrus; with no further increase in oestrus activity at the highest concentrations of milk oestradiol. Oestradiol concentrations were elevated on the day that levels of activity were indicative of behavioural oestrus. Enhancing oestradiol production by the ovulatory follicle may improve reproductive performance in lactating dairy cows. Further studies are required to determine whether on-farm assessment of milk oestradiol concentration can be developed to add to the repertoire of biomarkers of oestrus in lactating cattle, supporting activity sensors and milk progesterone concentrations as oestrous detection aids.

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Tables

Table 1. Mean number of steps per day (IceQube steps), GEA activity (AU) and milk yield (kg/d), from 4 days before, to 3 days after oestrus and between behavioural (n=29) and silent (n=8) oestrus in lactating dairy cows.

		days from oestrus									p value		
	Oe Exp	d-4	d-3	d-2	d-1	d0	d+1	d+2	d+3	sed	Oe Exp	days	Oe Exp x day
IceQube steps	B	982	995	1000	1181	2827	1237	991	981	277.0	0.105	<0.001	<0.001
	S	999	985	1066	975	1140	989	940	990				
GEA activity	B	365	360	365	403	1150	411	362	382	262.9	0.107	0.012	0.244
	S	303	314	320	328	407	325	302	314				
milk yield	B	34.8	34.7	34.4	35.3	31.4	35.0	34.8	34.3	3.67	0.528	<0.001	0.016
	S	37.3	36.3	36.4	36.3	36.6	36.8	35.1	35.5				

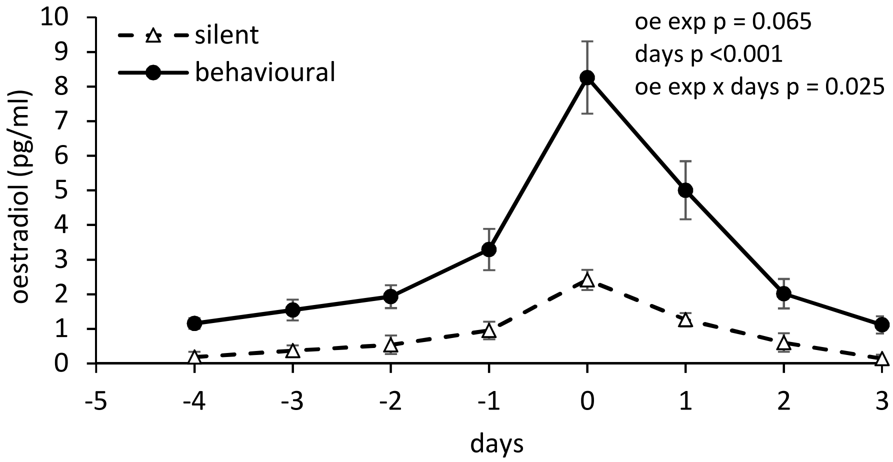
Oe Exp = Oestrous Expression, B = Behavioural oestrus and S = Silent oestrus, d0 = day of oestrus, sed = standard errors of differences

Figures

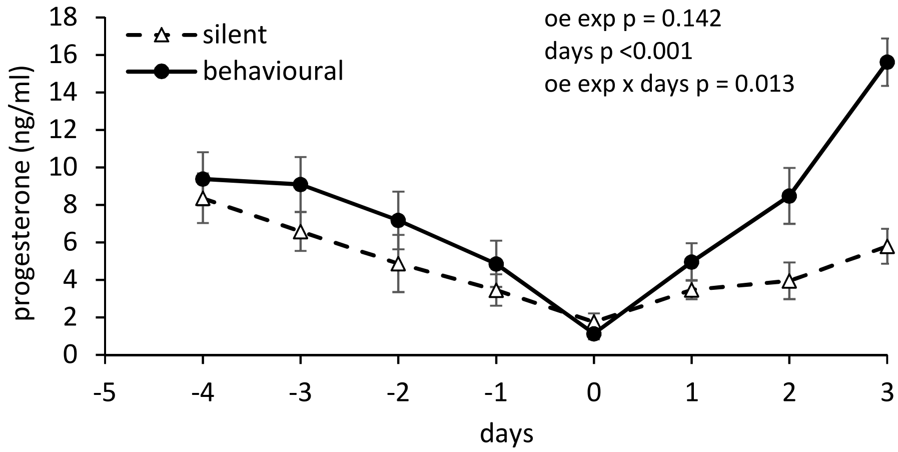
Fig. 1. Mean (± SEM) milk concentration of (a) 17β-oestradiol ng/mL and (b) progesterone ng/mL, from four days before to three days after behavioural (n=29) and silent (n=8) oestrus in lactating dairy cows. oe exp = Oestrous Expression, 0 = day of oestrus.

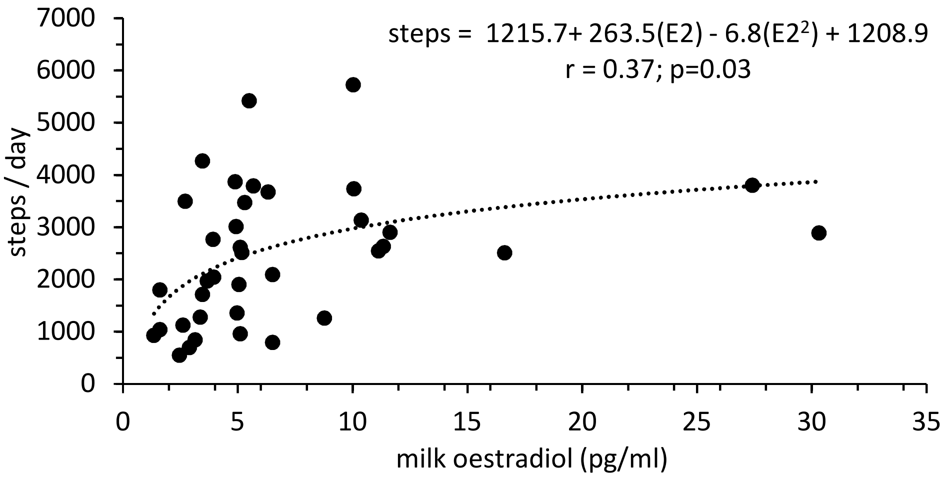
Fig. 2. The relationship between the concentration of milk oestradiol (pg/mL; E2) and the number of steps taken on the day of oestrus (steps) recorded by IceQube accelerometers in lactating dairy cows (n=37).

(a)


silent
behavioural
oe exp p = 0.065
days p <0.001
oe exp x days p = 0.025
oestradiol (pg/ml)
days

(b)


silent
behavioural
oe exp p = 0.142
days p <0.001
oe exp x days p = 0.013
progesterone (ng/ml)
days


steps = 1215.7+ 263.5(E2) - 6.8(E2²) + 1208.9
r = 0.37; p=0.03
steps / day
milk oestradiol (pg/ml)
7000
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
25
30
35