



**Harper Adams
University**

A Thesis Submitted for the Degree of Doctor of Philosophy at
Harper Adams University

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**Investigations of factors that influence oestrus
expression in dairy cattle**

By

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Abstract

Oestrus expression and detection are key in the reproductive management of dairy cows where AI is routinely used. Over the past 50 years, the percentage of dairy cows in oestrus that stand to be mounted has declined from 80% to 50% and the duration of oestrus has fallen from 15 h to 5 h. Furthermore, many cows show only the secondary signs of oestrus or do not show behavioural signs (silent oestrus). The first study was designed to determine whether cow time budgets were affected by behavioural and silent oestrus in lactating dairy cows. Of the 40 behavioural oestrus events that were detected, the number of steps were increased ($P < 0.001$) compared to three days before and three days after oestrus, whilst the percentage of lying time, the number of lying bouts, DMI, feeding duration and the number of visits to feed were reduced ($P < 0.001$). On the day of silent predicted oestrus, only the duration of feeding was reduced ($P < 0.03$).

The second study was designed to investigate factors affecting the strength of oestrus expression in dairy cows. The duration of oestrus was shorter ($P = 0.051$) in 1st oestrus postpartum (PP) with a lower intensity of oestrus expression on the day of oestrus compared to 2nd and $\geq 3^{\text{rd}}$ oestrus PP. More steps and a lower lying ($P < 0.001$) time with a longer oestrus duration ($P = 0.004$) were recorded when three cows or more were in oestrus (SG3+) simultaneously compared to one cow (SG1) in oestrus. Also a higher number of steps ($P < 0.001$) were taken when two cows (SG2) were in oestrus compared to SG1. More steps ($P < 0.001$) were recorded in body condition score (BCS) 2.75 cows compared to BCS ≤ 2.5 and BCS ≥ 3 . On the day of oestrus, more steps but a lower lying time and fewer lying bouts ($P < 0.001$) were recorded with a longer oestrus for cows of parity ≤ 2 . The number of steps taken was increased while lying time, and lying bouts decreased ($P < 0.001$) with increase locomotion score (LS). Oestrus duration was longer with a higher ($P < 0.001$) intensity in cows that had locomotion score one (LS1). This study also found cows spent more time ($P < 0.001$) walking with a longer oestrus duration in summer compared to other seasons.

To further investigate the factors that affect oestrus, the third study was designed to determine the relationship between milk oestradiol (E2) concentration and oestrus activity. Of the 39 oestruses detected from milk progesterone (P4) concentrations, 28 oestruses were behavioural and 11 were silent. Of the 28 behavioural oestruses, milk E2 concentrations increased from 2.0 ± 0.5 pg/mL to 8.2 ± 1.1 pg/mL on the day of oestrus. Milk E2 concentrations were significantly lower 1.3 ± 0.2 pg/mL during silent oestrus compared to behavioural oestrus. Overall there was a positive relationship between milk E2 concentrations and the number of steps taken ($r^2 = 0.73$; $P < 0.001$).

The fourth study was designed to determine the milk fatty acid profile of dairy cows during oestrus and day 14 of the dioestrus period and their relationship with oestrus activity. Milk samples were analysed for fatty acid concentrations using gas GC. On the day of oestrus, the concentration of acetic acid ($P < 0.001$), valeric acid ($P = 0.016$), caproic acid ($P < 0.001$) and myristoleic ($P = 0.035$) were higher in milk compared to day 14 after oestrus. However, on day 14 after oestrus, arachidonic acid concentrations in milk were higher ($P = 0.004$) compared to the day of oestrus.

In conclusion, from all these studies, approximately 59.9% of cows showed behavioural oestrus. Time budgets of the cows showing behavioural oestrus were disrupted with a lower lying time, feeding time but a higher number of steps per day. In cows undergoing silent oestrus, just feeding time was affected. Factors that affect oestrus intensity include the number of oestrus post-partum, SG, BCS, LS, parity, season and E2 concentrations. Concentrations of some milk FA were also affected. Further research is needed to determine whether these could become part of our oestrus detection arsenal.

Declaration

I hereby declare that this thesis has been composed entirely by me and all the studies described here has not been accepted in any previous application for any other degree of qualification. Sources of information and assistance have been specifically acknowledged by means of references.

Hawar M. H. Zebari

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Journal articles and conference proceedings

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- **Zebari, H M**, Rutter, S.M. and Bleach, E.C., 2017. Characterizing the activity and feed intake of lactating dairy cows during the peri-oestrus period. In: an annual international conference of British Society of Animal Science (BSAS) from 25th to 27th of April 2017 at Chester University, Chester, UK, (Abstract). It is published in *Journal of Animal*, April 2017, Volume **8**, Part 1, p69. ISSN: 2040-4700.
- **Zebari, H M**, Rutter, S.M. and Bleach, E.C., 2017. Characterizing changes in feeding behaviour of lactating dairy cows during the peri-oestrus period. In: an annual international conference of the International Society of Applied Ethology (ISAE) 2017 from 7th to 10th of August, Aarhus University, Aarhus, Denmark. (Abstract). Topic 04, p 124, *applied ethology 2017*.

Part of chapter 5 has appeared previously in:

- **Zebari, H M**, G L Charlton, S M Rutter and E C L Bleach, 2018. Effect of a number of cows in oestrus simultaneously on the activity of dairy cows in oestrus. In: an annual international conference of British Society of Animal Science (BSAS) from 8th to 11th of April 2018 at Cork Park, Dublin, South Ireland. (Abstract). Published in *Journal of Animal*, April 2018, Volume **9**, Part 1, p114. ISSN: 2040-4700.
- **Hawar M. Zebari**, S. Mark Rutter, Emma C.L. Bleach, 2018. Effect of body condition score on the activity of dairy cows in oestrus. In: an annual international conference of American Dairy Science Association (ADSA) from 24th to 27th of June 2018, Knoxville, Tennessee, USA. It is published in the *Journal of Dairy Science* Vol. **101**, p 27, Suppl. 2, 2018.

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Part of chapter 7 has appeared previously in:

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- **Hawar M. Zebari**, S. Mark Rutter, Emma C.L. Bleach, 2019. Fatty acid profile of milk for determining reproductive status in lactating Holstein Friesian cows. *Animal Reproduction Science*, Vol. **202**, 2019. Pp. 26-34.

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List of abbreviations

<u>Symbol</u>	<u>Name</u>
°C	Celsius
µg	Microgram
µL	Micro-litre
AI	Artificial insemination
AOD	Automated oestrus detection
AU	Arbitrary unit
BCS	Body condition score
vs	Versus
CL	Corpus Luteum
CP	Crude protein
d	Day
DIM	Day in milk
DM	Dry matter
E2	Oestradiol
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids
FSH	Follicle stimulating hormone
g	Gram
GC	Gas chromatography
GE	Gross energy
GnRH	Gonadotropin-releasing hormone
h	Hour
IS	Internal standard
Kg	Kilogram
LH	Luteinizing hormone
LS	Locomotion score
L	Litre
ME	Metabolisable energy
mg	Milligram

min	Minute
mm	Millimetre
mL	Millilitre
n	Number
NDF	Neutral detergent fibre
NEB	Negative energy balance
ng	Nano-gram
nm	Nano-metre
P4	Progesterone
PD	Pregnancy diagnosis
PD-	Non-pregnant cows
PD+	Pregnant Cows
pg	Picogram
PGF ₂ α	Prostaglandin F ₂ α
PGE	Prostaglandin E
PP	Post-partum
PRID	Progesterone-releasing intra-uterine
RIA	Radioimmunoassay
RICs	Roughage intake control system
rpm	Revolutions per minute
RRF	Relative response factor
SAG	Sexual activity group
SEM	Standard error of the mean
TMR	Total mixed ration
UK	United Kingdom
US	United State
VFA	Volatile fatty acid
3DA	Three days after
3DB	Three days before
4DB	Four days before

CHAPTER 1

General Introduction

Introduction

Globally the fertility of dairy cows has declined rapidly for the past two decades (Walsh *et al.*, 2011). The rate of fertility decline in the UK dairy herd is reported to be 1% per annum (Royal *et al.*, 2000; Homer, 2013). Similarly, it has been reported that the fertility of the US dairy herd is declining in the same pattern (Dolecheck, 2015). The fall in fertility of the European dairy cattle has been related to high milk yields per cow (Yániz *et al.*, 2008), because genetic selection in the dairy industry has focused more on higher milk yield and ignored selection for fertility (Lucy, 2001). Across Europe dairy herds, negative correlations between both milk yield and health, and milk yield and fertility have been reported (Barbat *et al.*, 2010). However, several factors affect fertility in modern dairy cows, besides genetic improvement for high milk yield, including disease, season, climate change, housing system, nutritional issues, management and herd environment (Cutullic *et al.*, 2009). Poor oestrous detection and expression are also important (Ranasinghe *et al.*, 2010).

In the UK, to get cows pregnant most dairy farmers use artificial insemination (AI) (Dobson *et al.*, 2008). Successful AI depends on delivery of semen into the cow's reproductive tract during the optimum time to fertilise the oocyte at ovulation and produce a viable embryo which is capable of establishing a pregnancy (Saacke *et al.*, 2000; Dalton *et al.*, 2001). The optimal time for AI is determined by the accurate detection of oestrus (Madureira *et al.*, 2015). Therefore, accurate oestrous detection has been identified as a key factor affecting dairy cow reproductive efficiency and improving pregnancy rate (Walsh *et al.*, 2011).

Over the past five decades, in accordance with high milk yield, the percentage of dairy cows in oestrus that show the definitive sign of standing oestrus has fallen from 74% (Van Vliet and Van Eerdenburg, 1996) to 44.9% (Dolecheck *et al.*, 2015). Thus detection of oestrus is recognised as one of the major problems in lactating dairy cows and remains a problem despite increased knowledge and understanding of dairy cow reproductive physiology and the development of automated oestrous detection (AOD) aids (Roelofs *et al.*, 2010). Where oestrus is accurately detected, calving intervals can be reduced from 389 to 365 days (Hanks and Kossaibati, 2017) and maximum conception rates within 85 days post-partum (PP) in lactating cows can be achieved (Roelofs *et al.*, 2010; Sveberg *et al.*, 2011). The failure to accurately determine the timing of oestrus leads to considerable economic losses for dairy farmers (Roelofs *et al.*, 2015). The cost of extended calving intervals is estimated to be between £2.50 and £5 per cow per day (Esslemont *et al.*, 2001).

In cows, the oestrous cycle varies from 18 to 24 days in length with an average of 21 days and the duration of behavioural oestrus is thought to range between 6 to 33 hours with an average of 8 h for the modern dairy cow (Lyimo *et al.*, 2000; Forde *et al.*, 2011). During behavioural oestrus, oestrogens produced from ovarian follicles affect the behavioural centres of the brain that can lead to oestrous expression (Domènech *et al.*, 2011) such as standing to be mounted, ano-genital sniffing, restlessness, bellowing, chin resting, head and side mounting, and attempting to mount (Gordon, 2011). In addition to the behavioural changes, lying time (Jónsson *et al.*, 2011), activity (as the number of steps; Dolecheck, 2015) as well as dry matter (DM) intake and feeding duration can be used as indicators of oestrous expression (Reith, 2016). The normal daily time budget of dairy cows is disrupted during oestrus (Valenza *et al.*, 2012) because cows in oestrus spend more time walking in comparison to rest and feeding time (Hurnik *et al.*, 1975).

Many factors affect oestrous expression such as the number of other cows in oestrus, nutrition, diseases (Gordon, 2011), management and housing system (Palmer *et al.*, 2010), type of floors such as dirt, clean and grooved concrete floor (Britt *et al.*, 1986), time of the day (Gwazdauskas *et al.*, 1983), and environmental temperature (Sankar and Archunan, 2012). Oestrous detection rate is affected by many factors including intensity of oestrus behaviour, the duration of oestrous expression (Lopez *et al.*, 2004), incidence of silent ovulation, the frequency and duration of monitoring (Valenza *et al.*, 2012) and the amount of time spent observing cows for oestrous signs (Van Eerdenburg *et al.*, 2002).

Around 70% of lactating Holstein Friesians were detected to be in oestrus when using an activity monitoring system as an aid to oestrus detection (Fricke *et al.*, 2014). Of the remainder, 20% of cows were considered as anovular and 10% ovulated without showing signs of oestrous activity (Fricke *et al.*, 2014). Research conducted on lactating Holstein cows by Dolecheck *et al.* (2015) achieved 62.1% and 65.5% accuracy of oestrus detection by visual observation (VO) and automated activity monitoring (AAM) technology, respectively. Increasing milk yield in Holsteins from 28 to 36 kg/d in early lactation may lead to an increase in the incidence of silent ovulations proportion from 0.7 to 1.6 ovulations, respectively (Harrison *et al.*, 1990). In the early PP period cows that lose more of their weight and condition take 30 days longer to show the first PP oestrus (Butler, 2003).

During the oestrous period, ovarian oestradiol stimulates the hypothalamus to release gonadotropin-releasing hormone (GnRH) which in turn stimulates the anterior pituitary gland to produce follicle stimulating hormone (FSH) and luteinizing hormones (LH) that lead to maturation of ovulatory follicles (Forde *et al.*, 2011). In general, it is well known that silent oestrus during 1st oestrus PP occurs due to a lack of exposure to progesterone (P4) before the rise in circulating oestradiol (E2) concentration (Allrich, 1994). In addition,

oestrogens play a key role in the regulation of the behavioural oestrus (Lyimo *et al.*, 2000). After a certain threshold of E2 is reached there will be an LH surge, which will result in ovulation. However, great variation exists concerning oestrous behaviour signs, which is also supposed to be induced by E2 concentration (Lyimo *et al.*, 2000).

Prior to ovulation E2 is secreted from developing follicles, and its concentration in blood will increase following the fall concentration of P4 at the end of the leuteal phase in non-pregnancy in dairy cattle (Domènech *et al.*, 2011). There is a strong negative correlation between E2 and P4 concentrations in blood and milk on the day of oestrus (Gorecki *et al.*, 2004). However, determining milk P4 concentrations alone is not enough to accurately predict the time of oestrus and ovulation because of a considerable variation in the time from the fall in P4 concentrations to ovulation (Roelofs *et al.*, 2006). Therefore, to increase oestrus detection rate and improve conception rate in dairy herds using AI, the determination of peak of E2 concentrations may more accurately indicate the time of oestrus before ovulation for achieving high fertilisation rates (Cavalieri *et al.*, 1997).

Pheromones are chemical signals which are released from one individual and induce specific endocrine and behavioural reactions in another individual of the same species (Vyas *et al.*, 2012). Pheromones have been detected in the urine (Ramesh Kumar *et al.*, 2000), faeces (Wiegerinck *et al.*, 2011) and vaginal secretions (Rekwot *et al.*, 2001) of cattle. Signalling pheromones from cows cause particular behavioural responses in bulls (Patra *et al.*, 2012). Bulls have differentiated oestrus and non-oestrus cows through the detection of urine pheromones (Vyas *et al.*, 2012). Bulls exhibit flehmen behaviour repeatedly after inhaling olfactory cues from cows to determine the stage of the reproductive cycle (Patra *et al.*, 2012). During oestrus, some studies found certain fatty acids (FA) at higher levels in bovine urine and faeces than other stages during the oestrous cycle (Kumar and Archunan, 2006; Gnanamuthu and Rameshkumar (2014). The appearance of certain FA in higher quantity in various body fluids during oestrus may be used as an alternative method of oestrus detection (Kumar and Archunan, 2006).

Strategies to improve oestrous detection rate and detection of factors affecting oestrous expression in modern dairy cows may therefore lead to improving reproductive performance and enhance the sustainability of the dairy industry.

CHAPTER 2

Literature Review

2.1. Oestrous cycle in cattle

Puberty occurs in heifers at 6 to 12 months of age and approximately 200 to 250 kg body weight (Forde *et al.*, 2011). At this age they initiate their first oestrous cycle (Forde *et al.*, 2011). The cyclical activity of the ovary is represented by the oestrous cycle during which female animals go from a non-receptive reproductive state to a receptive state that leads to mating and possible subsequent pregnancy (Cartmill *et al.*, 2001). The cow is poly-oestrus (cows ovulate several times throughout the year; unlike mares and ewes which only have oestrous cycles at a certain time of the year) and the length of oestrous cycles ranges from 18 to 24 days with an average of 21 days (Galina and Arthur, 1990; Forde *et al.*, 2011; see Figure 2.1). The oestrous cycle is divided into two phases (Ireland *et al.*, 1980; Sirois and Fortune, 1988; Cartmill *et al.*, 2001). The first phase is the follicular phase lasting 4 to 7 days (Ireland *et al.*, 1980). This phase follows the regression of the corpus luteum (CL) and lasts until ovulation (Forde *et al.*, 2011). It has two sub-divisions, pro-oestrus and oestrus (Cartmill *et al.*, 2001). Following ovulation, the oocyte is released into the oviduct from the mature ovulatory follicle (Forde *et al.*, 2011). The second phase is the luteal phase lasting 14 to 18 days. During this phase, the CL develops (Sirois and Fortune, 1988). This phase may be subdivided into met-oestrus and dioestrus (Forde *et al.*, 2011). The oestrous cycle in females occurs as a result of hormonal interaction resulting in cyclical changes in the ovaries (Cartmill *et al.*, 2001).

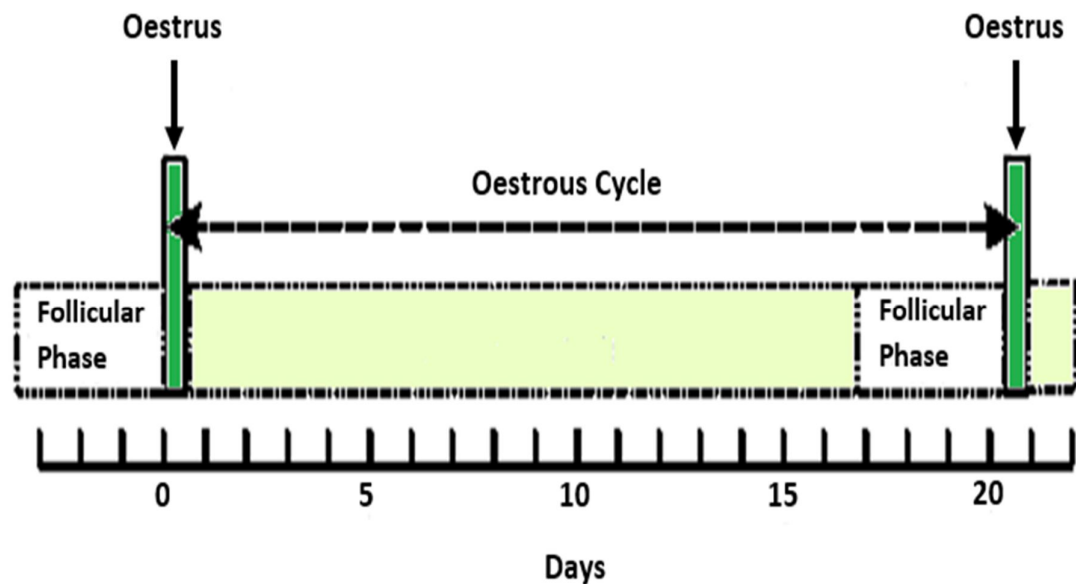


Figure 2. 1. The bovine oestrous cycle usually lasts 18 to 24 days so normal, non-pregnant cows should show signs of oestrous behaviour (heat) approximately every three weeks (King, 1996). Dark green shading indicates the time of oestrus, light green shading indicated the luteal phase.

2.1.1. The endocrine and physiological events leading to resumption of oestrous cycles postpartum

In cattle, following parturition the cow enters a post partum anoestrus period before oestrous cycles resume (Forde *et al.*, 2011). This typically lasts for 13 to 50 days post partum and ends with the first post partum ovulation (Stevenson, 2001). This first ovulation may or may not be preceded by any obvious signs of oestrus (Isobe *et al.*, 2004).

Production of large amounts of E2 and P4 by the placenta during late pregnancy exerts strong negative feedback effects on the hypothalamus, resulting in a decreased release of GnRH (Forde *et al.*, 2011). The resumption of normal PP ovarian cycles is regulated mainly by the rate of recovery of the hypothalamic-pituitary axis and normal secretions of GnRH and LH (Webb *et al.*, 1992). Failure of PP dominant follicles to undergo final maturation is related to the absence of appropriate GnRH and LH pulses, which is necessary for final follicular maturation and subsequent ovulation (Webb *et al.*, 1992). The lack of adequate GnRH and LH pulse frequencies is largely because of two main interrelated factors; 1) increased sensitivity of the hypothalamic GnRH pulse-generator to the negative feedback effect of E2, which results in the absence of GnRH pulses and subsequently LH pulse frequency remains low (Allrich, 1994), and 2) suckling stimulus which causes the release of endogenous opioid peptides from the hypothalamus that indirectly inhibits GnRH release (Gazal *et al.*, 1998). In addition, other factors can influence the length of the PP anoestrus period (Roelofs *et al.*, 2010), including uterine involution (Dobson-Hill, 2009) and nutritional status and negative energy balance (Gutierrez *et al.*, 2006; Roche *et al.*, 2009).

After parturition prostaglandin F_{2α} (PGF_{2α}) produced from the uterine endometrium causes regression of the CL and a fall in P4 concentrations (Forde *et al.*, 2011). This allows pulsatile LH release from the anterior pituitary gland (Webb *et al.*, 1992). The LH pulses stimulate the growth and development of waves of antral follicles (Forde *et al.*, 2011). The frequency of LH pulses and the size of the dominant follicles in each waves are affected by GnRH (Webb *et al.*, 1992; Webb and Campbell, 2007). Oestrus behaviour prior to the LH surge is more likely from the second oestrus PP (Garnsworthy *et al.*, 2008). The oestrous stage of the follicular phase marks the beginning of the oestrous cycle which is following by 1st ovulation PP and ending at the next episode of oestrus (Forde *et al.*, 2011). Once established in the PP period, oestrous cycles continue unless interrupted by pregnancy (King, 1996). This process is completed in approximately 20-40 days post-calving if no complications arise (Dolecheck, 2015).

2.1.2. Hormonal control of the oestrous cycle in cattle

The oestrous cycle is controlled through negative and positive feedback mechanisms involving several hormones (Wiltbank *et al.*, 2002). These hormones include gonadotropin-releasing hormone (GnRH) from the hypothalamus in the brain, follicle stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary gland, progesterone (P4), oestradiol (E2) and inhibins from the ovaries and prostaglandin F2 α (PGF2 α) from the uterine endometrium (Roche, 1996). The tonic centre of the hypothalamus secretes GnRH, and this hormone reaches the anterior pituitary gland via the hypophyseal portal blood system (Moenter *et al.*, 2003), controlling the secretion of both LH and FSH from the anterior lobe of the pituitary gland (Schally *et al.*, 1971). Figure 2.2. shows the pattern of hormone secretion throughout the oestrous cycle of cattle and the relationship of hormone secretion to the development of key structures on the ovaries. Follicle stimulating hormone stimulates the emergence of 2 or 3 follicle waves per oestrous cycle, while the growth and development of the dominant follicle are under the control of LH pulses which result in the secretion of E2 by the dominant follicle (Beam and Butler, 1999; Adams *et al.*, 2008). Oestrus is initiated as a result of increasing levels of LH in a positive feedback mechanism loop with E2 (Forde *et al.*, 2011). These increases in E2 (the dominant steroid hormone during the follicular phase of the oestrous cycle) result in increasing frequency of low amplitude LH pulses which in the absence of P4 (Vailes *et al.*, 1992) lead to a surge which culminates in ovulation (Forde *et al.*, 2011). After ovulation, during the luteal phases, the concentration of P4 increase as a result of its production by the developing CL which is formed from the theca cells of the ovulated dominant follicle (Ireland *et al.*, 1980). Production of P4 continues if a pregnancy is established and is required for the maintenance of pregnancy (Schwall *et al.*, 1986). During this time high amplitude LH pulses are released at low frequency (Schwall *et al.*, 1986).

In non-pregnant cows, the concentration of P4 decreases from approximately day 15 as a result of the regression of the CL caused by PGF2 α produced from the uterine endometrium (Ireland *et al.*, 1980). This allows LH pulse frequency to increase, increasing the concentration of oestrogen from pre-ovulatory follicles (Sirois and Fortune, 1988). Elevated E2 initiates oestrus activity and the LH surge which ovulates the dominant follicle (Crowe and Mullen, 2013).

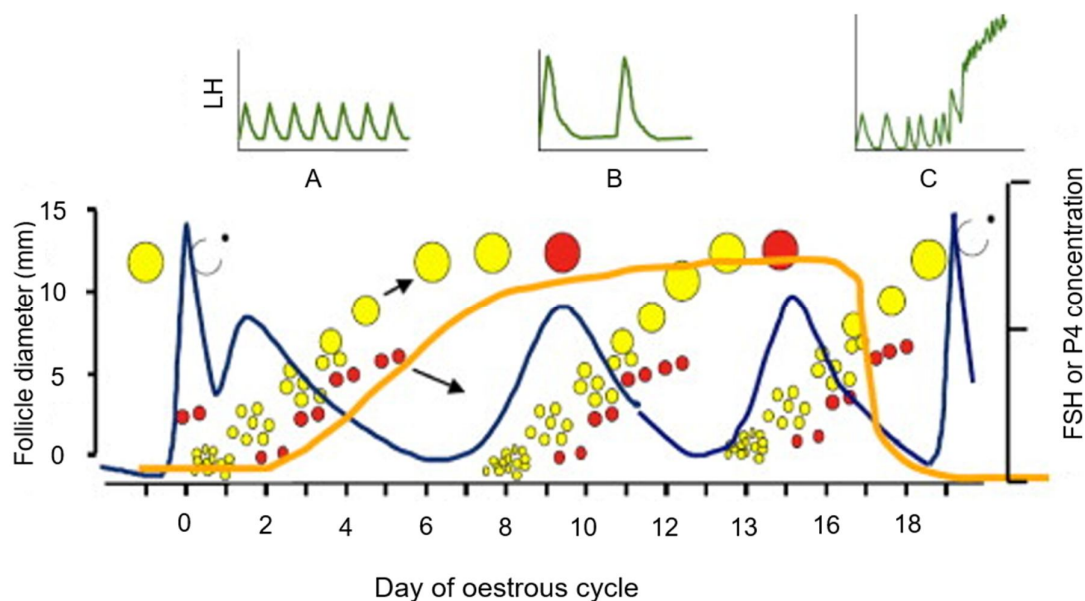


Figure 2. 2. Hormonal change and follicular waves throughout the oestrous cycle in cattle. A- Follicular phase, B- Luteal phase and C- Oestrus. The blue line shows the FSH secretion, green lines are the secretion of LH, and the orange line indicates the P4 hormone. The outline of the growth of three waves of ovarian follicles throughout the oestrous cycle in cattle is also shown. Yellow circles represent healthy growing follicles, and red circles are regressing follicles. LH pulses have a greater frequency but lesser amplitude through an 8h window in the early luteal stage while LH pulses have lesser frequency and greater amplitude in the mid-luteal phase. High frequency of LH pulses results in a surge LH during the follicular phase which causes ovulation (C) (Forde *et al.*, 2011).

2.1.3. Follicle development

The ovaries of sexually mature female mammals contain a mass of follicles at various developmental stages (Eppig, 2001). Primordial follicles contain an oocyte surrounded by a single layer of granulosa cells (the FSH receptor) then, after forming the follicular reservoir, the follicles gradually start to grow and form primary follicles (Rodgers and Irving-Rodgers, 2010).

For high reproductive efficiency in farm animals, follicle growth, development and maturation are considered vital processes (Forde *et al.*, 2011). During foetal development in cattle, approximately 150,000 primordial follicles develop in the ovaries (Bao and Garverick, 1998; Garnsworthy *et al.*, 2008), and each primordial follicle is composed of a single oocyte surrounded by a squamous follicular epithelium (Vendola *et al.*, 1999). In mono-ovular animals such as cattle, only about 0.1% of these follicles are ovulated

because most of the primary follicles which are recruited become atretic and do not develop into a dominant follicle (Webb *et al.*, 2003).

The growth of ovarian follicles takes about 3 to 4 months from recruitment to ovulation and is divided into two stages: a gonadotrophin dependent phase and an independent gonadotrophin phase (Webb *et al.*, 2004). During the gonadotrophin dependent phase, the pre-antral follicles are recruited to continue growing by FSH secretion from the anterior pituitary gland (Lucy *et al.*, 1992). Some follicles are selected to escape atresia, and one continues to grow as a dominant follicle and then ovulates (Lucy *et al.*, 1992). In cattle, 2-3 cohorts of follicles are recruited per oestrous cycle from gonadotrophin dependent follicles, as a result of an increase of FSH concentration (Savio *et al.*, 1990). While in some cows only one wave develops, in others as many as four waves can occur, with ovulation of the dominant follicle of the final wave (De Rensis and Peters, 1999; Aerts and Bols, 2010). The inherent lifespan of each follicle wave is 7 to 10 days (Diskin and Sreenan, 2000). Emergence, selection and dominance of follicles are involved in each cohort followed by either atresia or ovulation of the dominant follicle (Lucy *et al.*, 1992). Approximately 3 to 5 follicles emerge in each wave and grow to reach 6-8 mm in diameter (Webb *et al.*, 2004). Then one follicle continues to grow into a dominant follicle before ovulating or undergoing regression (Webb *et al.*, 2004). New waves of follicles emerge and are stimulated to develop by a transient increase in the concentration of FSH one to two days before emergence (Stagg *et al.*, 1998; Diskin and Sreenan, 2000).

The gonadotrophin independent phase of follicle development is following by the gonadotrophin dependent phase (Webb *et al.*, 2004). During the selection process, each wave of follicles is preceded by increases in the secretion of FSH for about one to two days, stimulating the growth of smaller (<2mm diameter) follicles (Webb *et al.*, 2003). Follicular stimulating hormone dependence occurs with cohorts of 5-20 follicles greater than or equal to 5 mm diameter (Webb *et al.*, 2003). Through the selection process, the concentrations of E2 and inhibin increase as a result of an increase of follicle size (Hillier, 1994). Secretion of FSH from the anterior pituitary gland falls back to basal concentrations due to negative feedback mechanisms involving E2 and inhibin produced by the developing dominant antral follicle (Ginther *et al.*, 2000). This basal level of FSH allows the growth and initiation of a new follicular cohort (Gibbons *et al.*, 1999). The dominant follicle is selected and continues to grow to reach an average of 8.5 mm in diameter then begins to emerge from the wave due to its increased receptiveness of the dominant follicle to LH (Ginther *et al.*, 2000). At this stage of the oestrous cycle the dominant follicle switches to LH dependency (Kulick *et al.*, 1999). The dominant follicle continues to grow, and produce increasing concentrations of E2 leading to an increased frequency of hypothalamic GnRH pulses and increased secretion of LH pulses from the anterior

pituitary gland (Homer, 2013). This stimulates further E2 production from the granulosa cells of the dominant follicle (Fortune, 1994). The high levels of E2 initiate oestrus then ovulation of the dominant follicle occurs approximately 24 hours later as a result of an LH surge (Homer, 2013).

2.1.4. Oestrus and ovulation

Oestrus refers to the period when a female cow is sexually receptive and is also called heat (Perry, 2004). In cattle, oestrus usually lasts about 15h with range of 4 and 24 h (Perry, 2004) when the cow or heifer stands to be mounted it is known as standing oestrus or true oestrus (Hurnik *et al.*, 1975). During oestrus, cows show behavioural signs as a result of the influence of E2, and such behavioural signs include restlessness, standing to be mounted, sniffing the ano-genital tract of other cows, presence of clear mucus from the vulva, swelling and reddening of the vulva (Sveberg *et al.*, 2011), bellowing, chin resting and head mounting (Gordon, 2011). In high yielding dairy cows, as milk production increases from 33.5 to 46.4 kg milk, this leads to a decrease in the duration of oestrus from 10.9 to 6.2 h, respectively (Lopez *et al.*, 2004). It appears that the duration of oestrus in heifers (12.4 h) is longer than cows (8.9 h; Roelofs *et al.*, 2005a). The duration of oestrus measured by continuous monitoring of Holstein cows by video recording were 7.5, 7.8 and 10.1 h, when one, two or three cows were in oestrus at the same time, respectively (Hurnik *et al.*, 1975). However, Kerbrat and Disenhaus (2004) found that the duration of oestrus in Holstein cows, housed in loose housing system with a concrete floor, monitored by continuous video recording was 14.1 ± 4.5 h. Sveberg *et al.* (2011) reported that the duration of standing oestrus was 7.1 ± 1.4 h, and mounting oestrus was 12.9 ± 1.8 h in lactating Holstein-Friesian cows monitored by camera recording. The frequency of the secondary signs of oestrus, which are initiated and received, significantly decreased 3 h after standing oestrous (Sveberg *et al.*, 2011). Kerbrat and Disenhaus (2004) reported that the number of mounts (6.6 vs 0.0 and 0.0), chin-resting (34.5 vs 0.6 and 0.3) and sniffs of the ano-genital region (18.6 vs 0.5 and 0.3) were significantly higher on the day of oestrus compare to the day before and the day after, respectively.

The principal signal to the brain which initiates oestrus expression is oestrogen, particularly E2 but only in the absence of P4 (Vailes *et al.*, 1992). An increase in E2, enhanced by LH, stimulates the theca cells (containing LH receptors) of the ovulatory follicle to produce androgen (Garverick *et al.*, 2002). The aromatase enzyme from granulosa cells converts this androgen into E2, and it is released into the blood causing oestrous expression (Fortune, 1994). LH pulse frequency increases to about one pulse per hour as a result of the positive feedback mechanism between E2 and LH (Roche, 2006). As a result of the increase in the concentration of LH, prostaglandin E (PGE) is

produced from the theca cells of the follicle, resulting in ovulation and release of the oocyte into the oviduct (Aerts and Bols, 2010). The rupture of the follicle wall at ovulation occurs by proteolytic enzymes that are produced from cells under the stimulation of prostaglandins (Sartori and Barros, 2011). The follicle itself controls this process by stimulating the surge in the release of LH (Roelofs *et al.*, 2010). The LH surge lasts for about 10 to 14 h after the onset of oestrus (Forde *et al.*, 2011).

2.1.5. Luteal phase

The CL originates from the cells of the ruptured ovulatory follicle under the control of LH which is considered the main luteotrophic hormone in bovines (Milvae *et al.*, 1996; Forde *et al.*, 2011). The theca and granulosa cells of the dominant pre-ovulatory follicle are luteinised into luteal cells then form the CL from the cells after ovulation of the mature follicle (Alila and Hansel, 1984; Forde *et al.*, 2011). Throughout the luteal phase of the oestrous cycle, sufficient concentrations of P4 are produced from the CL in order to sustain a pregnancy if conception has occurred (Forde *et al.*, 2011). The production of P4 during the luteal phase decreases the pulse frequency of GnRH and LH preventing the occurrence of behavioural oestrus as well as preventing ovulation of the dominant follicles (Forde *et al.*, 2011). During this period the dominant follicles which developed undergo atresia instead of ovulation (Forde *et al.*, 2011). During the first two to three weeks of pregnancy, the follicular waves occur regularly and stop about 21 days pre-partum (Ginther *et al.*, 2000).

If pregnancy has not occurred, the CL is luteolysed around day 16 of the cycle as the uterine endometrium of the non-pregnant cow produces PGF2 α (Lamothe *et al.*, 1977). Production of PGF2 α the main luteolytic hormone in the ruminant animal is brought about by oxytocin, produced by the CL (Ginther *et al.*, 2010). Receptors for oxytocin on the endometrium membrane of the uterus bind oxytocin (Forde *et al.*, 2011) which stimulates the pulsatile release of prostaglandin F2 α (Flint and Sheldrick, 1983). The luteolytic mechanism is mediated by PGF2 α through a counter-counter exchange of PGF2 α from the uterine vein to the ovarian artery leading to regression of the CL (Forde *et al.*, 2011). This causes the decline in circulating concentrations of P4 at the end of the luteal phase, following by increases in the frequency of pulses of GnRH from the hypothalamus and LH from anterior pituitary gland and so the follicular stage of the oestrous cycle can start again (Forde *et al.*, 2011; Homer, 2013).

2.1.2. Silent oestrus

The failure of high yielding cows to express oestrous behaviour during the postpartum period is defined as silent ovulation (Peter *et al.*, 2009). Globally, silent ovulation is considered one of the main reasons for poor oestrous detection efficiency (Lopez *et al.*,

2002; Yániz *et al.*, 2006) and it can significantly decrease reproductive performance in dairy cattle (Lucy, 2001). Silent oestrus results in mistimed AI which leads to poor conception rates in dairy cows (Ranasinghe *et al.*, 2010). Silent oestrus often occurs in the early PP period (Isobe *et al.*, 2004), particularly preceding first ovulation. This is thought to be caused by high levels of oestrogen pre-partum and low P4 concentrations during the early PP period (Allrich, 1994). The high level of E2 causes a refractory state in the hypothalamus and a lack of responsiveness to LH (Allrich, 1994). The release of P4, during the first luteal phase after silent oestrus and ovulation, removes this refractoriness leading to oestrus expression preceding the second PP ovulation (Allrich, 1994).

Several studies of silent oestrus, using different methods of oestrous detection, have indicated that the majority of first PP ovulations are preceded by silent oestrus (Senger, 1994; Shipka, 2000). King *et al.* (1976), using visual observation of tie-stall housed cows, found that the percentages of silent oestrus at the first, second and third PP ovulation were 80%, 56% and 36%, respectively. Similarly, Isobe *et al.* (2004), using milk P4 profiles and visual observation, showed that the incidence of silent oestrus at the first, second and third PP ovulation was 83%, 46% and 13%, respectively in free-stall housed Holstein-Friesian cows. In another study monitoring walking activity of cows using pedometers, it was reported that the occurrence of silent oestrus was 55.2%, 23.8% and 21.3% at the first, second and third ovulation PP in Holstein cows housed in a free-stall barn (Ranasinghe *et al.*, 2010).

2.2. Signs of oestrus

The period of sexual receptiveness within the oestrous cycle in cows is called behavioural oestrus and is considered to be the external and visible signs of physiological mechanisms of an internal, invisible event of ovulation (Roelofs *et al.*, 2010). This phenomenon occurs as a result of a specific effect of E2 and P4 from the ovary on the mammalian behavioural centres in the brain (Roelofs *et al.*, 2010). Oestradiol production from the pre-ovulatory follicle increases to a threshold 3-4 days before oestrus and, acting at the level of the hypothalamus, activates a series of programmed neurological events resulting in behavioural oestrus (Reames *et al.*, 2011). Other centres in the brain are also activated to release gonadotropin-releasing hormone at a surge level which is required for ovulation within 24 to 32 h after oestrus (Walker *et al.*, 1996).

2.2.1. Primary sign of oestrus

Standing to be mounted is considered as the primary and definitive sign that a cow is in behavioural oestrus (Van Eerdenburg *et al.*, 1996) see (Figure 2.3 (i)). Standing oestrus is

the period between the first and last time that the cow stands to be mounted and is also defined as 'true oestrus' when the cow is in a pre-ovulatory and sexually receptive state (Orihuela, 2000; Palmer *et al.*, 2010). In Figure 2.3 (i), the cow underneath is in oestrus, and it is allowing itself to be mounted by the other cow. Cow in oestrus remain stationary without resistance when mounting is attempted (Orihuela, 2000). In modern dairy cows, the duration and intensity of standing oestrus have decreased, and the mean duration of standing oestrus is about 6.2 h with 6.3 standing events (Lopez *et al.*, 2004). However, only about 50% of cows which are in oestrus express standing behaviour because not all cows which ovulate stand to be mounted (Van Eerdenburg *et al.*, 2002). Traditionally, for accurate oestrus detection and subsequently the proper time for AI, cows were visually observed for true oestrus (Dransfield *et al.*, 1998).

2.2.2. Secondary sign of oestrus

In addition to the primary sign, cows in oestrus exhibit some secondary signs of oestrus (Homer, 2013). In modern dairy cows, the secondary behavioural signs may be more useful for detecting oestrus accurately, because of the reduced standing behavioural signs (Dobson *et al.*, 2008). Secondary signs (see Figure 2.3, a-h) including different sexual, social and agonistic interactions that including the behavioural expression changes of the cow (Sveberg *et al.*, 2011). Sexual interaction includes attempts to mount other cows by raising the front limbs, mounting the head end of other cows with standing or without standing, mounting of their herd mates sideways, resting the chin on the rump of other cows, and licking or sniffing the ano-genital region of a cow (Sveberg *et al.*, 2011). Social interaction includes flank, head and neck licking of another cow (Kerbrat and Disenhaus, 2004). Aggression and butting others are considered agonistic interactions (Kerbrat and Disenhaus, 2004; Sveberg *et al.*, 2011). In addition, bellowing, flehmen, a pink and swollen vulva and clear mucus discharge from the vulva are all secondary signs of oestrus (Foote, 1975; Van Eerdenburg *et al.*, 1996).

Oestrous detection refers to the ability to accurately detect behavioural oestrus to maintain good reproductive performance in high yielding dairy cows when AI is used (Kinsel and Etherington, 1999). Low oestrous detection rates leads to loss of milk yield, a decrease in the number of calves born per lifetime, extended calving intervals and an increase in the number of cows culled as a result of infertility; therefore, this results in increased financial losses for dairy farmers (Walker *et al.*, 1996). Conversely, accurate oestrous detection before AI leads to an increased conception level because both the fertilisation rate and embryo quality depend on the correct timing of oestrus to detect ovulation (Roelofs *et al.*, 2010).

Sniffing the vulva of another cow.

- (a) Scuffed tail head dirty flanks and sweating.
- (b) Chin resting, both cows may be coming into oestrus.
- (c) Headbutting each other; both cows may be coming into oestrus.
- (d) Soliciting.
- (e) Bellowing and restlessness.
- (f) Licking— both cows may be in oestrus.
- (g) Mounting head to head.
- (h) Standing to be mounted, the underneath cow remaining stationary allowing the above cow to mount.

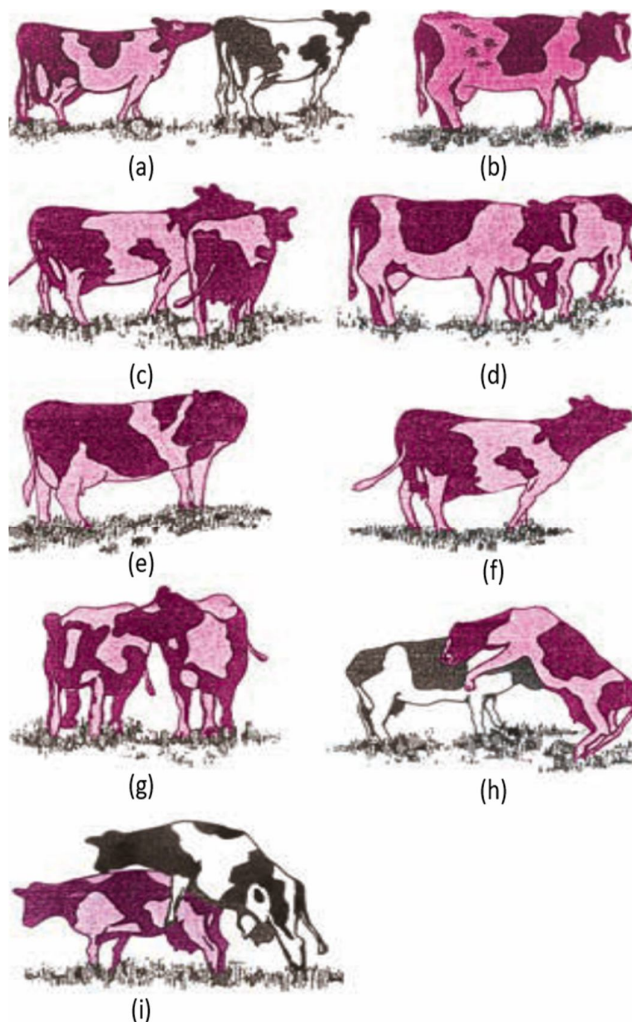


Figure 2. 3. The cows which are shaded red exhibit oestrous behavioural signs including primary (i) and secondary signs (a-h) toward the black and white cow, adapted from (DairyCo - Pd+ Section 6 - Managing heat detection - AHDB Dairy).

2.3. Methods of oestrous detection

Detection of a high percentage of behavioural oestrus in dairy cattle is important in maximising reproductive performance in dairy herds (Fricke *et al.*, 2014). For optimal fertility, oestrous detection aids must be highly correlated with the optimal time of insemination (Nebel *et al.*, 2000).

2.3.1. Visual observation

Visual observation is considered one of the best traditional and most accurate methods of oestrous detection and the most definitive method of detecting standing oestrus in dairy cows (Dolecheck, 2015). The optimal time for AI is approximately 12 h after the initiation of standing oestrus (Dransfield *et al.*, 1998). The accuracy of visual observation to identify true oestrus accurately is about 20% (Kiddy, 1977). To identify cows in oestrus, a secondary sign of oestrus can also be used although, in comparison to standing head, the secondary signs are not as reliable indicators of true oestrus (Diskin, 2008).

By visual observation, 56-58% of oestrus can be detected (Williamson *et al.*, 1972; Liu and Spahr, 1993). A scoring system has been developed to aid visual oestrous detection by Van Eerdenburg *et al.* (1996). According to the system, 100 points are given to a standing heat, but secondary signs of oestrus are also taken into account. Using this score, 74% of standing oestrus can be detected when cows were visually observed for about 30 minutes on 12 occasions per day and this reduced to 37% of standing oestrus when observed 3 times daily for 30 min, showing a low sensitivity of detection of oestrus using 3 times daily observation (Van Eerdenburg *et al.*, 1996). Using these scores, Palmer *et al.* (2010) detected about 20% of true standing oestrus in cubicle housed Holstein-Friesian cows when observing three times per day, with each observation period lasting 20 min. However, this method detected 70% oestruses when both the primary and secondary signs were considered by visual observation in 3 periods of 30 min per day (Van Eerdenburg *et al.*, 1996). Another study, using the same method, yielded 53% detection rate (Lyimo *et al.*, 2000).

Achieving efficient oestrus detection by visual observation requires experience, diligent attention and time (Harris *et al.*, 1989). Increasing herd size and increasing reliance on unskilled labour make this difficult to achieve (Michaelis, 2016). Observation of the cow at the wrong time or place, such as at feeding time or milking time, are other issues leading to lower accuracy of oestrus detection in lactating dairy cows (Diskin, 2008). Poor expression of oestrus in high yielding dairy cows is considered another problem that affects oestrus detection rate (Homer, 2013), due to an increase in the number of silent oestruses (Ranasinghe *et al.*, 2010). Intensity and duration of oestrous are reduced in modern dairy cattle, which is also a problem for the visual detection of oestrus (Homer,

2013). Therefore, alternative methods of oestrus detection have been developed to increase oestrous detection rates in modern dairy cattle.

2.3.2. Video camera recorder

The video recorder is considered one of the successful methods of recording standing mounts and the secondary oestrus sign for free-stall or tie-stall barn housed animals (Firk *et al.*, 2002). The most accurate methods of obtaining data on the duration of oestrus are continuous surveillance recording of the oestrous signs by video, in addition to continuous visual observation (Nebel *et al.*, 2000). In spite of their cost-effectiveness, the use of video camera to monitoring cow by continuous recording is limited, because it is too time-consuming to review recordings on a daily basis (Lehrer *et al.*, 1992).

2.3.3. Heat mount detectors

The most non-technical and cheapest methods of oestrus detection are pressure sensitive detectors of mounting behaviour (Foote, 1975). One such method of heat mount detector is tail paint which is applied to the cow's tail head before the expected onset of oestrus (Pennington *et al.*, 1986). The paint will be rubbed off totally or partially (>50%) when the cow which is in oestrus is mounted by other cows (Firk *et al.*, 2002). Other similar methods include KAMARS and scratch cards (when mounting activity occurs, the scratch cards is rubbed and changes colour to indicate that the cow is in oestrus (Diskin and Sreenan, 2000)).

Oestrous detection rates using tail paint vary from 44% to 96% in dairy cows (Pennington *et al.*, 1986; Diskin and Sreenan, 2000). Sawyer *et al.* (1986) reported oestrous detection rates of 78% using tail paint in pasture grazing dairy cows. In a more recent study a higher detection rate (91.7%) were recorded where tail paint was used on Holstein and Jersey cows in New Zealand with no falsely detected oestruses (Xu *et al.*, 1998). The sensitivity of tail paint for oestrous detection in an oestrous-synchronised dairy herd was significantly higher than for KAMARS and scratch cards and significantly better than pedometers (91.3 vs 85.7%) and (88.4 vs 81.4%), respectively (Cavalieri *et al.*, 2003). Macmillan and Curnow (1977) found also that approximately 95% of oestrous detection achieved using tail paint as an oestrous detector in three lactating dairy herds at pasture in New Zealand. However, Palmer *et al.* (2010) found that the efficiency of tail paint to detect standing oestrus was just 26% in cubicle housed Holstein Friesian cows. The lower proportion of oestruses detected by Palmer *et al.* (2010) using tail paint may be due to the effect of housing environment which resulted in mounting in the cubicles as a result of the slippery floor, lameness and close confinement stress.

Another method of oestrous detection by mounting is a scratch card (Holman *et al.*, 2011). These are fixed to the tail head of the cow, and when the weight of the mounting cow applies pressure, the colour of the scratch card (heat mount detectors) changes to indicate standing oestrus (Diskin, 2008). A study conducted on 67 housed Holstein Friesian cows in the UK found a sensitivity of 35.9% for the scratch card (Scratch card, Dairy mac, Hampshire, UK) in comparison to 74% for oestrus detection using the milk P4 profiles as an aid (Holman *et al.*, 2011). Heat mount detectors such as tail paint are ineffective for oestrous detection in cubicle-housed dairy cows in the UK (Ducker *et al.*, 1983). The efficiency of tail paint and scratch cards may be affected by the type of housing, the flooring and the other management system of the dairy farm (Lehrer *et al.*, 1992).

2.3.4. Teaser and androgenised bulls

For detection of oestrus, active vasectomised teaser or detector bulls are useful, particularly during the early PP period in dairy cattle (Diskin and Sreenan, 2000). Chin balls can be fitted under the chin of the teaser bull, leaving long, linear marks of paint on the back and rump of the oestrous cow (Foote, 1975). Blockey (1978) reported that the presence of a teaser bull may improve the percentage of cows detected in natural oestrus compared to synchronised under pasture conditions. In addition, teaser bulls may be able to predict the approach of oestrus by several days (Klemm *et al.*, 1994). The teaser bulls may initiate more interactions with pro-oestrous cows (days before oestrus), which were characterized by increased sexual behaviours at the early oestrus period compared to the presence of female herd mates (French *et al.*, 1989). Further to this, Stevenson (2001) reported that the treatment of cows or heifers with testosterone or E2 increased their sexual activity and made them useful as oestrus detecting animals in the dairy herd. This has been accomplished with injections of testosterone propionate or testosterone enanthate (Britt, 1980), subcutaneous implants containing testosterone propionate and E2 benzoate (Mortimer *et al.*, 1990), or E2 benzoate injection (Hackett and Lin, 1985). A combination of one injection of testosterone enanthate together with an implant containing testosterone propionate or E2 benzoate were most effective for producing oestrous detector females with greater sexual behaviour than in animals treated with the implants or hormone injections alone (Nix *et al.*, 1998). Stevenson and Britt (1977) found that accuracy of androgenised animals to detect oestrus was equal to visual observation with a slight increase in efficiency. Androgenised cattle selectively mounted only nonpregnant cows compared to other mounting herd mates (Mortimer *et al.*, 1990). Hackett and Lin (1985) reported that for continuously housed cows maintained indoors, hormonally treated cows were less effective in detecting oestrus than visual observation.

2.3.5. Milk progesterone (P4) profile

In the non-pregnant cow, the endometrium releases PGF₂α from day 18 after ovulation resulting in lysis of the CL and a decline in the concentration of the blood P4 before the next ovulation (Thatcher *et al.*, 2003). Declining P4 concentrations in milk can be detected at this time because there is a close relationship between milk and blood concentrations of P4 (Roelofs *et al.*, 2006). Gorecki *et al.* (2004) also reported a strong correlation ($r^2 = 0.9$; $P < 0.001$) between the P4 concentration in blood and milk. Domènech *et al.* (2011) found that the concentration of P4 in milk was reduced in the absence of pregnancy in dairy cattle. Desaulniers *et al.* (1989) reported that the concentration of milk P4 was 1.2 ng/mL around the time of oestrus in Holstein heifers. Milk P4 concentrations decline from >15 ng/ml on an average 97.7 ± 8 h before ovulation to <5 ng/ml and <2 ng/mL around ovulation (Roelofs *et al.*, 2006). Isobe *et al.* (2004) found that the milk P4 profile dropped to <5 ng/mL during silent oestrus in free-stall housed Holstein-Friesian cows. Cerri *et al.* (2011) reported a low concentration of P4 of approximately 1.7 ± 0.1 ng/mL in lactating Holstein cows. Roelofs *et al.* (2006) found that the concentration of P4 declined approximately two days before ovulation in dairy cows. However, there is a large individual variation in the time of the decline in P4 concentration in relation to the time of ovulation (Roelofs *et al.*, 2006). In addition, in the lactating dairy cows, circulating concentrations of P4 decreased around the time of oestrus and ovulation as a result of pattern of follicular wave development (Wiltbank *et al.*, 2006) and the release of PGF₂α from the endometrium (Shaham-Albalancy *et al.*, 2001).

Declining milk P4 concentration below 2 ng/mL can be used as an oestrous detection tool (Friggens and Chagunda, 2005). Using milk P4 as an aid to oestrous detection has the advantage over other oestrous detection aids in that silent oestrus can be detected (Dolecheck *et al.*, 2015). Furthermore, Ranasinghe *et al.* (2010) reported that about 55% of the first PP silent oestruses were detected by milk P4 concentration. Friggens *et al.* (2008) reported that more than 93% of oestrus could be detected by milk P4 resulted in pregnancy by AI. However, milk and blood P4 measurement have disadvantages including the need for serial collection and storage of samples, in addition to a relatively high cost per sample tested (Saint-Dizier and Chastant-Maillard, 2012).

2.3.6. Biosensors of milk progesterone profile

The need for serial collection and laboratory testing of samples for milk P4 concentrations could be overcome with the development of in-line milk P4 sensors (Homer, 2013). A fully automated system for monitoring milk P4 has been available in Denmark since 2009 (Saint-Dizier and Chastant-Maillard, 2012). This method uses dry stick technology (Herd Navigator; Lattec I/S, Hllirod, Denmark). Herd Navigator can be integrated into a DeLaval

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milking robot to monitor oestrus in dairy cows (Asmussen, 2010; Saint-Dizier and Chastant-Maillard, 2012). The system measures P4 concentrations over consecutive days (Friggens *et al.*, 2008; Saint-Dizier and Chastant-Maillard, 2012; Tse *et al.*, 2017). From the milk P4 curve, the algorithm developed in the Herd Navigator system classifies the cows into three groups: post-partum anoestrus, oestrous cycling and potentially pregnant (Friggens *et al.*, 2008). For oestrous cows, an alert is triggered by the software as soon as the P4 value drops below 4 ng/mL (Saint-Dizier and Chastant-Maillard, 2012). In the case of an oestrus alert, the algorithm also provides the probability of success of a prospective AI (between 0% and 100%) based on the duration of the previous luteal phase and the kinetics of the decrease in P4 concentration (Friggens *et al.*, 2008; Asmussen 2010). When tested for purposes of validation in an experimental herd, the in-line milk P4 measurements detected >90% of confirmed oestrus (Friggens *et al.*, 2008). An average oestrus detection rate of 95% was later confirmed in commercial herds in Denmark (Asmussen, 2010)

2.3.7. Milk oestradiol profile

Over the last 5 decades, oestrus duration and intensity in lactating dairy cattle has progressively reduced (Reames *et al.*, 2011). Therefore, the endocrine regulation of oestrous expression in dairy cattle has been subjected to intensive investigation, as a result of its particular importance in the successful application of AI (Reames *et al.*, 2011). In addition, the high incidence of silent oestrus (i.e. the inability to express oestrus behaviour) is considered the main cause of declining fertility in dairy cattle (Ranasinghe *et al.*, 2010). It has been reported that the occurrence of silent oestrus was 55.2%, 23.8% and 21.3% at the first, second and third ovulation PP of Holstein cows housed in a free-stall barn (Ranasinghe *et al.*, 2010).

In dairy cows, during oestrus developing follicles secrete E2 into the blood and its concentration in blood increases, with a decrease in the concentration of P4 (Domènech *et al.*, 2011). In cattle, E2 induces oestrus through its influence on the hypothalamus (Allrich, 1994). In addition, E2 affects the hypothalamus by inducing the release of GnRH that causes the surge of LH during the pre-ovulatory stage (Gazal *et al.*, 1998). There is a positive correlation between the concentrations of E2 in blood plasma and milk (Lopez *et al.*, 2002; Gorecki *et al.*, 2004). Measuring the concentration of E2 in milk indicates more precisely the time of oestrus prior to ovulation in comparison to the P4 profile in milk (Domènech *et al.*, 2011) because, there is a large variation in the time of the decrease in the level of P4 in relation to the time of oestrus (Roelofs *et al.*, 2006).

Many studies have measured E2 in skimmed milk using a radioimmunoassay (RIA) (Lopez *et al.*, 2002; Gorecki *et al.*, 2004; Yamanaka *et al.*, 2007). Using RIA for

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quantifying E2 in defatted milk during oestrus, Lopez *et al.* (2002) reported that the concentration of E2 was 38.8% higher compared to one milking before and 22.2% in comparison to two and three milkings before oestrus. However, RIA is not compatible with on-farm use, with only one study described the measurement of E2 in raw milk using commercially available non-radiolabelled enzyme immunoassay (EIA) kit (Domènech *et al.*, 2011). Using an EIA kit for E2 by Cayman Chemical Company (Ann Arbor, MI, USA), Domènech *et al.* (2011) reported that the E2 concentration in raw milk samples, collected manually at morning and afternoon milking, during oestrus ranged between 26.8 ± 8.6 to 52.9 ± 12.9 pg/mL.

Some studies have reported that standing behaviour does not display a linear dose-response relationship with plasma E2 concentration (Walton *et al.*, 1987; Coe and Allrich, 1989). Furthermore, Walton *et al.* (1987) found no relationship between oestrus behaviour and the concentration of plasma E2 in PP cows. Coe and Allrich (1994) also reported that the peak in the concentration of plasma E2 did not affect the oestrous behaviour of heifers. Madureira *et al.* (2015) reported that plasma E2 were 8.9 ± 0.2 and 8.1 ± 0.2 pg/mL during high and low peaks of oestrus activity in Holstein cow. Abeyawardene *et al.* (1984) have previously reported that milk lipid content is an important consideration when determining the E2 concentration in milk because approximately 52% of the E2 content in milk is distributed in the lipid fraction. In high yielding dairy cows, a high percentage of silent oestrus with a lower concentration of E2 on the day of silent oestrus may be related to increased hepatic blood flow and the clearance of steroidal hormones (Vasconcelos *et al.*, 2003) which leads to a decrease in the expression of oestrus behaviour.

2.3.8. Automated oestrus detection (AOD)

Alternatives to replace or supplement the visual detection of oestrus are automated oestrus detection technologies. The first study, carried out on Guernsey cows at pasture to report the relationship between cow physical activity and the stage of oestrus was documented by Farris (1954). Kiddy (1977) also reported that the physical activity of cows in oestrus is approximately 2 to 4 times higher than non-oestrus cows. Mounting events (Senger, 1994), level of activity (Saumande, 2002), number of steps (Roelofs *et al.*, 2005), standing time, lying time (Kerbrat and Disenhaus, 2004), rumination (Reith and Hoy, 2012), body temperature (Kyle *et al.*, 1998), can all be used as AOD technologies.

Automated oestrus detection technologies provide continuous monitoring, and individual cows can be identified accurately (Senger, 1994). Minimal labour is required to predict the time of ovulation accurately, and the identified cow can be inseminated at a specific time in relation to the time of ovulation (Senger, 1994). Several AOD devices are currently available such as pedometers (Roelofs *et al.*, 2005b), accelerometers (Dolecheck *et al.*,

2016), radiotelemetric devices, pressure sensitive mount count devices (Diskin and Sreenan, 2000) and rumination collar (Reith and Hoy, 2012). Activity monitoring using AOD devices is an efficient method of detection of oestrus in dairy cattle, more so than visual detection by visual observation because it allows continuous monitoring of activity and provides a reliable data signalling the onset of oestrus (Homer, 2013).

The IceQube is a device attached to the leg of the cow to measure activity and records variables such as motion index, steps, standing and lying behaviours using 3d-accelerometer technology (Jónsson *et al.*, 2011). A study conducted by Dolecheck *et al.* (2015), on Holstein cows, found that the number of steps were significantly increased but lying time and lying bouts were significantly decreased during the oestrous period compared with a non-oestrous period.

According to Dolecheck *et al.* (2015) when IceQubes (IceRobotics Ltd., Edinburgh, UK) were used, it was found that synchronised dairy cows in oestrus spent less time lying compared to non-oestrus cows (10.19 and 24.82 min/h respectively). Therefore, lying time can be used as an indicator of oestrus behaviour, as around the time of oestrus the amount of time spent lying by dairy cows was decreased as a result of an increase in activity and restlessness (Jónsson *et al.*, 2011). However, some of the secondary signs of oestrus are associated with other behavioural changes (Homer, 2013). For example, lying times are also altered by lameness (Chapinal *et al.*, 2009; Ito *et al.*, 2010).

Pedometers are automated devices that can be attached to the neck or the leg of the cow (Diskin and Sreenan, 2000; Roelofs *et al.*, 2005b). When the cow is in oestrus, the pedometer gives an alert over three consecutive 2 h periods (Hockey *et al.*, 2010). With the help of these devices, the relationship between increasing activity, ovulation and fertility in dairy cattle have been investigated (Roelofs *et al.*, 2005; Hockey *et al.*, 2010). In Holstein cows, ovulation occurs between 29 and 33 h after the onset of the increase in activity and on average 17 to 19 h after the end of an increase in cow activity (Roelofs *et al.*, 2005; Hockey *et al.*, 2010).

These studies indicate that automated systems are useful in detecting oestrus. However, the efficiency of AOD technologies for oestrous detection may be influenced by physiological and management factors (Diskin and Sreenan, 2000) such as housing system (Firk *et al.*, 2002), lameness and health (Roelofs *et al.*, 2005) and environmental conditions (Diskin and Sreenan, 2000; Hockey *et al.*, 2010).

2.4. The role of flehmen behaviours and pheromone in oestrus detection

Bulls use a combination of behavioural factors to detect cows in oestrus which include visual, tactile, auditory and olfactory stimulation (Zalesky *et al.*, 1984), then the bull retracts the upper lip and wrinkles the nose, which is defined as "flehen", followed by mating with the cow (Klemm *et al.*, 1987). The term "flehen" was recognised by German scientists (Hradecký *et al.*, 1983). Bull use visual, olfactory and auditory perception to detect cows in oestrus (Blaschke *et al.*, 1984). These attractions from the cow stimulate the olfactory apparatus of the bull and signal to the hypothalamus of the bull's brain during flehmen in the pre-oestrous period of the cow (Sankar and Archunan, 2004).

2.4.1. Pheromones

Pheromones are chemical signals which are produced by one individual and are released into the surrounding atmosphere to act on specific endocrine receptors (Gnanamuthu and Rameshkumar, 2014). The term 'pheromone' was first introduced by Karlson and Lüscher (1959). They are released as specific chemo-signals through vaginal mucosa, urine (Gnanamuthu and Rameshkumar, 2014), faeces (Sankar and Archunan, 2008), saliva, milk (Sankar and Archunan, 2004), sweat and specialised odour glands (Wyatt, 2010). Pheromones can be classified as volatile and non-volatile molecules (Dobson-Hill, 2009). These molecules are received by the male via the olfactory system (Tirindelli *et al.*, 2009). The olfactory system in mammals is divided into two forms: the main olfactory system and the accessory olfactory system and the detection and transmission of pheromone information are recognised by both of these two systems (Mucignat *et al.*, 2012).

Pheromones induce a behavioural reaction of another individual of the same species (Vyas *et al.*, 2012). In mammalian species, as well the importance of pheromone cues in the regulation of social interactions, pheromones also play an important role in sexual behaviour and the reproductive process (Brennan and Zufall, 2006). In some animals species, the male produces odoriferous compounds, especially pheromones, to influence the ovarian function and behavioural signs of oestrus in the female (Madej *et al.*, 2005). These effects are well reported in pigs (Madej *et al.*, 2005), goats (Gelez and Fabre-Nys, 2004) and sheep (Hawken and Martin, 2012). However, in cattle, the role of oestrus pheromones is not as clearly defined (Rekwot *et al.*, 2001).

It is well documented that olfactory signals, independent of visual queues, stimulate the reproductive behaviour of the male (Klemm *et al.*, 1987). Indeed, during oestrus females signal the stage of their oestrous cycle through the production of specific odours (pheromones) in urine and vaginal discharge that causes the stimulation of sexual

behaviour in the male (Vyas *et al.*, 2012) such as in bovine species (Kekan *et al.*, 2017). A bull can recognise oestrus and non-oestrus females via the pheromone signals released from the urine and vaginal fluid of the cows (Patra *et al.*, 2012). It has been reported that the maximum concentrations of pheromones in cow's urine is present before the onset of behavioural oestrus (Rekwot *et al.*, 2001).

Studies carried out by Sankar and Archunan (2004) and Archunan (2012) to investigate bull flehmen behaviour in response to different body fluids (such as vaginal fluid, saliva, faeces, urine and milk during oestrus and non-oestrus stages) of the cow, found a significantly higher flehmen response exhibited by bulls towards dummy cows sprayed with oestrus cow's fluid compared to other phases of the oestrous cycle. During pro-oestrus, flehmen behaviour in cows and bulls is considered one of the definitive signs of the onset of oestrus, integral to pre-mating in bovines (Hradecký *et al.*, 1983) that can be used as an indicator of oestrus in dairy cows (Van Eerdenburg *et al.*, 2002).

Pheromones in cattle may be composed of a single compound, or a mixture of several compounds (Sankar and Archunan, 2004). Each different pheromone may be involved at different stages of the reproductive cycle in dairy cattle (Sankar and Archunan, 2004). Pheromones related to oestrus are present only during pro-oestrus and oestrus stages of the oestrus cycle in cattle (Rekwot *et al.*, 2001). Pheromone compounds found in a variety of body fluids of oestrus cattle and summarised in Table 2.1.

Table 2. 1. Pheromones compounds found in a variety of body fluids in oestrus cattle.

Pheromone compounds	Sources	References
6-methyl-1-heptanol, 2-methyle-7-hydroxy-3-4-, and heptane	Vaginal fluid	Preti (1984)
Acetaldehyde	Milk	Klemm <i>et al.</i> (1987)
Acetaldehyde	Blood	Klemm <i>et al.</i> (1994)
Ethylbenzene, oxiranemethanol, n-propyl phthalate and 1-iodoundecane	Urine	Ramesh Kumar <i>et al.</i> (2000)
γ -12:2 lactone	Milk	Bendall (2001)
2-proenyl ester	Faeces	Sankar and Archunan (2004)
Trimethylamine and phenol 4-propyl,	Saliva	Sankar <i>et al.</i> (2007)
Trimethylamine, phenol and 3-hexanol	Vaginal mucosa	Rajnarayanan and Archunan (2011)
Phenol, 3-propyl phenol and 9-octadecenal	Urine	Rajnarayanan and Archunan (2011)
1-iodo undecane	Urine	Archunan and Rameshkumar (2012)
Indole	Faeces	Mozūraitis <i>et al.</i> (2017)

2.4.2. Fatty acids as pheromones

Recently, the knowledge of the chemistry of pheromone molecules produced during the oestrous cycle of dairy cattle has increased considerably (Gelez and Fabre-Nys, 2004; Rajanarayanan and Archunan, 2011). Dairy cow milk fat contains relatively high amount of fatty acids (Or-Rashid *et al.*, 2009). Fatty acids have an important role as pheromones in mammals and may act as an attractant for the bull (Kumar and Archunan, 2006). In a study where the day of AI was considered to be the day of oestrus (rather than using the monitoring for oestrus), Toledo-Alvarado *et al.* (2018) found that the milk concentrations of myristic acid (C14:0) and palmitic acid (C16:0) were lower on the day of oestrus compared to dioestrus. In addition, it was reported that the concentrations of stearic acid (C18:0) and oleic acid (C18:1 cis-9) were greater on the day of oestrus compared to other phases of the oestrous cycle. Furthermore, Mozūraitis *et al.* (2017) found significantly higher acetic acid (36 ± 8 ng) and pentanoic acid (125 ± 57.6 ng) in the 0.5 g faeces of oestrus cows compared (19 ± 5 ng and 22.96 ± 9.95 ng, respectively) to anoestrus Holstein cows. Sankar and Archunan (2008) found that acetic acid was present only in the faeces during oestrus compared to pro-oestrus and post-oestrus in Jersey cows.

Also Gnanamuthu and Rameshkumar (2014) found that valeric acid (C5:0) and caproic acid (C6:0) were present only in faecal samples of oestrus cows and not those in pro-oestrus or di-oestrus. The volatile FAs in milk may play a role in oestrus because urinary FA has a functional role as a pheromone in mammals (Kumar and Archunan, 2006). Increases in the concentration of acetic acid on the day of oestrus may be due to acetic acid acting as a precursor of E2 (Janowski *et al.*, 1988). In support of this, previous studies have found the E2 is synthesised by the bovine mammary gland and secreted into milk and mammary venous blood (Janowski *et al.*, 1988; Janowski *et al.*, 2002). Mozūraitis *et al.* (2017) found significantly higher butanoic acid in the faeces of oestrus cows compared to the faeces of anoestrus cows. Kumar and Archunan (2006) also found the concentration of tridecanoic, myristic and pentadecanoic acids were significantly higher in urine on the day of oestrus in comparison to the pre-pubertal and pregnancy periods in cattle. Gnanamuthu and Rameshkumar (2014) found that myristic acid (C14:0) and gadoleic acid (C20:1n9t) were only present in faeces on the day of oestrus, while they were not present during pro-oestrus and di-oestrus in Umblachery cattle. However, they also found that there were no differences in the concentrations of faecal stearic acid (C18:0) between pro-oestrus, oestrus and di-oestrus (Gnanamuthu and Rameshkumar, 2014). In addition, Gnanamuthu and Rameshkumar (2014) also reported the concentration of palmitic acid (C16:0), oleic acid (C18:1n9c) and behenic acid (C22:0) in cattle faeces did not differ between pro-oestrus and di-oestrus. In another study conducted in cattle by Kumar and Archunan (2006), lauric acid (C12:0), tridecanoic acid

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(C13:0), myristic acid (C14:0) and linoleic acid (C18:2) were present at similar concentrations in samples collected during pro-oestrus, oestrus and di-oestrus. The presence of FA in cows' milk at higher concentrations in the oestrous period compared to di-oestrus may act as chemical signals for the attraction of the bull because the FA acts as pheromones in mammals (Brahmachary *et al.*, 1992). The findings of the studies discussed above are summarised in Table 2.2.

Table 2. 2. Fatty acids found in various body fluids of oestrus cattle.

Fatty acids	Sources	References
Butanoic acid, carboxylic acid and pentanoic acid	Faeces	Sankar and Archunan (2004)
Tridecanoic acid, myristic acid, pentadecanoic acid, palmitic acid, cis oleic acid, heptadecanoic acid and linolenic acid	Urine	Kumar and Archunan (2006)
Acetic acid, pentanoic acid and propionic acid	Saliva	Sankar <i>et al.</i> (2007)
Acetic acid and propionic acid	Faeces	Sankar and Archunan (2008)
Acetic acid and propionic acid	Vaginal mucosa	Rajanarayanan and Archunan (2011)
Valeric acid, caproic acid, myristic acid, gadoleic acid and pelargonic acid	Faeces	Gnanamuthu and Rameshkumar (2014)
Acetic acid, propionic acid and pentanoic acid	Faeces	Mozūraitis <i>et al.</i> (2017)
Myristic acid, palmitic acid, stearic acid and oleic acid	Milk	Toledo-Alvarado <i>et al.</i> (2018)

2.5. Factors affecting oestrous behaviour expression

In order to maintain high reproductive efficiency, accurate oestrous detection is important in dairy herds which are using AI (Kinsel and Etherington, 1999). The duration and intensity of oestrous expression are highly variable among individuals cow (Løvendahl and Chagunda, 2006; Madureira *et al.*, 2015). Many factors contribute to this and decline in oestrous expression in dairy cattle, which results in poor oestrous detection (Orihuela, 2000). For example, management factors, social interaction, feed and leg problems (Diskin and Sreenan, 2000), environmental factors, age and parity, nutrition and BCS and genetic factors (Roelofs *et al.*, 2010). These factors are discussed below.

2.5.1. Management and environmental factors

2.5.1.1. Housing design and type of floor

Housing design and type of floor effect behavioural expression of oestrus in dairy cows (Dolecheck, 2015). Cows require adequate space and comfortable conditions for the satisfactory expression of oestrus (Orihuela, 2000). Hurnik *et al.* (1975) reported that low ceilings and a deep muddy floor might affect oestrous expression behaviour. Cows in a free-stall barn exhibit greater expression of oestrous behaviour (Phillips and Schofield, 1990) compared to those housed under tie-stall barn conditions (Roelofs *et al.*, 2010). However, De Silva *et al.* (1981) found that cows in cubicle housing displayed a lower mounting frequency (7 mounts/h) compared to open barn housing (11 mounts/h). Palmer *et al.* (2010) reported that standing oestrus in Holstein Friesian cows was significantly higher at pasture than cubicle housed cattle (91% vs 52%, respectively). In addition, Palmer *et al.* (2012) also reported that fewer cows were standing to be mounted in cubicle housing (0.5 mount/h) than at pasture (2.5 mount/h). While De Silva *et al.* (1981) reported that barn housed cows exhibited more mounts/h during oestrus (11.2 mounts/h) than cow housed primarily in free stalls (6.5 mounts/h) or at pasture (5.4 mounts/h). This may have been because cows at pasture spend more time grazing and have less frequent contact with other cows than animals maintained in barns, thus have less opportunity to express oestrus behaviours (Phillips and Leaver, 1986).

Regarding the type of floor, Britt *et al.* (1986) documented that dairy cows show more mounting behaviour on a soft surface covered by grass, dirt or straw bedding compared to slippery concrete floor. Diskin and Sreenan (2000) also reported that mounting activity was increased by 25% when cows were kept on softer under-foot conditions compared to cows on concrete. However, others found the frequency of mounting by cows during oestrus was more on the slatted floor than in a straw yard, especially by older cows (Hurnik *et al.*, 1975; Rodtian *et al.*, 1996). Oestrus duration was longer when cows were kept on a dirt floor (by around 25%), in comparison to the concrete floor (Britt *et al.*, 1986).

Furthermore, Boyle *et al.* (2007) found a similar number of mounts and oestrus duration in dairy cows maintained on rubber covered slats, straw and at pasture, while significantly fewer mounts and a shorter oestrus duration was seen in cows that were kept on concrete. It has been reported that cows with foot problems, and when the floor is slippery or coarse, displayed less mounting behaviour, especially when kept on a wet or worn rubber (Blowey, 2005). Furthermore, Boyle *et al.* (2007) reported that the number of mounts during oestrus were higher on the rubber covered floor with grooves and raised ridges because it reduced slipping and improved resistance in addition to cushioning, as well as decreases foot injuries which lead to increased oestrous expression.

2.5.1.2. Social interaction and stock density

Dominant cows have a major role in the expression of behavioural oestrus (Galina *et al.*, 1996). Orihuela (2000) found dominant animals inhibited the mounting activity of subordinate cows during oestrus. In addition, Orihuela *et al.* (1988) found that 60% of the mounting time was initiated by herd mates, which were larger and heavier than smaller herd mates. The number of cows in oestrus at the same time (i.e. sexual group; SG) also has a major influence on the overall expression of oestrus activity in the dairy herd (Roelofs *et al.*, 2005b). In addition, Yániz *et al.* (2006) found that walking activity during oestrus increases by about 6.1% with each additional cow in oestrus. Furthermore, the manifestation of behavioural oestrus in oestrous-synchronised cows was significantly influenced by the number of cows in oestrus at any given time (Castellanos *et al.*, 1997).

The duration of oestrus and mounting activity are lowest when only one cow is in oestrus (Hurnik and King, 1987). The number of mounts per cow increases with increasing numbers of cows that are in oestrus at the same time for oestrous synchronised cows (Dolecheck, 2015). The number of mounts initiated increased from 11 to 36.6 and 52.6 mounts when one, two or three cows were in heat simultaneously, respectively (Hurnik *et al.*, 1975). However, Castellanos *et al.* (1995) showed that even with a PGF2 α treatment the number of cows in oestrus at the same time and the average time to the onset of oestrus varied according to the period when dominant cows displayed behavioural signs of oestrus, suggesting that factors such as the social order in the herd influence reproductive behaviour possibly overriding underlying physiological mechanisms.

The intensity and duration of behavioural oestrous expression can also be affected by increasing the number and familiarity of dairy cows in oestrous simultaneously (Orihuela, 2000). A sexual activity group (SAG) is formed by oestrus synchronisation, and SAG members are more active, and mobile compared to cows that come into oestrus spontaneously at the same time (Williamson *et al.*, 1972; Chenoweth, 1981). The intensity of oestrus and mounting expression was increased with increasing numbers of cows in

oestrus at the same time in high yielding, Holstein Friesian cows (Gilmore *et al.*, 2011). The SAG stimulated and encouraged more partner cows in the herd to come into oestrus and enhanced increased oestrous expression which included both primary and secondary signs (Orihuela, 2000). In addition, Orihuela (2000) also reported that the accessibility of sexual partners is greater with an increasing number of cows in oestrus at the same time and this increased the sexual activity of the SAG of cows.

The increase in the number of steps and a reduction in the lying time observed with an increased number of cows in oestrus at the same time may be due to increased sexual stimulation by other animals (Roelofs *et al.*, 2005). This leads to an increase in walking around by sexual partners during oestrous periods as a result of sniffing the ano-genital region of fellow herd mates, chin-resting, flehmen, attempts to mount and mounting (Van Eerdenburg *et al.*, 1996).

Otherwise, in over crowded herd situations, cows may not have enough space to display signs of oestrous behaviour and the expression of oestrus could be undetected by visual observation with larger numbers of cows in close proximity (Diskin, 2008). In addition, insufficient area per cow leads to indiscriminate mounting activity, directed towards any herd mates as a result of the close confinement (Metz and Mekking, 1984). High stocking densities of dairy herds can affect oestrous expression through many factors such as aggression (Metz and Mekking, 1984), over crowding which minimizes lying times of dairy cows, resulting in a higher incidence of lameness (Metz and Mekking, 1984; Blowey, 2005). Over crowding may also decrease feed intake as a result of increased cow displacement from the area of feeding (Hosseinkhani *et al.*, 2008). These factors all have a negative effect on the expression of oestrous signs for a number of reasons including cow stress (Sood and Nanda, 2006).

2.5.1.3. Effects of lameness on oestrous expression in dairy cattle

One of the chronic painful and stressful conditions associated with poor reproductive performance and reduced intensity of oestrous expression in dairy cows is lameness (Walker *et al.*, 2008b). The intensity of oestrous expression in dairy cattle can be influenced by many physiological, psychological and environmental factors (Sood and Nanda, 2006; Orihuela, 2000). Lameness stress has an influence on oestrous expression (Walker *et al.*, 2008a) through its negative impact on reproductive hormones from the hypothalamic-pituitary-ovarian axis that are necessary for expression of oestrous behaviour in dairy cattle (Allrich, 1994; Dobson *et al.*, 2003). In addition, lameness reduces physical activity during the oestrous period in dairy cows (Walker *et al.*, 2008a) as oestrous behaviours in cattle include increased walking or restlessness with a sexually active group and mounting behaviour (Van Vliet and Van Eerdenburg, 1996).

Several previous studies revealed that lame cows express less oestrus intensity by reducing the frequency of both primary and secondary signs of oestrus (Sood and Nanda, 2006). A study conducted on 110 cows of native Indian breeds (Sahiwal, Hariana and Red Sindhi) by Sood and Nanda (2006) found that lame cows show fewer standing to be mounted events than non-lame cows (2.4 ± 0.4 vs 8.0 ± 2.5 events, respectively). This study also found the head side mounting proportion in lame cows were lower (35%) compared to healthy cows (53%). Another study conducted on 59 Holstein Friesian cows on a commercial dairy farm in the UK by Walker *et al.* (2008a) found that walking activity was reduced significantly in lame cows compared to non-lame cows. Walker *et al.* (2008a) also found that during the oestrous periods lame cows spent more time lying compared to healthy cows. A further study, using visual observation for oestrous detection in oestrus-synchronised cows, conducted by Morris *et al.* (2011) found that lame cows had less oestrous intensity compared to non-lame cows (1368 vs 2254 visually observed points) and lame cows began oestrus earlier and stood to be mounted earlier than non-lame cows. This study also found that about 21% of oestrus-synchronised lame cows failed to express primary and secondary signs of oestrus. Oestrous expression can also be affected by lameness as a result of lower levels of activity in lame cows and less interaction with their herd mates (Sood and Nanda, 2006). Lameness could also affect activity monitoring by accelerometer and pedometer methods of oestrous detection because lameness leads to display fewer primary and secondary signs of oestrus behaviour in dairy cattle (Homer, 2013).

However, other studies have reported that expression of oestrous behaviour in dairy cattle is similar in lame cows compared to non-lame cows and found a similar daily milk E2 profile during oestrus (Walker *et al.*, 2008b; Walker *et al.*, 2010). In addition, Hassall *et al.* (1993) show that lame cows at pasture spent the same amount of time walking on the day of oestrus compared to healthy cows, suggesting that walking at pasture was less painful for cows than walking on concrete (Walker *et al.*, 2010). Furthermore, based on a behavioural scoring system for three-hour recording during oestrus, Walker *et al.* (2008b) found that lame cows were just as restless (walking, bunting and playful) as healthy cows.

2.5.1.4. Time of day

The peak of the behavioural oestrus intensity may be related to the time of day that oestrus starts (Orihuela, 2000). Some studies showed that during the evening and at night dairy cows display more frequent of mounting activity than at other times of the day (Orihuela *et al.*, 1983; Nebel *et al.*, 2000), while others reported that the onset of behavioural oestrus occurs more frequently during daylight hours (Gwazdauskas *et al.*, 1990). However, Hurnik *et al.* (1975) and Hackett and McAllister (1984) found that 65% more cows came into oestrus during darkness and they displayed more frequent of

mounting activity in the early morning and late evening. De Silva *et al.* (1981) observed that cows during the morning in oestrus displayed more mounts per hour (11.4 mounts/h) compare to those cows in oestrus during the evening (7.6 mounts/h). Other studies have suggested no differences in oestrus activity and the onset of oestrus in dairy cattle during day or night (Alexander *et al.*, 1984; Xu *et al.*, 1998).

In the Zebu breed of cattle, it has been reported that the start of oestrus and the peak of oestrous activity are more likely to occur during the hours of darkness or in the early hours of the morning (Orihuela *et al.*, 1983; Galina and Arthur, 1990). Hurnik *et al.* (1975) also found that Holstein cows in oestrus displayed more behavioural activity between 1800 and 0600 h. However, in a study using HeatWatch® (DDx Incorporated, Denver, CO) conducted by Dransfield *et al.* (1998) found that there was no difference in the percentage of standing events (28.4%) during the period between 06:00 to 12:00 compared to another periods of the day. Sankar and Archunan (2012) also reported that the time of day did not affect oestrous activity and found that mounting activity was evenly distributed across 24h of the day. Furthermore, Orihuela (2000) reported that the time of the day did not affect the oestrous duration. In another study using the HeatWatch® system (HW; DDx Inc., Denver, CO) system of oestrous detection in New Zealand, Xu *et al.* (1998) also found no variation in oestrus at different times of the day in Holstein and Jersey cows. Daily management activities like feeding, milking, cleaning and other factors such as location, season and environmental condition may influence the distribution of oestrus activity between day and night time (Nebel *et al.*, 2000; Sankar and Archunan, 2012).

2.5.1.5. Time of the year

Season and related environmental factors, such as ambient temperature (Gwazdauskas *et al.*, 1983; Landaeta-Hernández *et al.*, 2002) can affect oestrous expression in cattle. Firk *et al.* (2002) reported that during late autumn and early winter the intensity of oestrus in dairy cows is lower compared to intensity during summer months. More recently, Homer (2013) reported that the intensity and duration of oestrous activity in Holstein Frisian cows were higher in late spring and summer in comparison to autumn and winter. This may be related to longer days during late spring and summer than other times of the year. Sankar and Archunan (2012) also found that the mean duration of oestrus of dairy cows reared under natural conditions in India, was longer in summer (21.0 h) compared to winter (18.7 h) and spring (18.0 h), while the average number of mounts was more in winter (61.5 mounts) than summer (39.7 mounts). In another study conducted in the USA by Nebel *et al.* (1997) found that Holsteins mounted more (8.6 mounts) during winter months compared to summer (4.5 mounts). However, a study conducted in the USA by Walker *et al.* (1996) found that season did not affect oestrus duration (9.5 h) in Holstein cows.

Gwazdauskas *et al.* (1983) reported that when the ambient temperature was lower than 25°C, dairy cows express greater oestrous behaviour than when the temperature was higher than 30°C. This is confirmed by Arthur and Abdel Rahim (1984) who found that oestrous duration of Holstein cows in Saudi Arabia was reduced in hot summer weather (5.3h) in comparison to cold weather in winter (10.2h), which may be due to heat stress as a result of very high summer temperatures of 45-50°C in Saudi Arabia. In dairy cows, the duration and intensity of oestrous behaviour reduced further when the period of high temperature was extended (Thatcher *et al.*, 1986; Chicoteau *et al.*, 1989). Furthermore, Landaeta-Hernández *et al.* (2002) reported that a high temperature-humidity index suppressed both oestrous duration (9 ± 1 h vs 16 ± 1 h) and a total number of mounts (8 ± 4 vs 34 ± 4 mounts) during spontaneous oestrus compared with synchronised-oestrus, respectively.

Reduction in oestrous expression at high temperature may be due to the effect of heat stress on follicular development and steroidogenesis (De Rensis and Scaramuzzi, 2003). Another study conducted by Sankar and Archunan (2012) found that the oestrus duration during summer was longer (21.0 ± 0.4 h) than the oestrus duration in winter (18.7 ± 0.2 h) in dairy cattle in India. Landaeta-Hernández *et al.* (2002) reported that a high and low-temperature humidity index affected the duration of spontaneous oestrus (9 ± 1 h vs 16 ± 1 h) in Senepol and Brahman cattle. The incidence of specific behavioural signs of oestrus was significantly lower in dairy cattle according to in summer (night length 6 h) compared to winter (16 h) (Phillips and Schofield, 1990). Sankar and Archunan (2012) found that the activity as the number of mounts during oestrus in dairy cows was higher in winter (61.5 ± 0.4 mounts) compared to summer (49.5 ± 0.3 mounts) or spring (39.7 ± 0.4 mounts). The peripheral concentration of E2 is influenced by heat stress and the effect on oestrus activity (Wilson *et al.*, 1998). The effect of different times of the year on oestrous behavioural expression could also be due to the environmental effect on the oestrus cow's willingness to express primary or secondary signs of oestrus, rather than the influence of temperature on the physiological status of the cows in oestrus (Sankar and Archunan, 2012).

2.5.2. Spontaneous oestrus versus synchronised oestrus

One of the most important concerns of dairy cattle breeder using AI is the accurate detection of oestrus (Diskin and Sreenan, 2000). Therefore in modern dairy herds using AI oestrous synchronisation strategies have been used to maximise oestrous detection rate and increase conception rates (Dolecheck, 2015). While methods of oestrus synchronisation were developed with the objective of achieving compact calving, methods of synchronised oestrus can play an important role in reducing the problem of oestrous detection (Diskin and Sreenan, 2000). However, other reports indicated that oestrous

synchronisation by hormonal treatment affects oestrous expression (Hurnik *et al.*, 1975; Roelofs *et al.*, 2010).

The accuracy of oestrous detection was decreased, and the proportion of false negative was increased by about 61% when prostaglandin was used for oestrous synchronisation (Slennig and Farver, 1990). However, Kilgour *et al.* (1977) reported that in an oestrus synchronised dairy herd, the proportion of cows in oestrus increased due to the increased number of cows in the SAG during oestrus as a result of sexual stimulation which may lead to increase oestrous expression. Furthermore, Lyimo *et al.* (2000) found that during spontaneous oestrus a low proportion of cows (53%) in oestrus stand to be mounted.

Jaume *et al.* (1980) reported that oestrous duration in Zebu heifers was approximately 2-hours longer (21.7 h) during spontaneous oestrus in comparison to synchronised oestrus (19.8h). A similar difference in oestrous duration was reported by Vaca *et al.* (1985), although in their study spontaneously occurring oestrus lasted 15.3 h compared with 13.3 h during PGF2 α synchronise oestrus. However, Walker *et al.* (1996) found a highly variable duration and frequency of oestrous expression with no differences between natural and PGF2 α treated Holstein cow. Furthermore, a study conducted on an Indian native breed by Lokhande *et al.* (1983) showed that the efficiency of oestrous detection in dairy cows was 70% when using the progesterone-releasing intra-vaginal device (PRID) compared to cows treated with PGF2 α where only 44% of oestruses were detected.

Using activity as an oestrous detection method in Holstein Friesian dairy cows, López-Gatius *et al.* (2005) concluded that there was no difference between PRID oestrous-synchronised cows and cows in spontaneous oestrus. In addition, Roelofs *et al.* (2005) also reported no difference in oestrus intensity and duration between spontaneous oestrus compared to synchronised-oestrus in dairy cows.

2.5.3. Days in milk and number of oestrus PP

Oestrus expression in dairy cows can be affected by the number of days PP (Roelofs *et al.*, 2010). The onset of oestrous cyclicity is delayed by the presence of a nursing calf, and the display of sexual behaviour is also inhibited (Peters, 1984). This finding is supported by that of Masilo *et al.* (1992) who reported that the interval from calving to the first oestrous cycle in the milked dairy breed are shorter compared to suckled beef breeds.

In general, during the first three months after calving, the average number of mounts per cows per oestrus increases with the increasing number of oestrus PP (Esslemont *et al.*, 1980). Therefore, during the first 100 days of lactation, the rate of oestrous detection was around 25% lower than cows after 100 days PP (Mayne *et al.*, 2002). Furthermore, Sharma and Luktuke (1983) showed that about 15% of a crossbred herd (Holstein x

Brown Swiss x Jersey with Harianas) had no effect in behavioural oestrus activity by 90 days PP.

A study conducted by Isobe *et al.* (2004) based on milk P4 profiles and visual observation found that the incidence of silent oestrus' in 32 Holstein Friesian cows in Japan were 83, 46 and 13% at first, second and third oestrus. Similarly, a study that used radio-telemetry to record mounting activity continuously, also found that the occurrence of silent oestrus was higher at first oestrus (42.1%) than at second oestrus (12.5%) (Shipka, 2000). In addition, it has been found that the rates of silent oestrus detection using pedometers in first, second and third oestrus were 43, 9 and 5%, respectively, but by visual observation, the detection rates of oestrus in first, second and third oestrus were 19, 37 and 79%, respectively (Firk *et al.*, 2002). Furthermore, López-Gatiús *et al.* (2005) showed that the activity of dairy cows in oestrus increases after day 50 PP. In addition, a study also conducted on Holstein-Friesian cows in north-eastern Spain by Yániz *et al.* (2006) found that walking activity decreased with increased days in milk during oestrus.

The physiological mechanism associated with the lower incidence of behavioural oestrus during the first oestrus PP is unknown (Roelofs *et al.*, 2010). A lower oestrous activity at first oestrus may be due to a negative energy balance (NEB) in high yielding dairy cows during early lactation, which may lead to lower E2 by production in the pre-ovulatory follicle (Isobe *et al.*, 2004). However, it is also proposed that high concentrations of E2 during late pregnancy and parturition may cause refractoriness of the hypothalamus to E2 produced by first ovulatory follicle PP (Allrich, 1994). After the first silent oestrus and ovulation, the production of P4 from the CL resets the hypothalamus leading to behavioural expression of oestrus preceding the subsequent ovulation (Allrich, 1994).

2.5.4. Age and parity

Generally, most reports indicate age and increasing parity lead to decrease expression of oestrous behaviour (Orihuela, 2000). The oestrous behaviour scoring system of Van Eerdenburg *et al.* (2002) demonstrates this with higher scores recorded in primiparous compared with multiparous cows (Roelofs *et al.*, 2005; Yániz *et al.*, 2006). In addition, Peralta *et al.* (2005) found that standing events significantly decreased with increasing parity, in Holstein cows under heat stressed conditions, with 9.2 standing events observed in first parity, 6.2 events in second parity and 5.6 events in third parity cows.

However, it has also been reported that the intensity of oestrous behaviour increased with parity from 5.5 to 6.3 and 7.9 mounts per hour in oestrous maiden heifers, first-calf heifers and older cows, respectively (De Silva *et al.*, 1981). Similarly, (Orihuela, 2000) reported that the number of standing mounts increases with parity.

Concerning walking activity on the day of oestrus, Løvendahl and Chagunda (2006) found that oestrous activity was higher in first parity cow in comparison to older cows. Furthermore, studies conducted by Arney *et al.* (1994), López-Gatius *et al.* (2005b) and Yániz *et al.* (2006) showed that walking activity decreases about 21.4% with increase lactation number in dairy cows.

Using neck mounted activity tags (Alpro, version 6.60, DeLaval, 2007) a study by Løvendahl and Chagunda (2010) found that activity in lactating heifers lasted 9.2 h and in multiparous cows 8.12 h. In agreement with this Roelofs *et al.* (2005) reported that oestrus was 3 h longer in primiparous than multiparous cows. However, Van Vliet and Van Eerdenburg (1996) reported a shorter oestrus duration in primiparous cows. Other studies found that there was no difference in oestrus duration between primiparous and multiparous cows (Lyimo *et al.*, 2000). This finding was supported by that of Esslemont and Bryant (1976) who showed that the average oestrous duration was similar in 4th parity (14.6 h) and 2nd parity cows (15.1 h).

These differences in expression of oestrus and their relation to parity may be due to increases in milk yield with increase parity (Dolecheck, 2015). Generally higher milk yields produced by a dairy cow increase with each successive lactation (Garnsworthy, 2006). Metabolic changes with increasing numbers of lactations is thought to result in reduced fertility (Dolecheck, 2015). It has also been reported that the relationship between fertility, metabolic and endocrine traits change with lactation number in dairy cattle and this could explain the effects of parity on the expression of oestrus (Wathes *et al.*, 2007; Dolecheck, 2015).

2.5.5. Milk yield

Oestrus detection rates vary from dairy herd to herd and breed to breed is highly variable from 30 to 70% (Diskin and Sreenan, 2000; Chagas *et al.*, 2007). It has been reported that the over-focus on genetic improvement of the dairy cow for milk production, especially in the Holstein breed, may have the consequence of reducing heat detection rate and declining fertility in the dairy herd (Homer, 2013). In addition to genetic improvement, there has also been more focus on milk yield through improved diets (Gutierrez *et al.*, 2006), which may result in changes to the energy metabolism of the cow altering endocrine signals, that affect oestrus expression (Roche, 2006).

In high yielding dairy cows, there is thought to be a weak antagonistic relationship between milk yield and oestrus expression (Van Eerdenburg, 2008). In a study conducted by Lopez *et al.* (2004) they found that high yielding dairy cows (≤ 39.5 kg/d) had significantly shorter oestrous durations (6.2 h), fewer standing events (6.3 events) and shorter standing time (27.1 seconds) compared to lower yielding dairy cows (10.9 h, 8.8

events and 28.2 seconds, respectively) during the day of oestrus. In another study, López-Gatius *et al.* (2005b) reported that an increase of 1 kg in milk production leads to a reduction of about 1.6% in walking activity on the day of oestrus. However, Patton *et al.* (2007) reported no interaction between the level of milk production and the expression of oestrus and conception rate. This may be due to lower concentrations of E2 in higher yielding (6.8 pg/mL) than lower yielding cows (8.6 pg/mL) on the day oestrus (Lopez *et al.*, 2004).

High yielding dairy cows require a high plane of nutrition as there is close correlation ($r^2 = 0.88$) between milk yield and DMI (Harrison *et al.*, 1990). Therefore, for high yielding dairy cows to meet the requirements for milk production, they need an energy dense diet (Lopez *et al.*, 2004). This leads to increase blood flow through the liver and more rapid clearance of steroid hormones from the blood stream by the liver (Sangsrivong *et al.*, 2002). This reduced behavioural oestrus expression as a result of lower circulating E2 concentrations (Roelofs *et al.*, 2010). Therefore, the higher rate of metabolic clearance of steroids provides a possible mechanism by which high milk yield can affect oestrous duration and expression (Lopez *et al.*, 2004).

2.5.6. Nutrition, negative energy balance and BCS

Oestrous expression can be affected by nutrition (Gutierrez *et al.*, 2006). Feeding a high energy diet to maximise milk yield may decrease hormonal signals (Gutierrez *et al.*, 2006; Roche *et al.*, 2009). Nutrition can also influence the expression of oestrus through the effect on NEB during early lactation (Macmillan *et al.*, 1996). As milk yields increase, if they cannot meet their requirement for lactation through feed intake, cows will mobilise their body fat and protein (Roche *et al.*, 2009). This causes a change in metabolic status and energy partitioning (Wathes *et al.*, 2007), leading to NEB and loss of up to 75 kg body weight in the early PP period which forces the cow to mobilize mainly fat but also protein to meet the energetic demands of milk production. (Roche, 2006) and loss of BCS more than 1 BCS (Scale 1-5) (Garnsworthy, 2006).

It has been reported that oestrous detection rate over the first 100 days of lactation, increases from 58.7% to 84.2% in cows that lost less body condition in early lactation (0.3 vs 0.6 BCS units), respectively (Mayne *et al.*, 2002). During the pre-ovulatory stages, there is a positive relationship between NEB and the concentration of E2 which effects the behavioural expression of oestrus (Mackey *et al.*, 1999; Lyimo *et al.*, 2000). Furthermore, low circulating concentrations of glucose, insulin and IGF-1 are strongly related to the loss of body reserves during NEB in PP period (Homer, 2013). The regulation of the LH receptors on follicles is associated with the level of glucose and insulin in the blood. This

leads to an alteration in the sensitivity of the pituitary gland to GnRH that affects LH pulses and so can limit the production of E2 by the dominant follicle (Butler, 2003).

Cutullic *et al.* (2009) reported that for a group of Holstein cows fed *ad libitum* a mixed ration of 65% maize silage, 5% hay and 30% concentrate (high diet), exhibited approximately 8 times more secondary signs of oestrus with fewer standing oestrus, in comparison to a similar group fed 85% grass silage and 15% concentrate as a low diet designed to produce lower milk yield while mobilizing more adipose tissue. This study concluded that less intense standing heats in the high diet group may be due to greater adipose tissue mobilisation and higher milk yields, rather than a direct effect of diet (Cutullic *et al.*, 2009).

A study conducted on lactating Holstein cows by Madureira *et al.* (2015) using two types of activity monitors, mounted accelerometer-collar (Heatime, SCR Engineers, Netanya, Israel) and a leg mounted pedometer (Boumatic Heat-seeker-TX, Boumatic Dairy Equipment, Madison, WI) found that oestrous-related activity during the day of oestrus was significantly higher in medium BCS (2.75 to 3.0; 345.7 ± 6.0 relative increase) and high BCS (≥ 3.25 ; 339.2 ± 14.9 relative increase) compared to low BCS (≤ 2.5 ; 294.7 ± 11.3 relative increase) cows. Similarly, Aungier *et al.* (2012) showed that an increase of BCS by 0.25 point was positively correlated with an increase in dairy cow activity before ovulation. Buckley *et al.* (2003) and Roche *et al.* (2007) agreed that BCS in early lactation is negatively correlated with days to the first oestrus, and positively associated with the possibility of oestrus detection because of an increase behavioural activity.

Law *et al.* (2009) reported that cows fed three different levels of protein showed no difference in oestrous expression, but with progressive stages of lactation cows supplied with a diet high in protein showed increased secondary signs of oestrus such as mucous discharge from the vulva, chin resting and heat-to-head mounting. Similarly, Gilmore *et al.* (2011) found that four nutritional strategies (low level of protein, medium level of protein, high level of protein or high starch) had no effect on primary oestrous behaviour, while secondary signs were expressed more frequently in Holstein Friesian dairy cattle fed a high level of protein and high starch.

2.5.7. Genetics and breed

Reducing fertility has been related to the high milk yield of cows that have been extensively genetically selected for yield (Dobson *et al.*, 2008). Dairy cows genetically selected for high milk yield are more susceptible to NEB (Boer *et al.*, 2010). Furthermore, Chagas *et al.* (2007) suggested that the changes in the genotype of high yielding modern dairy cow have a significant role in declining fertility.

In addition, oestrus duration and intensity significantly differed among different breeds of cattle (Cutullic *et al.*, 2009). Rae *et al.* (1999) reported that the duration of oestrus in Angus, Brahman and Angus X Brahman cross heifers were 8.5 ± 1.2 h, 6.6 ± 1.2 h and 11.9 ± 1.2 h, respectively. The number of mounts per oestrus were also higher in the cross breed heifers (Angus, Brahman and Angus x Brahman cross 19 ± 3.6 , 25 ± 5.4 and 37 ± 5.5 mounts, respectively) (Rae *et al.*, 1999). Ranasinghe *et al.* (2010) and Cutullic *et al.* (2009) reported that Holstein cows had less intense oestrus' and poorer reproductive performance compared with Normande cows. This may be due to higher milk yields in Holstein cows (Lopez *et al.*, 2004). Furthermore, Reith (2016) reported that the rate of occurrence of first behavioural oestrus after parturition in Danish Red cows was greater than in Jersey and Holstein cows. Reith (2016) also reported that inter-oestrous intervals varied considerably between different breeds of cows. Diskin and Sreenan (2000) reported that the oestrous cycle length of Holstein cows averaged 21 days. However, Negussie *et al.* (2002) reported the mean oestrous cycle length of tropical Fogera cows 29.2 ± 1.7 days, while the duration of oestrus of Holstein cows was averaged 10.6 ± 4.5 h (ranging from 2.2 h to 21.0 h) which was in close agreement with Lamothe-Zavaleta *et al.* (1991) who reported a mean duration of oestrus in Zebu cattle of 10.3 ± 4.5 h.

2.6. The effect of oestrus on-time budgets of dairy cows

The net behavioural response of a cow to her environment is represented in the 24 h time budget (Grant, 2011). A normal time budget of free-stall Holstein dairy cow is 3 to 5 h/d eating, 14 feeding bouts per day, 12 to 14 h/d lying time (resting), 2 to 3 h/d social interaction, 7 to 10 h/d rumination during both standing and laying time, 0.5 h/d drinking and 2.3 to 3.5 h/d spending time outside of the pen for milking and other management practices (Grant and Albright, 2000). The normal time budget is influenced by oestrus (Yániz *et al.*, 2006). One of the most representative indicators of oestrus in dairy cattle is restlessness (Diskin and Sreenan, 2000) as a result of the increase in E2 release (Sumiyoshi *et al.*, 2014). A study conducted on Holstein Friesian dairy cows, assuming the day of AI was the day of oestrus (rather than observing for oestrous behaviour), found that time spent feeding on the day of oestrus was reduced by about 20% in comparison to a normal day (2.82 vs 3.54 h/d, respectively; Halli *et al.*, 2015). In relation to this other recent studies of Holstein-Friesian cows have shown reduced DMI on the day of AI (14.6%; Reith *et al.*, 2014, 10.3%; Halli *et al.*, 2015). Reith and Hoy (2012) showed that rumination time was reduced on the day of oestrus from 429 to 355 min/d. Reith *et al.* (2014) using pedometers for oestrous detection found that during oestrus, 22 from 33 of studied Holstein-Friesian dairy cows drank 15% less water than on a reference day.

During oestrus, the time spent lying by dairy cows was decreased as a result of an increase in activity and restlessness (Jónsson *et al.*, 2011). According to Dolecheck *et al.*

(2015), oestrus-synchronised cows in oestrus also spent less time lying than non-oestrus cows (10.19 vs 24.8 min/h, respectively) when accelerometer was used to monitor activity. Kiddy (1977) reported that the activity of dairy cows increases by about 2 to 4 times when in oestrus compare to non-oestrus cows. Arney *et al.* (1994) found that this activity of dairy cows increases linearly during the period from 72 h to 16h before oestrus and increases further from 16h before to oestrus. Roelofs *et al.* (2005) reported that during behavioural oestrus the number of steps taken was higher (841 ± 254 steps/2h interval) when more cows were in oestrus simultaneously in comparison to 10 days before oestrus (179 ± 50 steps/2h interval).

As discussed in this literature review, the studies related to oestrous expression and detection published to date have a number of gaps in the knowledge specifically relating to the variation in oestrous expression, factors affecting oestrous expression and oestrous detection rate in lactating dairy cows. The remainder of this thesis will attempt to address some of these gaps.

2.7. Thesis objectives

The objectives of the thesis were to:

- 1- Characterise oestrous related changes in activity level, as the number of steps, lying time and lying bouts, during behavioural and silent oestrus.
- 2- Evaluate oestrous-related changes in feeding behaviour, dry matter intake, feeding duration and the number of visits to feed, during behavioural and silent oestrus.
- 3- Evaluate methods of oestrous detection such as visual observation, video camera recording, tail paint, IceQube accelerometer and GEA pedometer.
- 4- Investigate factors that are responsible for the variability in oestrous behaviours in dairy cattle.
- 5- Evaluate E2 and P4 concentrations in milk and their relation to oestrous activity during behavioural and silent oestrus.
- 6- Evaluate milk fatty acid profile during oestrus in lactating dairy cows.

CHAPTER 3

General materials and methods

3.1. Experimental animals

The animals used in each of the studies were lactating Holstein Friesian cows undergoing spontaneous oestrous cycles. Cows were chosen from about 398 cows of the Harper Adams University dairy herd. The cows were assessed at a post-natal check by the herd veterinarian and the selected cows had no abnormal signs in their reproductive tract. The study cows were milked twice a day at approximately 05:00 h and 16:30 h through a 40-point internal rotary milking parlour, with the milk yield of each animal recorded at each milking by an automatic recording system (Westfalia, GEA Milking System, Germany).

3.2. Ethical considerations

The Harper Adams University Research Ethics Committee approved all research protocols.

3.3. Proximate analysis of feed samples (Nutrient analysis of TMR)

3.3.1. Dry matter (DM)

For the determination of DM, feed samples were collected directly from the feed supplied to the cows at feeding time. The DM content of the diet was determined according to the Association of Official Analytical Chemists (AOAC, 2012; 934.01). An accurate weight of the sample was oven dried (Bider, Coel-Palmers, UK) overnight at 105°C to constant weight. A desiccator was used to cool down the samples after being taken from the oven and reweighed. The DM was calculated according to the following equation:

$$\text{DM (g/kg)} = \left[\frac{\text{Weight of dried sample (g)}}{\text{Weight of fresh sample (g)}} \right] \times 1000$$

3.3.2. Crude protein (CP)

Crude protein concentration in the samples was determined according to (AOAC, 2012; 990.03). An element auto LECO FP 528 N analyser (Leco Corporation, Stockport, UK) was used to determine CP concentration in the samples by combustion method. Dried milled samples were accurately weighed (150 mg) onto aluminium foil. They were then placed into the auto analyser, and the CP content of the feed was calculated according to the following equation:

$$\text{CP (g/kgDM)} = \text{Nitrogen Content (g/kg DM)} \times 6.25$$

3.3.3. Neutral detergent fibre (NDF)

The concentration of NDF of dried feed was measured using a Fibertec apparatus (Tecator Fibretec 1020 Hot Extractor, FOSS, Warrington, UK) in accordance to the methods described by Van Soest *et al.* (1991). The NDF reagent was previously prepared by mixing 93 g of disodium ethylene diamine tetraacetic acid dihydrate. Approximately 34 g sodium borate, 150 g sodium lauryl sulphate and 50 mL of tri-ethylene glycol with 22.8 g anhydrous di-sodium hydrogen phosphate were mixed and dissolved in distilled water then diluted to 5 litres with pH adjusted from 6.9 to 7.1.

Approximately 2.8 g of α -amylase enzyme from *Bacillus subtilis* spp (Sigma, Gillingham, UK) was dissolved in 90 mL of distilled water then 10 mL of tri-ethylene glycol was added for preparation of the α amylase solution.

Dried ground feed samples (0.5 to 0.6 g) was accurately weighed into previously dried and weighed crucible (sinter porosity 1, Suham Scientific, Ely, UK). The crucibles were placed into Fibretech® 1020 hot and 1021 cold extractor (Foss UK Ltd, Cheshire, UK) then about 25 mL of the cold neutral detergent reagent with a few drops of alcohol reagent grade (Sigma, Aldrich, Dorset, UK) were added to each of the crucibles. The samples were heated until starting of boiling; then the heat was reduced for about 30 min for digestion of samples. Following this the heat was switched off. Approximately 2 mL of α -amylase solution with an additional 25 mL of cold neutral detergent reagent were added, the samples were digested by boiling for an extra 30 min. Digested samples were then filtered and washed with 20 to 30 mL of hot distilled water (80°C). Additional α -amylase solution (2 mL) and distilled water (25 mL) were added to each sample and allowed to stand for 15 min. Then each sample was filtered and washed with hot distilled water 3 times. Crucibles were removed from the hot, and cold Fibretech® extractor then dried at 105°C overnight. After cooling using desiccator, crucibles were weighed and then placed in a muffle furnace for 4 h at 550°C. The crucible were cooled again to room temperature using the desiccators and NDF determined as follows:

$$\text{NDF (g)} = (\text{crucible} + \text{dry fibre weight}) - (\text{crucible} + \text{ash weight})$$

$$\text{NDF (g/kg DM)} = \left[\frac{\text{NDF weight (g)}}{\text{Weight of dried sample (g)}} \right] \times 1000$$

3.3.4. Ether extract (EE)

The ether extract content of feed samples was determined using the Soxtec apparatus (HT extraction apparatus, FOSS, Warrington, UK) according to the solvent procedure of Foss (1987). Approximately one gram (g) of dried sample was accurately weighed into a previously weighed cellulose extraction thimble (Warrington plc, Maidstone, UK). The cellulose extraction thimble was plugged with defatted cotton wool and fitted into the extraction unit. A cold petroleum ether (25mL) at 30–40°C (AnalaR, VWR International Ltd, Lutterworth, UK) was added to a pre-weighed extraction cup and placed under the extraction thimbles then boiled for one hour before additional rising 15 min. The final traces of the solvent were evaporated off, and the extraction cups were removed from the apparatus and moved into a fume cupboard. After the extraction cups were cooled, they were reweighed and the EE content determined:

$$\text{EE (g/kg DM)} = \left[\frac{\text{Weight of fat (g)}}{\text{Weight of dried sample (g)}} \right] \times 1000$$

3.3.5. Gross energy

The gross energy (GE) of feed was determined using an adiabatic bomb calorimeter apparatus (Parr 6200 Instrument Company, Moline, IL, 61265, USA). To make pelleted dried ground samples a pellet pressor (Parr 2811 pellet press, Parr Instrument Company, Moline, USA) was used. Pelleted samples were accurately weighed then placed into a crucible. Approximately 10 cm of fuse wire was inserted through the holes of the bomb, connected to both heads of the bomb without contact between the wire and the pelleted sample. The bomb was assembled and filled with O₂ gas pressure, then placed into a bucket containing water (2L). The energy content of the sample was measured by burning the sample in the bomb calorimeter under enclosed conditions and a constant volume of O₂ gas. The GE produced from the burning was measured as MJ/kg DM.

3.3.6. Ash and organic matter

Ash and organic matter were analysed according to AOAC (ash, AOAC, 2012; 942.05). Approximately 2g of dried and milled feed samples were weighed accurately into a labelled porcelain crucible and ashed by muffle furnace (Gallenkamp Muff Furnace, Size 3, GAFSE 620, Gallenkamp, Loughborough, UK) at 550°C for 4 h. A desiccator was used to cool down the samples and reweighed. The ash content of the feed was calculated according to the following equation:

$$\text{Ash (g/kg DM)} = \left[\frac{\text{Weight of ash (g)}}{\text{Weight of dry sample before ashing (g)}} \right] \times 1000$$

Organic matter (g/kg DM) content of the feed was calculated as 1000 minus ash content.

3.4. Hormone assays

3.4.1. Milk samples

Milk samples were collected from each cow at the afternoon milking according to Walker *et al.* (2008) and stored in a freezer at approximately -20°C until the E2 and P4 assays were completed. Milk samples were brought to room temperature and mixed well before analysis using an enzyme immunoassay.

3.4.1.1. Milk progesterone assay

Milk samples were brought to room temperature (22°C) and vortexed thoroughly to ensure homogeneity of the milk sample. Milk samples were analysed for P4 concentrations using a commercial 96 well microtitre plate based enzyme-linked immunosorbent assay (ELISA; Ridgeway Science Ltd., Gloucestershire, UK). The sensitivity of the assay was 0.15 ng/mL. The cross-reactivity of the antibody used in the kit is shown in Table 3.1. The assay procedure was as follows:

Microplates, coated with a polyclonal antibody, and the whole milk standards (0, 1, 2, 5, 10, 20 and 50 ng/mL) were warmed to room temperature prior to use. The foil seal was stripped from the wells, and they were emptied and tapped dry. Standards, milk samples and quality controls (10 µL; µL = Micro-litre) were added to the wells in duplicate. Then P4 horse radish peroxidase enzyme conjugate (200µL) was added to each well except two blank wells (A1 and B1), and the plate was incubated at room temperature for 1h and 30mins. After the incubation, all of the wells were emptied, and the plate was washed by filling with cold water, emptying and tapping dry 3 times. During the incubation period, the phenolphthalein monophosphatebis was dissolved in 25 mL substrate buffer.

Phenolphthalein monophosphatebis (cyclohexylammonium) substrate solution (200µL) was then added to all wells and incubated at 22°C for 20 min in the dark for a pink colour to develop. The absorbance of each well was determined at 570 nm using a microplate reader (BMG LABTECH, Allmendgrün 8, 77799 Ortenberg, Germany). Progesterone concentrations (ng/ml) were estimated from the absorbances using a four parameter logistic standard curve generated by MARS data analysis software (Version: 2.30 R3, Omega, BMG LABTECH, 2007-2012). A typical standard curve is shown in Figure 3.1.

Table 3. 1. Cross-reaction values for the polyclonal antibody in the P4 kit used for milk and serum P4 analysis.

Steroids	Cross-reactivity %
Progesterone	100
5 α -Pregnane-3,20-dione	5.3
5-Pregnane-3,20-dione	4.9
Epipregnenelone	5.4
Pregnenelone	0.5
17 α OH-Progesterone	0.3
21OH-Progesterone	3.2
Cortisol	<0.1
Cortisone	<0.1
Pregnanediol	<0.1
17 α OH-Pregnenelone	<0.1
Prednisolone	<0.1
Etiocholanolone	<0.1
DHA	<0.1
Oestrone	<0.1
Oestradiol	<0.1

Ridgeway Science Ltd., Rodmore Mill Farm, Alvington, Gloucestershire, UK

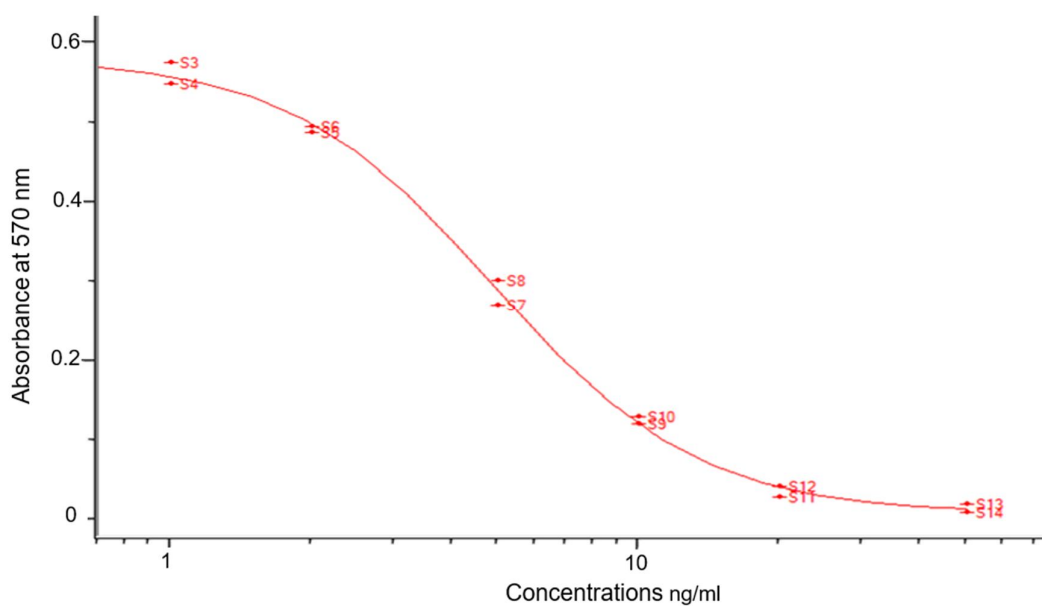


Figure 3. 1. The typical standard curve for the milk P4 enzyme immunoassay. Absorbance at a wavelength of 570 nm plotted against standard concentrations 0, 1, 2, 5, 10, 20 and 50 ng/mL.

3.4.1.2. Milk oestradiol assay

Whole milk samples were brought to room temperature (22°C) and mixed well to ensure homogeneity of milk sample (Domènech *et al.*, 2011). Duplicate milk samples were analysed for E2 concentration using a commercially available plasma and serum E2 ultrasensitive 96 well microtiter, plate-based, enzyme-linked immunosorbent assay kit (ELISA; ALPCO, Salem, NH 03079, US). The microtiter plates were previously coated with polyclonal antibody. The cross-reactivities of the antibody used in this kit are shown in Table 3.2. The sensitivity of the assay was <1.399 pg/mL. Milk E2 was determined according to the procedure provided by ALPCO with slight modification; an additional standard (50 µL of standard 0 pg/ml mixed with 50 µL of standard 3 pg/ml to give 1.5 pg/mL standard). Microplates coated with polyclonal antibody and standards; 0, 1.5, 3, 10, 50 and 200 pg/mL were also brought to room temperature approximately 3 h prior to use.

The foil seal was stripped from wells, and 100 µL of standards (containing 0.03% proclin 300 and 0.005% gentamicin sulfate as preservatives) were added to the first 12 wells. Milk samples and two samples of quality controls were added to the remaining wells in duplicate. A volume of 200 µL of E2 *enzyme conjugate* (Oestradiol horseradish peroxidase) was added to each well then all of the wells on the plate were thoroughly mixed for approximately 10 seconds. The plate was incubated at room temperature for 4 h 30 min. During the incubation period, 30 ml of concentrated Wash Solution (containing 5-bromo-5-nitro-1,3-dioxane; BND and 2-methyl-2H-isothiazol-3-one; MIT) were diluted (40X) with 1170 mL of deionized water to a final volume of 1200 mL and shaken about 10-20 min to mix properly.

After the incubation, all of the wells were emptied, and the plate was washed 3 times by adding diluted Wash Solution (400 µL per well), then emptying the wells and banging the plate on absorbent paper to remove residual droplets. A volume of 200 µL of Tetramethylbenzidine (TMB) Substrate Solution was then added to each well and incubated at room temperature for 30 min. Then 100 µL of Stop Solution (containing 0.5M H₂SO₄) was added to each well to stop the enzymatic reaction. Within 10 min of the stop solution being added, the absorbance of each well was determined at 450 nm using a microplate reader (BIOTEK Instrument Limited, Bedfordshire England, UK). The results were calculated automatically using a 4 parameter logistics curve fit by Gen5 All-In-One Microplate reader software (Version: 2.01.14, 2006-2012 Bio-Tek Instruments). A typical standard curve is shown in Figure 3.2.

Table 3. 2. Cross-reaction values for the polyclonal antibody in the E2 kit used for milk and serum P4 analysis.

Steroids	Cross-reactivity %
Oestradiol	100
Oestron	0.2
Oestriol	0.05
Androstendione	0
Corticosterone	0
Epiandrosterone	0
16-Epiestriol	0
Oestradiol-3-sulfate	0
Oestradiol-3-glucuronide	0
Oestradiol-17 α	0
Oestriol-16-glucuronide	0
Oestrone-3-sulfate	0
Dehydroepiandrosterone	0
11-Desoxycortisol	0
11-Desoxycorticosterone	0
21-Desoxycortisol	0
Dihydrotestosterone	0
Dihydroepiandrosterone	0
20-Dihydroprogesterone	0
11-Hydroxyprogesterone	0
17 α -Hydroxyprogesterone	0
17 α -Pregnenolone	0
17 α -Progesterone	0
Pregnanediol	0
Pregnantriol	0
Pregnanolone	0
Progesterone	0
Testosterone	0
Fulvestrant	0.3

ALPECO, Salam, NH 03079, USA

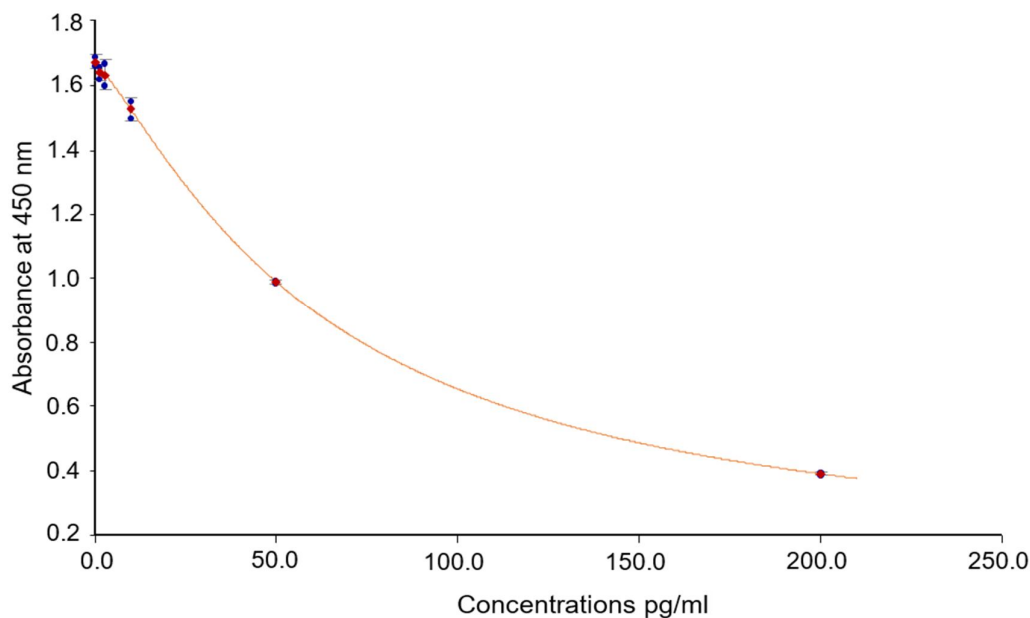


Figure 3. 2. A typical standard curve for the E2 enzyme immunoassay. Absorbance at a wavelength of 450nm plotted against standard concentrations 0, 1.5, 3, 10, 50 and 200 pg/mL. The blue dot representing the concentration of each of the standards in duplicate and red diamond represented the mean of each standards.

3.4.2. Blood samples

To determine the relationship between milk and blood E2 concentrations, milk samples were collected from 38 Holstein Friesian cows being blood sampled as part of another study from 78 ± 16.1 days in milk, with a live weight of 664 ± 52.5 kg. The average body condition score (BCS; Scale 1-5; AHDB Dairy, 2014) and locomotion score (LS; Scale 1-5; as described by Chapinal *et al.*, 2009) of the selected cows were 2.9 ± 0.2 and 2.3 ± 0.4 , respectively. The Milk samples (30 mL) were collected at morning and evening milking and stored at -20°C until analysed for E2 and P4 concentration. Blood samples (5 mL) were collected at approximately 11 a.m. from the jugular vein using (18G; 1.1/2 inch) vacutainer needles into vacutainer tubes (BD, Plymouth, UK). Blood samples were also stored at 4°C for 24 h and centrifuged at 1000g for 15 min at 4°C using a SIGMA centrifuge (SIGMA Osterode am Harz, Germany). Serum was separated and stored at -20°C until analysed for E2 and P4 concentration. Blood samples were collected in compliance with the United Kingdom Home Animals (Scientific Procedures) Act 1986 (amended 2012).

3.4.2.1. Serum progesterone assay

Serum samples and standards (0, 1, 2, 5, 10 and 20 ng/mL) were removed from the freezer (-20°C) and placed into an incubator to bring them up to 22°C. Duplicate serum samples were analysed for P4 concentration using a commercial 96 well microtitre plate ELISA (Enzyme-linked immunosorbent assay Ridgeway Science Ltd., Gloucestershire, UK). The sensitivity of the assay was 0.04 ng/mL. The assay procedure was as follows:

Microtitre plates coated with a polyclonal antibody and the serum standards were warmed at room temperature prior to use. The foil seal was stripped from wells, and they were emptied and tapped dry. Standards and serum samples (10 µL per well) were added to each well in duplicate. A volume of 200 µL of Wash Buffer was added to two blank wells (A1 and B1). A volume of 200 µL of P4 horseradish peroxidase enzyme conjugate was added to all another wells. The reagents were mixed, and the plate was incubated at 22°C for 2 h 20 min. After the incubation, all of the wells were emptied, and the plate was washed 3 times by filling Wash Buffer (200 µL) per well per wash, then dried on paper between each washing time. During the incubation period, the substrate was dissolved in 30 ml of the substrate solution.

A volume of 200 µL of the phenolphthalein monophosphatebis (cyclohexylammonium) substrate solution was then added to all wells including the blank wells and left for 20 to 40 min in the dark for the pink colour to develop. At a wavelength of 570 nm, the absorbance of each well was recorded by a microplate reader (BMG LABTECH, Allmendgrün 8, 77799 Ortenberg, Germany). Progesterone concentrations (ng/mL) were determined from the absorbance using a standard curve generated by MARS data analysis software (Version: 2.30 R3, Omega, BMG LABTECH, 2007-2012).

3.4.2.2. Serum oestradiol assay

Serum samples (38 samples) were brought to room temperature (22°C) approximately 3 h before analyses. Serum E2 was determined in duplicate according to the procedure provided by ALPCO without modification using E2 Ultrasensitive ELISA kit coated a polyclonal antibody (ALPECO, Salam, NH 03079, USA; See Milk E2 assay; section 3.4.1.2.) with sensitivity <1.399 pg/mL and standards: 0, 3, 10, 50 and 200 pg/mL.

3.5. Cow's activity

3.5.1. IceQube accelerometer

To monitor cow activity, IceQubes (IceRobotics Ltd., Edinburgh, UK) were attached to the back left leg of each cow using a Velcro® hook and loop strap (Dolecheck *et al.*, 2015). The IceQube incorporates a 3-axis accelerometer and reports cow activity summarised in 15-minute blocks (Dolecheck *et al.*, 2015). The data were downloaded each time the cow entered the milking parlour. The Cow Alert system (IceRobotics Ltd., Edinburgh, UK) uses the data from IceQubes and alert when activity is sufficiently increased. IceQubes generate data to show the number of steps taken, lying time and the number and length of lying bouts for each cow, every day.

3.5.2. GEA pedometer

Cow activity was also measured using GEA pedometers (GEA Rescounter II; Farm Technologies, Düsseldorf, Germany). This is the standard activity monitor used on the HAU dairy unit as an oestrous detection aid. All cows were fitted with a GEA Rescounter II pedometer on their right front leg, according to the manufacturer recommendations. The activity of the cow recorded by the Rescounter II is based on a 2h cycle measured as AU, the data were transferred to a computer through the receivers at the entrance to the milking parlour. Data were collected when cows came into the parlour to be milked twice a day.

3.6. Definitions

3.6.1. Body condition score and locomotion score

The body condition scores (BCS) of the study the cows were determined every fortnight by the same technician according to the method described by AHDB dairy (2014; scale 1-5). The locomotion scale (LS; scale 1-5) were determined as described by Chapinal *et al.* (2009).

3.6.2. Definition of oestrus based on milk progesterone profile

A cow was considered to be in oestrus when milk P4 concentrations were <3 ng/mL for two days before a period when P4 rose to >5 ng/mL for at least 5 days (Isobe *et al.*, 2004), see Figure 3.3. If no signs of oestrus were observed, the silent oestrus was taken as the day of the lowest P4 concentration (Ranasinghe *et al.*, 2010).

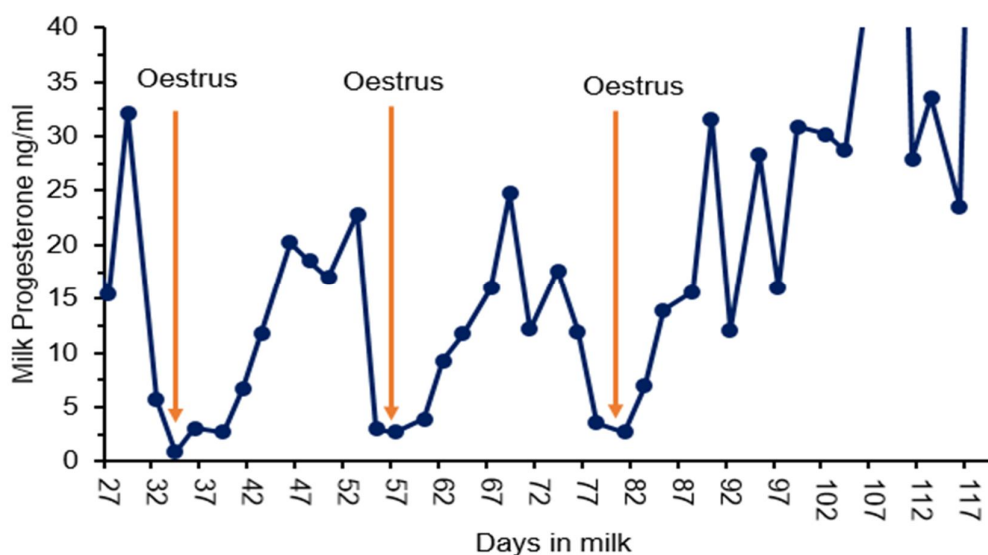


Figure 3. 3. A typical milk P4 profile ng/mL of a lactating Holstein Friesian dairy cow.

3.6.3. Definition of behavioural and silent oestrus based on IceQube

Each oestrus event identified from the P4 profile was classified as *behavioural* or *silent* oestrus. A cow was defined to be in *behavioural* oestrus when the Cow Alert system (IceRobotics) produced an oestrus alert as a result of increased to >80% above the mean number for the preceding three days followed by a decrease to <80% of the following two days (López-Gatius *et al.*, 2008), in addition to the change in milk P4 profile described in section 3.6.2. A cow was considered to be in *silent* oestrus when the Cow Alert system did not produce an alert on the day of assumed oestrus according to the milk P4 profile.

3.6.4. Duration of oestrus according to IceQube and Rescounter II (GEA)

Oestrus duration was defined as the time (h) between an increase in activity (Rescounter II, GEA in arbitrary unit; AU), and the number of steps taken (IceQube; IceRobotics) to >80% above the mean number for the preceding three days and the decrease to <80% of the following two days (López-Gatius *et al.*, 2008).

3.6.5. Number of oestrus post-partum

Each oestrus event that was detected by the milk P4 profile and generated an alerted from the Cow Alert system was assigned as the first, second or \geq third observed oestrus PP. The cows that conceived at their 3rd or 4th oestrus PP were considered \geq 3rd oestrus post-partum. Thus the number of oestrus post-partum were grouped as 1st, 2nd and \geq 3rd oestrus and data were analysed for the effect of oestrus number PP on oestrus expression in dairy cows.

3.7. Artificial insemination and pregnancy diagnosis

All cows which showed behavioural oestrus were artificially inseminated 12 h after oestrous detection. All AI were performed by one trained dairy herd technician by transcervical fixation, using frozen-thawed semen from one of six Holstein Friesian bulls. All cows that did not return to oestrus within 30 days of AI 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). Cows were designated pregnant (PD+) or non-pregnant (PD-). Cows that had returned to oestrus 18 to 30 days after AI were also considered to be non-pregnant (PD-) and these cows were not presented for pregnancy diagnosis by transrectal ultrasound.

CHAPTER 4

Characterising changes in activity and feeding behaviour of housed, lactating dairy cows during behavioural and silent oestrus

4.1. Introduction

The normal time budget of a Holstein dairy cow fed a total mixed ration (TMR) and in free-stall housing is 3 to 5 h/d eating, with an average 14 feeding bouts per day, 12 to 14 h/d lying time, 2 to 3 h/d social interaction, 7 to 10 h/d rumination during both standing and lying time, 0.5 h/d drinking and 2.3 to 3.5 h/d spent outside of the yard for milking and other management practices (Grant and Albright, 2000).

In mammals, oestrus is a behavioural sign that ensures that the female is ready to be mated close to the time of ovulation (Perry, 2004). Mounting behaviour with standing to be mounted is the definitive sign of oestrus (Roelofs *et al.*, 2010). However, over the past 30 to 50 years, the incidence of mounting behaviour has decreased from 80% to 50% in dairy cows (Dobson *et al.*, 2008) and over the last 50 years the duration of oestrus in dairy cattle has also declined from 18 to 8 h (Dolecheck *et al.*, 2015). Oestrus is the period of maximum sexual activity; it has been shown to range from 2 to 30 h (Hanzen *et al.*, 2000). Standing oestrus is often defined as true oestrus when the cow makes no effort to escape when mounted by other cows and its duration is defined as “the interval between the first and last standing events” (Hurnik *et al.*, 1975). Other signs of oestrus include mounting of other cows, increased activity and mucus discharge from the vulva (Sveberg *et al.*, 2011). While standing to be mounted is considered as the primary behavioural sign of oestrus, other behaviours such as ano-genital sniffing, restlessness, bellowing, chin resting, head mounting, and an attempt to mount are considered secondary signs (Gordon, 2011).

The cows' normal time budget can be influenced by oestrus (Yaniz *et al.*, 2006). During oestrus, the activity of dairy cows increases about 2 to 4 times compared to non-oestrus cows (Kiddy, 1977). In addition, during the period from 72 to 16 h before standing oestrus, dairy cow activity increases linearly with further increases during the 16 h before standing oestrus (Arney *et al.*, 1994). In dairy cows, ovulation occurs from 8 to 30 h after the onset of increased activity (Hockey *et al.*, 2010). With the availability of activity monitoring on commercial dairy farms, restlessness has become an important indicator of oestrus (Diskin and Sreenan, 2000).

During oestrus, the time spent lying by dairy cows decreased as a result of increased activity and restlessness (Jónsson *et al.*, 2011) driven by increased secretion of E2 (Sumiyoshi *et al.*, 2014) from the developing ovulatory follicle (Allrich, 1994). According to Dolecheck *et al.* (2015), oestrus-synchronised cows spent less time lying than non-oestrus cows (10.19 vs 24.82 min/h, respectively) when IceQubes were used to monitor activity, and Reith *et al.* (2014) found that dairy cows drank 15.3% less water during oestrus. In a study where the day of AI was assumed to be the day of oestrus (rather than observing for oestrus behaviour), Halli *et al.* (2015) found that cows spent approximately

21% less time feeding on the day of oestrus in comparison to other days of the oestrous cycle (2.82 vs 3.54 h/d, respectively), but it was unclear whether the cows were synchronised or naturally cycling. In addition, Reith and Hoy (2012) showed that rumination was reduced on the day of oestrus from 7.2 to 5.9 h/d.

However, 35 % of cows show no obvious behavioural signs of oestrus and are defined as showing silent oestrus (Palmer *et al.*, 2010). This means that despite the use of oestrus detection aids such as activity monitors, judging the correct time for AI in naturally cycling cows is difficult.

The present study was designed to:

- 1- Investigate whether the activity and feeding behaviour of lactating Holstein Friesian cows undergoing spontaneous oestrous cycle is affected by behavioural and silent oestrus.
- 2- Investigate the effect of the number of oestrus PP on the activity and feeding behaviour of lactating Holstein Friesian cows.
- 3- Evaluate methods of oestrous detection.

4.2. Materials and methods

The experiment was undertaken between June and August 2016 at the dairy unit of Harper Adams University, Newport, Shropshire, TF10 8NB, UK.

4.2.1. Ethical considerations

See section 3.2.

4.2.2. Experimental animal, housing and management

Thirty Holstein Friesian cows (parity 2.5 ± 1.1 mean \pm SD) with an initial body weight of 637 ± 60.0 kg and daily milk yield of 35.8 ± 1.8 kg/d, were used at Harper Adams University dairy unit (see section 3.1). At the start of the study, the cows were 20 ± 6.3 days in milk and 2.9 ± 0.28 BCS (see section 3.6.1). The average LS of the selected cows was 2.0 ± 0.58 (see section 3.6.1). They were submitted for assessment of any abnormal signs in the reproductive tract by the herd veterinarian approximately 21 days PP. Cows were kept as a separate group, housed in a covered yard with 34 cubicles (2.7 x 1.2 m, with 3 cm thick rubber mattresses) and two grooved concrete passageways (6 x 50 m) giving approximately 10.8 m² area per cow. The cubicles were bedded with sawdust three times per week. The passageways were scrapped by an automatic scraper 4-5 times per day. Study cows were milked twice a day.

4.2.3. Diet composition

The cows were fed a total mixed ration (TMR) *ad libitum* via 30 Roughage Intake Control System (RICs) feed bins with automated weighing system (1.0 x 0.9 x 0.8 m) manufactured by Insentec B. V. (Marknesse, the Netherlands). The composition of the TMR is shown in Table 4.1.

They were moved into the study area on 6th June 2016 and data were collected until 19th August 2016. All the cows used in the study were trained to feed through RICs bins over a one week period in order to ensure that each cow could access feed without assistance. Water was provided from water troughs *ad libitum* at the end of each passageway area.

4.2.4. Nutrient analysis of TMR

For the determination of nutrient supplied of the ration, feed samples were collected directly from RICs bins at feeding time (Table 4.2).

Table 4. 1. Dietary composition of the trial total mixed ration. DM = dry matter.

Ingredient	g/kg DM	kg DM/hd
Maize silage	342.20	7.20
Lucerne	161.60	3.40
Blend	200.57	4.22
Soda wheat	113.12	2.38
Sweet starch	73.19	1.54
Soya hulls	53.23	1.12
Spey syrup	26.62	0.56
Megalac	7.13	0.15
Butterfat extra	7.13	0.15
Dairy minerals	7.13	0.15
Salt	3.33	0.07
Acid buff	3.80	0.08
Saccharomyces cerevisiae	0.95	0.02
Total	1000	21.04

(Profeed Nutrition Consultancy, UK, 2016)

Table 4. 2. Predicted nutrient content of the total mixed ration. DM = dry matter, ME = metabolisable energy, CP = crude protein, NDF = neutral detergent fibre, GE = Gross energy, OM = organic matter.

Nutrient content	
DM (g/kg fresh)	395.3
ME (MJ/kgDM)	11.8
CP (g/kg DM)	176.0
NDF (g/kg DM)	363.5
Fat (g/kg DM)	35.6
GE (MJ/kgDM)	18.8
Ash (g/kg DM)	76.7
OM (g/kg DM)	923.3

4.2.4.1. Dry matter (DM)

For determination of DM, feed were samples collected directly from RIC bins at feeding time, and immediately oven dried overnight at 105°C to constant weight (AOAC, 2012; 934.01) see section 3.3.1.

4.2.4.2. Metabolisable energy (ME)

Samples of TMR were sent to Sciantec Analytical Services (Stockbridge Technology Centre, Cawood, North Yorkshire YO8 3SD) for ME (MJ/kg DM) determination.

4.2.4.3. Crude protein (CP)

Crude protein was determined using LECO FP 528 N analysers (Leco Corporation, St Joseph, MI, USA) by combustion method (AOAC, 2012; 990.03) see section 3.3.2.

4.2.4.4. Neutral detergent fibre (NDF)

The concentration of NDF was measured in accordance with the methods described by AOAC (2012; 2002.04), see section 3.3.3.

4.2.4.5. Fat

The fat content of TMR was determined by the Soxtec system (Foss UK Ltd, Warrington, UK) using petroleum ether as described by AOAC (2012; 920.39), see section 3.3.4.

4.2.4.6. Gross energy (GE)

The GE of feed was determined using a bomb calorimeter apparatus (Parr 6200 Instrument Company, Moline, IL, 61265, USA) see section 3.3.5.

4.2.4.7. Ash and organic matter

For Ash and organic matter determination, milled feed samples were ashed by muffle furnace at 550°C for 4h (AOAC 2012; 942.05) see section 3.3.6.

4.2.5. Milk samples

Milk samples (30 mL) were collected from each cow daily at the afternoon milking according to Walker *et al.* (2008) see section 3.4.1.

4.2.6. Milk progesterone assay

Oestrous periods were identified by measuring the concentration of P4 in whole milk. Milk samples (40 mL) were collected from each cow 3 times per week on Monday, Wednesday and Friday afternoon. Immediately after sampling one preservative tablet (Broad Spectrum Microtabs II, Advanced Instrument, INC. USA; containing 8 mg Bronopol and 0.30 mg Natamycin) was added to each milk sample. Sample pots were inverted to mix

until the tablet was dissolved. The samples were stored in a refrigerator at 4°C until the P4 assay which was completed within one week of collection. Milk samples were brought to room temperature and mixed well before analysis using an enzyme immunoassay (Ridgeway Science Ltd., Rodmore Mill Farm, Alvington, Gloucestershire, UK) see section 3.4.1.1.

4.2.7. Data collection

4.2.7.1. Visual observation of oestrus behaviour

Cows were visually observed for signs of oestrus three times per day at 07:30, 12:30 and 19:30 for about 30 min using the scoring system of Van Eerdenburg *et al.* (2002) see Table 4.3. All observation was carried out by the same researcher or technician. Study cows were identified by numbers from 1 to 30 and an individual combination of coloured tape applied to the back of each cow (Kerbrat and Disenhaus, 2004). The assigned scores were recorded each time a symptom was observed. A cow was considered to be in oestrus if the sum of the points scored exceeded 100 during one observation period. Cows were inseminated in the afternoon if behavioural oestrus was observed in the morning, whereas they were inseminated the following morning if they were seen in oestrus in the afternoon (Trimberger and Davis, 1943).

Table 4. 3. Scoring scale for visual observation of oestrous signs.

Oestrous signs	Scores
Flehmen	3
Mucous discharge from the vulva	3
Cow restlessness	5
Sniffing the vulva of another cow	10
Mounting but not standing	10
Resting the chin on the back of another cow	15
Mounting or attempt to mount other cows	35
Mounting or attempt to mount head side other cows	45
Standing heat	100

(Source: Van Eerdenburg *et al.*, 2002)

4.2.7.2. Video recording of oestrous behaviour

The cows were monitored to detect spontaneous behavioural oestrus using four video cameras (Voltek, KT&C Co Ltd, Seoul, South Korea) for approximately 19.46 ± 1.7 h/d except for milking time, AI time, foot trimming and vet observation. The four cameras were placed at about 5.25 m above the trial cubicles and passageways to give a clear view of the area in which the cows were housed. Viewing angle of the camera was 35-90 degrees to give a large field of view. The cameras were connected to an external hard drive video recorder (Sentient 960H, Digital Video Recorder HDMI, Output 1080p Full HD, England, UK). Cows were identified by numbers from 1 to 30 on both sides of the cow and an individual combination of coloured tape on each cow (Kerbrat and Disenhaus, 2004). Video recordings were retrospectively reviewed to determine the time and intensity of oestrus. The scores of Van Eerdenburg *et al.* (2002) were allocated and recorded each time a sign of oestrus was observed on the video recording. The total number of points scored in a day indicated oestrous intensity.

Behavioural interactions were recorded (Table 4.4) such as standing to be mounted, mounting without standing, attempt to mount, chin-resting and sniffing the ano-genital region were categorised as sexual interaction (Kerbrat and Disenhaus, 2004).

The following equation was used to correct the number of behavioural signs which were missed during milking time

$$X = (1440 - T) (N/T)$$

X = number of observed signs in milking time

N = number of observed signs observed outside milking

T = observed time (mins)

4.2.7.3. Tail paint

Fluorescent pink tail paint (All-Weather Paint-Stik, LA-CO Industries, Inc., 1201 Pratt Blvd. Elk Grove Village, USA) was first applied to the tail head of each cow at an average of 27 days PP. Tail paint was inspected and recorded each morning for oestrous signs. When more than 75% of paint was removed it was considered all removed, between 10 and 75% of total paint removed was recorded as some removed but when less than 10% was removed it was considered as no paint removed.

4.2.7.4. IceQube accelerometer

Cow activity was monitored by IceQubes (IceRobotics Ltd., Edinburgh, UK) see section 3.5.1.

4.2.7.5. GEA pedometer

Cow activity was also measured using GEA Rescounter II pedometers (GEA Farm Technologies, Düsseldorf, Germany) see section 3.5.2.

Table 4. 4. Behavioural signs which were recorded.

Classification	Behavioural sign	Definition
Sexual interactions	Stand to be mount	The animal stands to be mounted by another cow from behind. The mounted cow does not try to move away from the other cow but may move a few steps over the period of the mount to balance the weight of the mounting cow.
	Mounting without standing	The mounted cow moves away forwards, sideways or backwards when mounted from behind by another cow.
	Mount	The mounting cow orientates herself behind another cow, then raises her body above that of the other cow and clasps the cow with her front legs in front of the other cow's pelvic bone. The mounting cow may or may not push forwards and backwards.
	Attempt to mount	Cow attempts to mount any part of the body of another cow but the cow being mounted avoids mounting.
	Mounting head end on	The cow mounting of another cow from the head (heat-to-head mounting).
	Chin-resting	The animal rests or rubs its chin on the rump area of another cow.
	Sniffing the ano-genital region	Cow brings its head within approximately 10 cm of the ano-genital area of another cow for more than approximately 5 seconds in order to sniff the region.
Social interactions	Caressing	Cow caressing any part of another cow, using the top or underside of the head or neck.
	Licking	Cow licking the head, the flank or the neck of the other cow.
Agonistic interactions	Head to head butt	The animal stands to face another and butts the head of another animal, using its own head.
	Butting another part	The cow butts any part of another cow, using its own head.
	Sniffing the udder	The cow sniffing the udder of another cow.

Adapted from Kerbrat and Disenhaus (2004)

4.2.7.6. Feed intake

All cows used in the study were trained to feed through RICs bins over a week period in order to ensure that each cow could access RICs bins without assistance. About 65 kg of fresh TMR per cow was provided daily at approximately 08:30. The RICs identifies each cow by scanning the individual transponders, installed within the cow's collar. Once the cow has been identified, the gate of the RICs drops to permits access to the feed trough and the amount of feed consumed at each visit is automatically recorded. Therefore, feed intake and the duration of each visit to the feed trough was recorded by the system for each cow. The previous days leftover feed were removed on Monday, Wednesday and Friday morning at 08:00 for about 20 to 30 min and RICs were cleaned.

4.2.8. Definitions of oestrus

4.2.8.1. Definition of oestrus based on milk progesterone profile

A cow was considered to be in oestrus when milk P4 concentrations were less than <3 ng/mL (See section 3.6.2).

4.2.8.2. Definition of oestrus based on IceQube and GEA Rescounter II

Oestrus was defined as an increase in walking activity and the number of steps (see section 3.6.3).

4.2.8.3. Definition of *behavioural* and *silent* oestrus

Each oestrus identified by the P4 profile was classified as *behavioural* or *silent* oestrus. A cow was defined to be in *behavioural oestrus* when the sum of the points scored for oestrus behaviour observed by the video recording exceeded 100 (Van Eerdenburg *et al.*, 2002). A cow was considered to be in *silent oestrus* when the cow did not display any behavioural signs of oestrus, or the oestrus score was <100 points at or around the day of oestrus as defined by her milk P4 profile (Van Eerdenburg *et al.*, 2002).

4.2.8.4. Number of oestrus post-partum

Each oestrous event that was detected by the milk P4 profile and visually observed and/or successfully alerted by the Cow Alert system was included in the first, second and third or more oestrus PP (see section 3.6.5).

4.2.9. Duration of oestrus

4.2.9.1. Duration of oestrus according to camera data

The duration of oestrus recorded by the camera was defined as the interval between the time that cows showed the first observed signs of oestrus and the time that the last observed signs of oestrus were observed.

4.2.9.2. Duration of oestrus according to IceQube and GEA

Oestrus duration was defined as an increase in activity (See section 3.6.4).

4.2.10. Statistical analysis

Statistical analyses were performed using the Genstat statistical software package (Genstat V 17th.17.1.14713 supplied by VSN International Ltd, UK). The datasets were analysed by repeated measures ANOVA to compare between groups (behavioural and silent oestrus) days before and after oestrus and the group x day interaction. The datasets were analysed by factorial one way ANOVA to compare sexual, social and agonistic frequency between 1st, 2nd and ≥3rd oestrus. Factorial one way ANOVA was used to compare *behavioural* and *silent* oestrus on the day of oestrus. When there was a significant interaction a paired t-test was used to compare the changes in the variable such as lying time h/d, steps per day, lying bouts, DM intake (kgDM/d), feeding duration (h/d) and number of visiting to feed occurs from day -3, -2 and -1 to day of oestrus (0) and from day of oestrus to +1, +2 and +3. Tukey test was used to compare between 1st, 2nd and ≥3rd oestrus PP and silent versus behavioural oestrus. Chi-Square tests were used to compare expression (0/1) of flehmen, mucous discharge from vulva, restlessness, mounting but not standing, mounting or attempt to mount and standing to be mounted between 1st, 2nd and ≥3rd oestrus PP. Kappa statistical analyses were used to detect the percentage of sensitivity and specificity between Camera as a 'gold standard' and other methods of monitoring oestrus in cows.

Sensitivity% = (Oestrus undetected by other methods agreement/Total undetected by the gold standard and other methods) *100

Specificity% = (Oestrus detected by other methods agreement/Total detected by the gold standard and other methods) *100

(Cavalieri *et al.*, 2003; Sim and Wright, 2005)

Linear regression analyses were used to determine the relationship between the numbers of steps taken/d and DM intake (kg DM/d) on the day of behavioural oestrus and lying time h/d. It was also used to determine the relationship between a number of steps and the number of signs observed and the total score of observed symptoms on the day of behavioural oestrus. Simple linear regression and multiple linear regression were also used to compare the relationships between the number of steps on the day of oestrus and SG, parity, DPP and LS at oestrus.

The relationship between oestrus expression (response variable; behavioural = 1, silent = 0) and each of the explanatory variables: steps, SG, LS, DPP, parity and BCS was explored using logistic regression analyses. Additionally, the influence of other explanatory variables (LS, parity and BCS) on the relationship between oestrus and steps were assessed using forward stepwise logistic regression. This involved assessing the change in deviance following the addition of each of the selected variables in turn to a model including the constant term alone. Differences were reported as significant at $P < 0.05$ and trends were reported when P is between <0.1 and >0.05 .

4.3. Results

4.3.1. The percentage of oestrus detection, the duration of oestrus and scores of behavioural activity

For all cows, approximately 61 oestruses were detected during the study period by milk P4 profile. For the 61 oestruses, the percentage of oestrus detection by camera, IceQube, GEA, visual observation and tail paint were 65.5, 52.4, 41.1, 35.0, and 15.0% respectively. From Kappa analysis and camera as a gold standard, the percentage of sensitivity and specificity between camera and IceQube, camera and GEA, camera and visual observation, and camera and tail paints were 100% and 80%, 100% and 63%, 100% and 53% and 100% and 23%, respectively. The average duration of oestrus was 9.1 ± 3.1 h from the camera, based on the number of steps recorded by IceQube, the oestrous duration was 12.9 ± 2.5 h, and the duration of GEA oestrus was 13.0 ± 3 h. The number of points scored during *behavioural* oestrus determined from the camera recordings was between 225 and 2921 points. However, during *silent* oestrus, the number of points scored was between 0 and 32 points.

4.3.2. Behavioural and silent oestrus

4.3.2.1. Oestrus activity

Of the 61 oestrus events detected, 40 were defined as *behavioural* (65.5%) and 21 defined as *silent* (34.5%) oestrus. Regarding time budgets during behavioural versus silent oestrus in comparison to the average of 3 days before (DB) and 3 days after (DA) oestrus, a number of steps, lying time and lying bout, steps were increased about 146.8% vs 10.4% and 115.9% vs 15.9% but lying time was reduced around 29.7% vs 7%, and 32.2% vs 5.4% and lying bouts were also reduced by 30% vs 8.1% and 28.3% vs 5.8%, respectively. In addition, on the day of behavioural oestrus the number of the steps (2095 ± 217 steps; mean \pm SEM) were higher ($P < 0.001$) compared to 3DB (849 ± 60 steps) and 3DA (971 ± 61 steps) while on the day of silent oestrus the number of steps (984 ± 73.5 steps) were not significantly different in comparison to 3DB (891 ± 63 steps) and 3DA (849 ± 50 steps). From factorial one way ANOVA, cows took more ($P < 0.001$) steps during behavioural oestrus compared to silent oestrus. There was an interaction ($P < 0.001$) between oestrus expression and day of oestrus on the number of steps taken (Figure 4.1). In addition, there was a positive correlation ($P < 0.001$) between the number of points scored and the number of steps taken ($y = 0.348x + 486$; $r^2 = 0.32$) during behavioural oestrus.

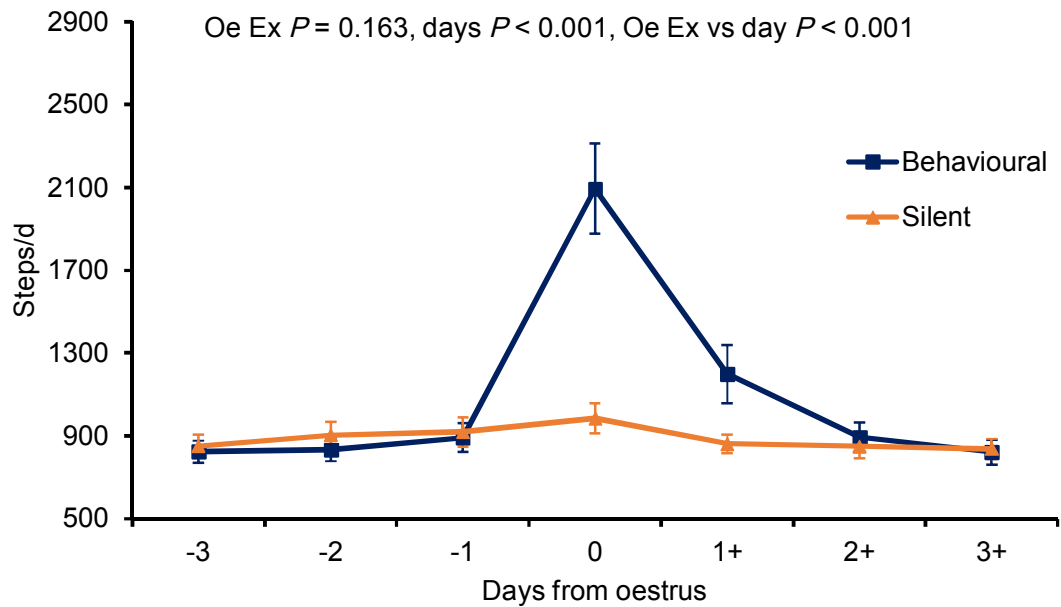


Figure 4. 1. Effect of oestrus on the number of steps, 3 days before, on the day of oestrus (0) and 3 days after and during silent ($n = 21$) and behavioural ($n = 40$) oestrus in lactating Holstein Friesian dairy cows. Oe Ex = Oestrus Expression, 0 = day of oestrus, error bars = SEM.

Lying time and the number of lying bouts (7.1 ± 0.3 h/d and 9.1 ± 0.5 bouts, respectively) were reduced ($P < 0.001$) on the day of behavioural oestrus in comparison to 3DB (10.0 ± 0.3 h/d and 13.0 ± 0.7 bouts, respectively) and 3DA (10.1 ± 0.3 h/d and 12.7 ± 0.8 bouts, respectively). However, lying times (9.3 ± 0.5 h/d) and the number of lying bouts (13.0 ± 1.1) bouts were not significantly affected by the day of silent oestrus compared to 3DB (10.0 ± 0.4 h/d and 14.0 ± 1.2 bouts, respectively) and 3DA (10.4 ± 0.4 h/d and 13.7 ± 1.3 bouts, respectively). Furthermore, from a factorial one way (ANOVA), lying times were lower ($P < 0.001$) on the day of behavioural oestrus compared to silent oestrus and the number of lying bouts were also lower ($P < 0.001$) on the day of behavioural oestrus compared to silent oestrus. With regard to lying time, there was an interaction ($P < 0.001$) between oestrus and day with lying time significantly reduced during behavioural oestrus but not silent oestrus (Figure 4.2). Similarly, the number of lying bouts was reduced during behavioural oestrus but not during silent oestrus as well as there being an interaction between oestrus expression and day ($P < 0.01$) see Figure 4.3. In addition, there was a negative correlation ($P < 0.001$) between the number of steps taken and lying time ($y = 0.0001x + 9.78$; $r^2 = 0.37$) during *behavioural* oestrus.

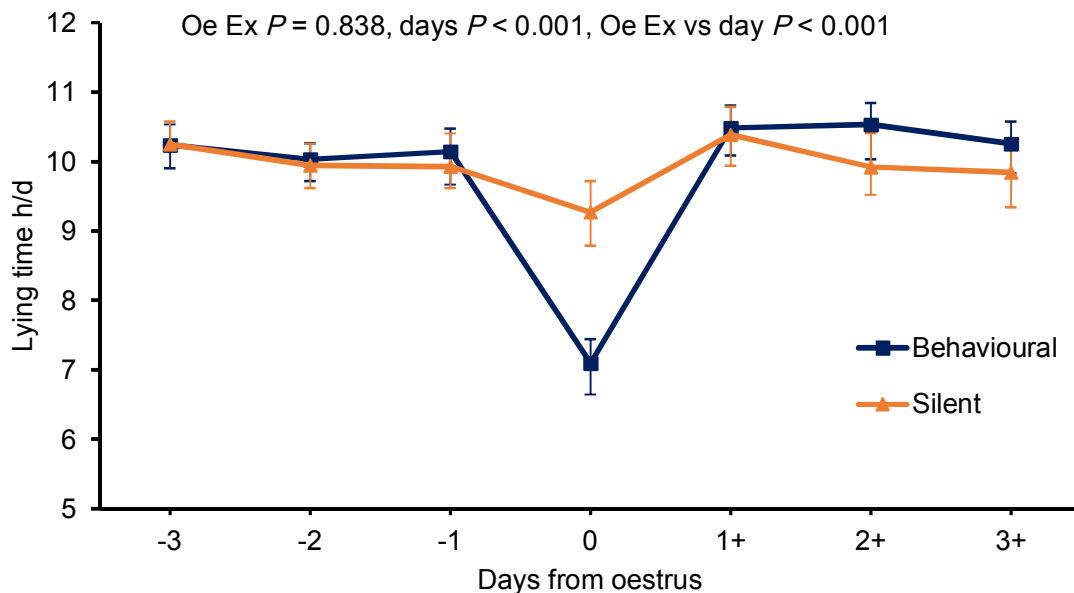


Figure 4. 2. Effect of oestrus on lying time, 3 days before, on the day of oestrus (0) and 3 days after and during silent ($n = 21$) and behavioural ($n = 40$) oestrus in lactating Holstein Friesian dairy cows. Oe Ex = Oestrus Expression, 0 = day of oestrus, error bars = SEM.

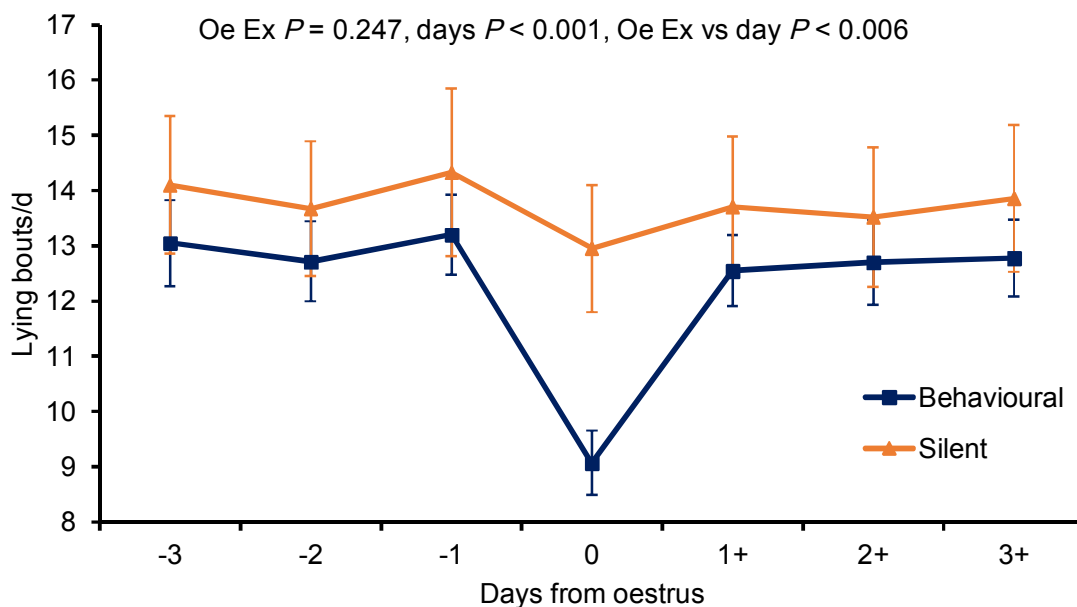


Figure 4. 3. Effect of oestrus on a number of lying bouts, 3 days before, on the day of oestrus (0) and 3 days after and during silent ($n = 21$) and behavioural ($n = 40$) oestrus in lactating Holstein Friesian dairy cows. Oe Ex = Oestrus Expression, 0 = day of oestrus, error bars = SEM.

4.3.2.2. Feeding behaviour

Dry matter intakes were lower ($P < 0.001$) on the day of behavioural oestrus (19.8 ± 0.41 kg/d) in comparison to 3DB (22.4 ± 0.5 kg/d) and 3DA (22.6 ± 0.5 kg/d) Table (4. 5). There was a negative correlation ($P < 0.001$) between the number of steps taken and DMI ($y = -0.0014x + 22.46$; $r^2 = 0.46$) during behavioural oestrus. However, DMI was not significantly lower on the day of silent oestrus compared to other days. There was also no interaction between oestrous expression and day.

The occurrence of behavioural oestrus reduced ($P < 0.001$) the mean duration of feeding (2.4 ± 0.09 h/d) in comparison to 3DB (3.4 ± 0.17 h/d) and 3DA (3.2 ± 0.12 h/d) Table (4. 5). Duration of feeding (2.9 ± 0.15 h/d) was also reduced ($P < 0.03$) during silent oestrus when compared to one day before (3.4 ± 0.2 h/d) and one day after (3.6 ± 0.2 h/d) the predicted day of oestrus. There was a tendency for an interaction between oestrous expression and day ($P = 0.06$) effect on feeding duration.

The mean number of visits to the RICs feed bins during behavioural oestrus was less (25.3 ± 1.26 visits/d; $P < 0.01$) compared with 3DB and 3DA oestrus (Table 4. 5). However, there were no differences ($P = 0.211$) in the number of visits on the day of silent oestrus (29.0 ± 1.71 visits/d) in comparison to other days. There was also no interaction ($P = 0.588$) between oestrus expression and day with regard to the number of visits to feed. Analysing the number of visits to feed with regard to oestrous expression, there was also no difference ($P = 0.318$) between behavioural and silent oestrus.

4.3.3. First, second and third or more oestrus PP

The percentage of first, second and third or more oestrus from 61 oestrus detected by P4 profile were 47.5% ($n = 29$), 36% ($n = 22$) and 16.3% ($n = 10$), respectively. Moreover, the percentage of behavioural oestrus during the first, second and third or more oestrus PP were 55.2%, 72.7% and 80% respectively.

4.3.3.1. Oestrus activity during 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP

Regarding the number of steps, there were more ($P < 0.001$) steps taken (1502 ± 192 steps) on the day of oestrus at first oestrus PP than 3DB and 3DA oestrus. At second oestrus there was higher ($P \leq 0.01$) number of steps (1730 ± 285 steps) at day (0) in comparison to other days. There was also a higher ($P < 0.001$) number of step count (2285 ± 480) on the day of oestrus compared to 3DB and 3DA, while no difference ($P = 0.07$) compared to the day two after oestrus (1234 ± 273 steps). There was no difference ($P = 0.233$) in the number of steps on the day of oestrus between 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus. There was no interaction ($P = 0.220$) between oestrus number and time (Figure 4.4).

Table 4. 5. Means of DM intake (gkDM/d), feeding duration (h/d) and the number of visiting to feed/d, 3 days before, on the day of oestrus (0) and 3 days after oestrus, during behavioural ($n = 40$) and silent ($n = 21$) oestrus in lactating Holstein Friesian dairy cows.

Feeding behaviour	Oe	Time/d							SED	P-value			
		Ex	-3	-2	-1	0	1+	2+		3+	Oe Ex	Days	Oe Ex vs days
Dry matter intake kg/d	B		22.5	22.1	22.5	19.8	22.5	22.5	22.7	0.91	0.314	<0.001	0.371
	S		21.9	21.8	21.6	20.5	21.8	21.8	22.0				
Feeding duration h/d	B		3.5	3.4	3.3	2.4	3.2	3.2	3.3	0.18	0.306	<0.001	0.06
	S		3.1	3.4	3.4	2.9	3.6	3.4	3.4				
Number of visits to feed/d	B		29.2	28.8	27.2	25.3	27.8	28.3	28.3	3.75	0.318	<0.01	0.588
	S		29.6	29.9	29.0	29.0	30.5	30.6	30.8				

Oe Ex = Oestrus Expression, B = Behavioural oestrous and S = Silent oestrus, 0 = day of oestrus, SED = standard errors of differences

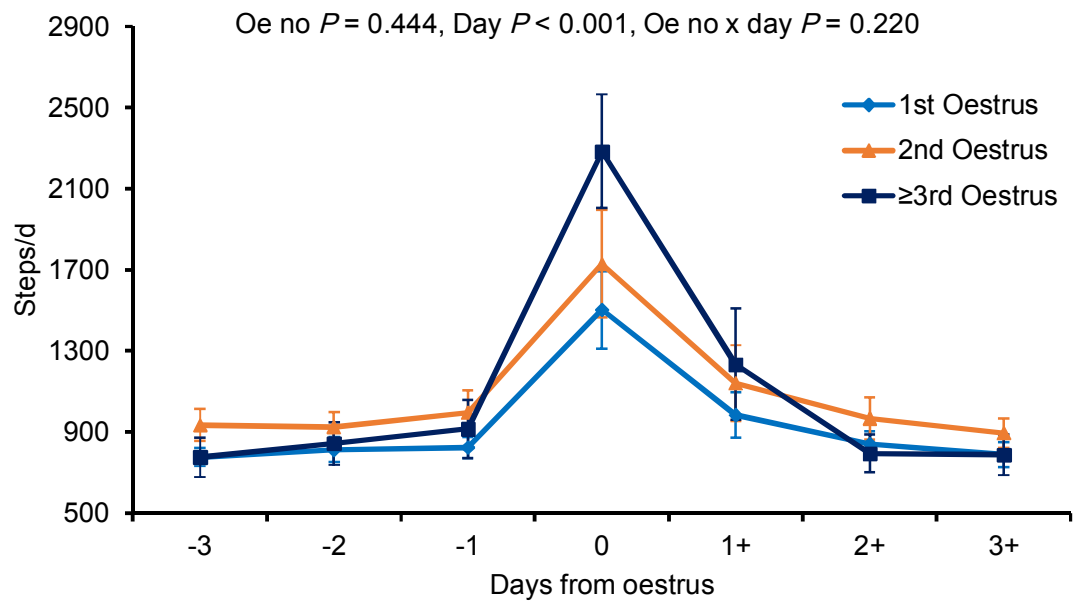


Figure 4. 4. Mean (\pm sem) number of steps, 3 days before, the day of oestrus (0) and 3 days after oestrus and between 1st ($n = 29$), 2nd ($n = 22$) and $\geq 3^{\text{rd}}$ ($n = 10$) oestrus PP in lactating Holstein Friesian dairy cows. Oe no = number of oestrus PP, 0 = day of oestrus.

On the day of oestrus, cows spent less ($P < 0.001$) lying time at first (8.11 ± 0.5 h/d), second (7.77 ± 0.45 h/d) and third or more (7.25 ± 0.58 h/d) oestrus PP than on other days. However, lying time was not influenced ($P = 0.60$) by oestrus PP on the day of oestrus. There was no interaction ($P = 0.440$) between oestrus PP and day on the lying time (Figure 4.5). The number of lying bouts was affected ($P < 0.001$) by day; fewer lying bouts were recorded on the day of oestrus of the 1st (11.6 ± 1.09 bouts), 2nd (9.55 ± 0.6 bouts) and $\geq 3^{\text{rd}}$ (8.6 ± 1.5 bouts) oestrus PP in comparison to 3DB and 3DA oestrus, while on the day of oestrus, there was no difference between ($P = 0.105$) first, second and third or more oestrus PP. Numerically, more lying bouts were found at first compared to second or third or more ($P = 0.479$) oestrus PP. There was also no interaction ($P = 0.567$) between oestrus PP and day (Figure 4.6).

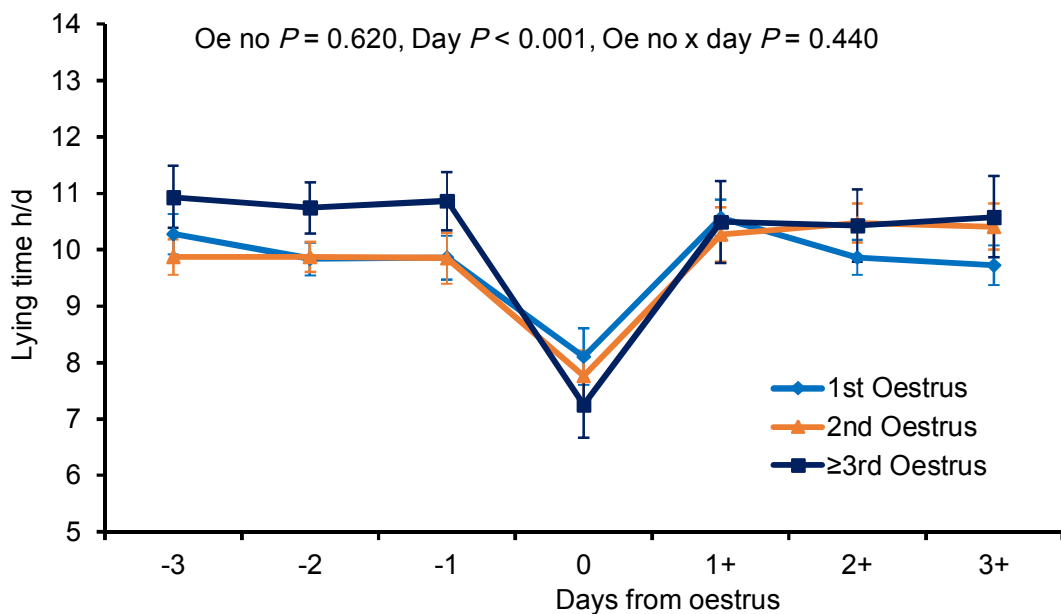


Figure 4. 5. Mean (\pm sem) of lying time, 3 days before, the day of oestrus (0) and 3 days after oestrus and between 1st ($n = 29$), 2nd ($n = 22$) and $\geq 3^{\text{rd}}$ ($n = 10$) oestrus PP in lactating Holstein Friesian dairy cows. Oe no = number of oestrus PP, 0 = day of oestrus.

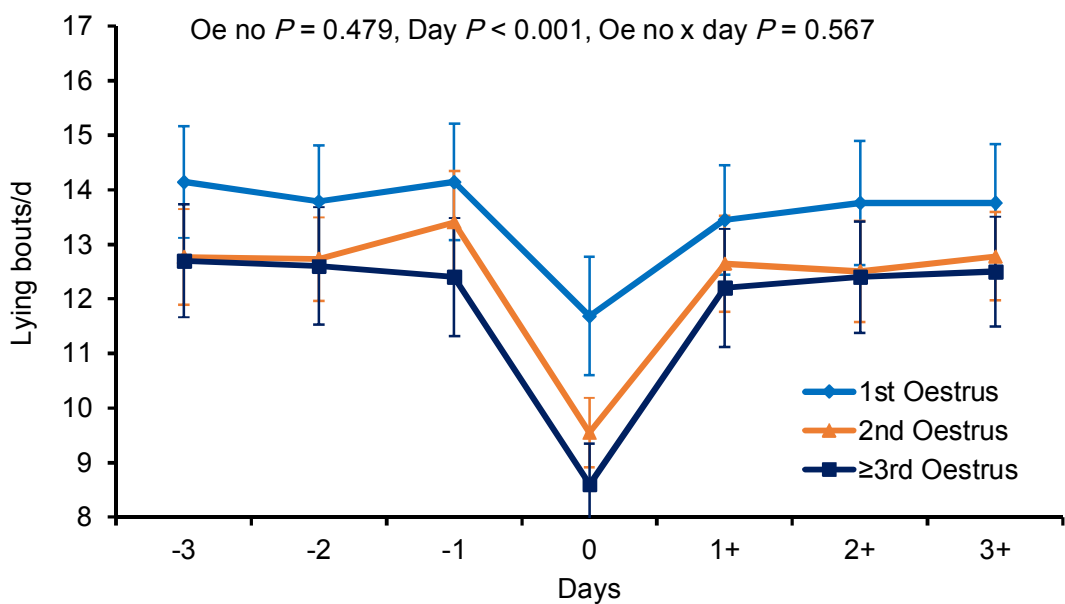


Figure 4. 6. Mean (\pm sem) number of lying bouts, 3 days before, the day of oestrus (0) and 3 days after oestrus and between 1st ($n = 29$), 2nd ($n = 22$) and $\geq 3^{\text{rd}}$ ($n = 10$) oestrus PP in lactating Holstein Friesian dairy cows. Oe no = number of oestrus PP, 0 = day of oestrus.

4.3.3.2. Feeding behaviour during the first, second and third or more oestrus PP

Dry matter intake and time spent feeding by cows were reduced ($P < 0.001$) by the occurrence of oestrus during first, second and third or more oestrus PP and number of visits to feed were also affected ($P \leq 0.049$) by oestrus compared to the 3DB and 3DA oestrus (Table 4.6). Regarding the number of oestrus PP, there were no differences ($P = 0.485$) of day of oestrus on DM intake (19.9 ± 0.49 , 20.0 ± 0.59 and 20.7 ± 0.62 kg/d), feeding duration (2.7 ± 0.16 , 2.7 ± 0.17 and 2.7 ± 0.15 h/d) and number of visits to feed (25.2 ± 1.39 , 25.6 ± 1.77 and 26.6 ± 2.08 visits/d) on the day of 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP, respectively. Furthermore, there was no oestrus PP by time interaction ($P = 0.184$).

4.3.3.3. Expression of behavioural oestrus signs during first, second and third or more oestrus PP

Regarding first, second and third or more oestrus PP, cows were more ($P = 0.002$) restless during 2nd (87.5%) and $\geq 3^{\text{rd}}$ (93.8) oestrus compared to 1st oestrus PP (Table 4.7). During $\geq 3^{\text{rd}}$ oestrus showed a higher ($P = 0.013$) proportion of cows stood to be mounted (75%) compared to second (56.3%) and 1st (25%) oestrus PP. However, there were no differences in the proportion of cows expressing the flehmen response ($P = 0.655$), with mucous discharge from the vulva ($P = 0.367$), mounting but not standing ($P = 0.264$) and mounting head to head ($P = 0.448$) between 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP. Furthermore, all cows equally expressed the following behaviours: sniffing the vulva, resting the chin on the back of another cow and mounting or attempt to mount other cows.

4.3.3.4. The frequency of behavioural oestrus signs during first, second or third or more oestrus PP

There were no differences in the frequency of sniffing vulva ($P = 0.184$), resting the chin on the back of another cow ($P = 0.162$) and mounting or attempting to mount other cows ($P = 0.218$) during 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP (Table 4.8). There were no differences ($P = 0.104$) in the frequency of total social interactions during first, second and third or more oestrus PP. There was a tendency ($P = 0.071$) for a higher frequency cows expression of licking during $\geq 3^{\text{rd}}$ oestrus compared 1st and 2nd oestrus PP. There was no difference ($P = 0.248$) in the frequency of caressing expression between 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP. The frequency of butting another part of other cows was higher ($P = 0.010$) in $\geq 3^{\text{rd}}$ oestrus compared to 1st and 2nd oestrus PP. The frequency of sniffing the udder was also more ($P < 0.001$) in $\geq 3^{\text{rd}}$ oestrus (2.8 ± 0.8) than 1st (0.5 ± 0.3) or 2nd (0.8 ± 0.4) oestrus. Total frequency of expression of agonistic interaction behaviours was higher ($P = 0.016$) in $\geq 3^{\text{rd}}$ (10.8 ± 1.9) oestrus in comparison to 1st (6.5 ± 1.0) and 2nd (6.1 ± 0.7) oestrus PP.

Table 4. 6. Means of DM intake kg/d, feeding duration (h/d) and number of visiting to feed during day 3 days before, the day of oestrus (0) and 3 days after and between 1st ($n = 29$), 2nd ($n = 22$) and $\geq 3^{\text{rd}}$ ($n = 10$) oestrus PP in lactating Holstein Friesian dairy cows.

Feeding behaviour	Oe No.	Days from oestrus							SED	P-value		
		-3	-2	-1	0	1+	2+	3+		Oe no.	Days	Oe no. x Days
DM intake kg/d	1 st Oe	22.0	21.8	22.0	19.9	22.1	21.8	21.6	0.612	0.485	<0.001	0.184
	2 nd Oe	21.8	22.4	21.8	20.0	22.2	22.8	22.9				
	$\geq 3^{\text{rd}}$ Oe	24.1	22.0	23.7	20.7	22.8	22.3	22.9				
Feeding duration h/d	1 st Oe	3.5	3.2	3.3	2.7	3.2	3.1	3.4	0.415	0.847	<0.001	0.390
	2 nd Oe	3.1	3.4	3.5	2.7	3.4	3.6	3.2				
	$\geq 3^{\text{rd}}$ Oe	3.8	3.7	3.3	2.7	3.4	3.3	3.3				
No. of visiting to feed/d	1 st Oe	28.1	26.7	26.2	25.2	27.0	27.6	29.2	4.958	0.756	0.049	0.243
	2 nd Oe	28.8	30.3	29.8	25.6	31.6	30.5	28.5				
	$\geq 3^{\text{rd}}$ Oe	29.9	27.2	27.9	26.6	27.3	27.5	28.8				

Oe = oestrus, SED = standard errors of differences, 1st, 2nd and $\geq 3^{\text{rd}}$ oe = number of oestrus PP

Table 4. 7. Expression of behavioural oestrus signs of first ($n = 16$), second ($n = 15$) and third or more ($n = 8$) oestrus PP in lactating Holstein Friesian dairy cows.

Expression (0/1) of behavioural oestrus signs	oestrus post par-tum			P value
	1 st Oe	2 nd Oe	≥3 rd Oe	
Flehmen	5/16 (31.3%)	4/16 (25%)	3/8 (37.5%)	0.655
Mucous discharge from vulva	2/16 (12.5%)	4/16 (25%)	3/8 (37.5%)	0.367
Cow restlessness	10/16 (62.5%)	14/16 (87.5%)	15/8 (93.8%)	0.002
Mounting but not standing	9/16 (56.3%)	10/16 (62.5%)	7/8 (87.5%)	0.264
Mounting or attempt to mount head side other cows	10/16 (62.5%)	11/16 (68.8%)	7/8 (87.5%)	0.448
Standing to be mounted	4/16 (25%)	9/16 (56.3%)	6/8 (75%)	0.013
Sniffing the vulva of another cow	16/16 (100%)	16/16 (100%)	8/8 (100%)	N/A
Resting the chin on the back of another cow	16/16 (100%)	16/16 (100%)	8/8 (100%)	N/A
Mounting or attempt to mount other cows	16/16 (100%)	16/16 (100%)	8/8 (100%)	N/A

Oe = Oestrus; 1st Oe = first oestrus PP, 2nd Oe = second oestrus PP and ≥3rd Oe = third or more oestrus PP.

Table 4. 8. The frequency of behavioural oestrous signs during first ($n = 16$), second ($n = 15$) and third or more ($n = 8$) oestrus PP in lactating Holstein Friesian dairy cows.

The frequency of behavioural oestrus signs	oestrus post-partum			P value
	1 st Oe	2 nd Oe	3 rd Oe	
Sexual Interaction				
Sniffing the vulva of another cow	19.6 ± 5.2	28.3 ± 7.3	34.6 ± 7.4	0.184
Resting the chin on the back of another cow	12.9 ± 3.8	23.6 ± 6.8	21.9 ± 3.3	0.162
Mounting or attempt to mount other cows	5.8 ± 1.8	8.1 ± 2.0	10.4 ± 2.1	0.218
Social Interaction				
Caressing	2.6 ± 0.4	2.4 ± 0.6	4.4 ± 1.6	0.248
Licking	4.8 ± 0.8 ^b	3.7 ± 0.6 ^b	6.2 ± 1.1 ^a	0.071
Total Social Interaction	9 ± 1.1	6.1 ± 1.1	10.5 ± 2.6	0.104
Agonistic Interactions				
Head to head butt	3.7 ± 0.8	2.8 ± 0.5	4.5 ± 1.0	0.129
Butting another part	2.3 ± 0.4 ^b	2.3 ± 0.3 ^b	3.5 ± 0.8 ^a	0.010
Sniffing the udder	0.5 ± 0.2 ^b	1.0 ± 0.3 ^b	2.8 ± 0.8 ^a	0.001
Total Agonistic Interaction	6.5 ± 1.0 ^b	6.1 ± 0.7 ^b	10.8 ± 1.9 ^a	0.016

Means within rows with different superscript letters are significantly different ($P \leq 0.05$).

Oe = oestrus, 1st Oe = first oestrus PP, 2nd Oe = second oestrus PP and ≥3rd Oe = third or more oestrus PP.

4.3.4. The relationship between explanatory variables and the number of steps taken on the day of oestrus

A summary of the relationships between factors affecting oestrus activity and the number of steps taken during the day of oestrus are presented in Table 4.9. The results show a positive ($P < 0.001$) correlations between SG and DPP and the number of steps on the day of oestrus. In addition there were negative ($P < 0.001$) correlations between parity and LS and the number of steps. There was high multi colinearity between oestrus PP and DPP ($r^2 = 0.89$) and between parity and age ($r^2 = 0.99$), therefore both oestrus PP and age were excluded from multiple linear regression analysis.

Using step-wise multiple regression analysis, factors affecting oestrus activity on the day of oestrus that minimised the residual mean square of the number of steps taken, are shown in the Table 4.10. The number of cows in oestrus at the same time (SG) was chosen as a first independent variable added to the model as it had the lowest residual value in relation to the number of steps. For oestrus activity, the statistically significant ($P < 0.001$) explanatory variables were SG (1, 2 and 3+), LS (1,2 and 3), DPP and parity (2, 3 and ≥ 4). The addition of other explanatory variables did not further reduce ($P > 0.05$) the residual mean squares in the dependent variables.

Table 4. 9. The relationships between factors affecting oestrus expression using IceQubes and the number of steps taken during the day of oestrus in lactating Holstein Friesian dairy cows.

Factors	Regression	Constant	SE	P-value	r^2
Parity $\leq 2,3$ and ≥ 4	3278	-488	1095	<0.001	0.21
SG 1, 2 and ≥ 3	24	1068	963	<0.001	0.39
DPP	499	25.9	1068	<0.001	0.25
LS at oestrus	3878	- 976	1002	<0.001	0.34

SG = number of cows in oestrus simultaneously, DPP = days post-partum, LS = locomotion score and SE = standard error of observation.

Table 4. 10. Relationships between the number of steps on the day of oestrus and the number of cows in oestrus simultaneously (SG; 1, 2 and 3+), locomotion score at oestrus (LS), DPP and parity (2, 3 and ≥ 4) in lactating Holstein Friesian dairy cows.

Response variate	Explanatory variates					r^2	SE	P-value
	Constant	SG	LS	DPP	Parity			
Steps	24 (SE=405)	1068 (SE=170)				0.39	963	<0.001
Steps	1811 (SE=619)	751 (SE=184)	-597 (SE=179)			0.48	890	<0.001
Steps	775 (SE=678)	714 (SE=173)	-412 (SE=180)	14.3 (SE=4.8)		0.54	836	<0.001
Steps	1059 (SE=709)	690 (SE=173)	-310 (SE=195)	13.8 (SE=4.8)	-140 (SE=109)	0.55	831	<0.001

SE = standard error of observation.

4.3.5. The relationship between explanatory variables and oestrous expression (behavioural versus silent oestrus)

Step-wise logistic regression analysis indicated that on the day of oestrus, the number of steps taken were positively ($P < 0.001$) associated with the oestrous expression (Table 4.11). In addition, oestrus expression was also positively associated with SG ($P = 0.012$) and DPP ($P = 0.015$). However, oestrous expression was negatively associated with LS at oestrus ($P = 0.054$) and parity ($P = 0.037$). When SG and DPP ($P = 0.09$) were added to the model that already included steps as a constant, only the LS at oestrus, parity and BCS significantly reduced the deviance further.

The regression equation for this model is:

$$\text{Logit}(p) = 0.0035 (\text{steps}) + 1.880 (\text{LS}) - 0.264 (\text{parity}) - 0.138 (\text{BCS}); P < 0.001$$

Table 4. 11. Regression coefficients (and s.e.) for the explanatory variables (plus constant) assessed for association with oestrous expression (behavioural (1) or silent (0)) in lactating Holstein Friesian dairy cows by stepwise logistic regression analysis.

Variable	Estimate	Odds ratios	s.e.	Constant	P-value
Steps/d	0.0019	1.002	0.00066	-1.992	<0.001
SG	1.048	2.852	0.458	-0.973	0.012
LS	-0.226	0.798	0.368	1.148	0.054
DPP	0.0283	1.029	0.012	-0.639	0.015
Parity	-0.479	0.619	0.236	2.205	0.037
BCS	0.887	2.429	0.659	-1.63	0.171

SG = number of cows in oestrus in the same time, LS = locomotion score, DPP = days post-partum, BCS = body condition at oestrus and s.e. = standard error of observation

4.4. Discussion

4.4.1. The percentage of oestruses detection by different methods

Overall 65.5% of oestrus were associated with behavioural signs detected by the camera, and the percentage of oestrus detected by GEA and IceQube were 41.1 and 53.3%, respectively. This was within the range 51 to 87% found by Roelofs *et al.* (2005) using pedometers for oestrous detection. The results of the present study also agree with the previously reported 52% detection rate in cubicle housed Holstein-Friesian cows studied by Palmer *et al.* (2010) and At-Taras and Spahr (2001) who detected approximately 54% of oestrus by visual observation. Conversely, this finding was lower than the 70% of oestrus events detected recorded by Fricke *et al.* (2014). However, this study used oestrus-synchronised Holstein cows fitted with activity monitors attached to the neck (Heatime, SCR Engineer Ltd, Netanya, Israel), so may related to the number os cows in oestrus in the same time. The results of the present study found that using both the IceQube accelerometers and GEA pedometers can detect 100% of oestruses that were identified by 'gold standard' camera. These results concurred with those found by Mayo *et al.* (2019) who found that both IceQube accelerometer and AfiAct pedometer (AfiMilk, Kibbutz Afikim, Israel) were capable of detecting 100% of observed oestrus in synchronised Holstein cows. This high detection rate was attributed to the high number of cows in oestrus at the same time (Gilmore *et al.*, 2011). However, in the present study cows were all undergoing spontaneous oestrous cycles. Low percentage of oestrous detection in high yielding dairy cows might be due to NEB during the early lactation period that results in a lower production of E2 by the pre-ovulatory follicle and decreases the sensitivity of the hypothalamus to E2 which leads to a high incidence of silent oestrus (Isobe *et al.*, 2004).

4.4.2. Duration of oestrus activity and observed oestrus activity

Previously, the duration of standing oestrus in dairy cows has been considered to be 18 h (Valenza *et al.*, 2012). The average duration of oestrus measured using the video camera in the present study was approximately half this (9.1 h). However, this was 2 h longer than the duration reported by Sveberg *et al.* (2011) of (7.1 ± 1.4 h) detected in Holstein Friesian cows kept on an outdoor wood chip-pad. Based on the number of steps recorded by the IceQubes, the duration of oestrus in the present study was 12.9 (± 2.5 h) very similar to that seen in the study of Roelofs *et al.* (2005) who found that the duration of oestrus detected by pedometer lasted for 12.3 h, while a study by Silper *et al.* (2015), reported longer oestrus (14.3 ± 4.1 h) using neck mounted accelerometers; however, this study used 12-month-old Holstein heifers rather than adult cows. The duration of oestrous activity in the present study was 3.2 h shorter than that reported by Valenza *et al.* (2012)

of 16.1 (\pm 4.7 h) also using an activity monitoring system. The present study found that the duration of oestrus determined by activity monitors was 3 h longer than that detected by observation. The difference between the duration of oestrous activity in high yielding dairy cows may be due to the disconnection of secondary signs of oestrus behaviour detected by activity monitors (restlessness) and standing oestrus (Valenza *et al.*, 2012). Our finding is supported by a reported increase in activity, detected by pedometers in dairy cows, in the 1 to 3 h before the onset of standing oestrus (Sveberg *et al.*, 2011).

In the present study, standing behaviour was observed in 50% of those cows expressing behavioural oestrus. Similarly, Van Eerdenburg *et al.* (2002) detected 50% of standing oestrus events in Holstein Frisian cows. However, Kerbrat and Disenhaus (2004) observed standing events by a video camera in 32% of Holstein cows housed in a loose housing system with a concrete floor.

The results of the present study indicate that there is great variability between cows in the total points scored with 225 to 2921 points during behavioural oestrus and also the number of steps taken during oestrus (754 to 6008 steps). The results of the current study agree with those reported by Van Eerdenburg *et al.* (1996) who continuously monitored cows and another study conducted on Holstein cows by Kerbrat and Disenhaus (2004) who reported the total number of behavioural signs (rather than points score) which ranged from 27 to 239 signs. As expected, the oestrus scores of the present study were higher than the scores (approximately 50 - 1000 points) reported by Van Eerdenburg *et al.* (2002) who observed cows during two time periods of about 30 min in the morning at 5:00 before milking and 30 min in the afternoon at 17:00.

In dairy cows, oestrus often takes place without clear changes in behaviour (Kyle *et al.*, 1992). Indeed this was the case in 44.8% of first PP oestruses observed in the present study. Low expression of oestrus behaviour at the first oestrus PP in lactating dairy cows is thought to be an effect of high concentrations of E2 from foetal origin during late gestation, which induces refractoriness of the hypothalamus to E2 (Kyle *et al.*, 1992). Other studies suggested poor expression of oestrus in the early PP may also be caused by lower frequency of LH pulses as a result of NEB in early lactation (Lucy, 2001) which results in lower E2 synthesis (Butler, 2000; Isobe *et al.*, 2004) by the pre-ovulatory follicle leading to a high incidence of silent oestrus.

4.4.3. Behavioural and silent oestrus

4.4.3.1. Oestrus activity detected by activity monitor

Restlessness is one of the most important secondary indicators of oestrus in cattle (Firk *et al.*, 2002). In the current study, on the day of behavioural oestrus, the number of steps was increased by 146.8%, while during silent oestrus, step count was only 10% higher. Similarly, Sakaguchi *et al.* (2007) recorded a 100% increase in the number of steps on the day of behavioural oestrus using radiotelemetric pedometers on grazing dairy Holstein heifers in Japan. Using pedometers, ultrasound and visual observation, Roelofs *et al.* (2005) recorded a 5.5 fold increase in the number of steps taken on the day of visually observed oestrus. Environmental conditions, the type of housing and management conditions may affect the extent of walking activity (López-Gatius *et al.*, 2005b; Yániz *et al.*, 2006).

Alongside the increase in activity, on the day of behavioural oestrus in the present study, cows spent significantly less time lying down (32.2%) and had 28.3% fewer lying bouts during spontaneous oestrous events. Previously, on the day of behavioural oestrus, continuously observed Friesian cows housed in cubicles were also found to spend less time lying down (approximately 5 h/d) and more time standing than non-oestrus cows (Esslemont and Bryant, 1976).

A recent study at the University of Kentucky conducted by Dolecheck *et al.* (2015) using oestrus-synchronised Holstein cows, found a 50% decrease in lying time and also a reduction in lying bouts (56.0%) during oestrus. The greater reduction in lying behaviour may be because oestrus synchronisation meant there were more cows in oestrus at the same time (Hurnik *et al.*, 1975) resulting in greater restlessness and activity on the day of behavioural oestrus (Roelofs *et al.*, 2005; Jónsson *et al.*, 2011).

4.4.3.2. Feeding behaviour

In the present study, cows consumed approximately 22 kg DMI/d during a normal day, similar to other published studies of early lactation dairy cows: e.g. Dado and Allen (1994) who reported average DMI/d of 22.8 kg during a normal day. However, on the day of behavioural oestrus, the increase in activity observed was associated with a 12% reduction in DMI. Furthermore, both feeding duration and the number of visits to feed per day were lower on the day of behavioural oestrus compare to 3DB and 3DA. These data suggest that increased activity at oestrus diverts cows from their normal time budget with more steps replacing both feeding and resting time (Walker *et al.*, 2008b). This is exacerbated in more active cows which had a greater reduction in DMI demonstrated by the negative correlation between the number of steps taken and DMI during the day of

behavioural oestrus. Other studies of Holstein-Friesian cows have shown reduced DMI on the day of AI (14.6%; Reith *et al.*, 2014, 10.3%; Halli *et al.*, 2015). Cows spent a similar amount of time feeding (2.8 h/d) but had many more visits to the feed troughs (46.2 visits/d) than in the present study (Halli *et al.*, 2015). This may be because Halli *et al.* (2015) only determined feeding behaviours in relation to AI rather than behavioural oestrus. However, in other previous studies conducted by Vasilatos and Wangsness (1980) and Dado and Allen (1995) it was found that lactating dairy cows housed in a tie-stall barn consumed approximately 12 meals. These values are considerably less from those found in the present study. This may be due to that with less social disruption in tie stalls housing or could be due to fewer within meal disruptions, translating into a less frequent number of visits to the feed, as seen in both of these previous studies

As far as I am aware, the present study is the first to report feeding behaviour during silent oestrus in dairy cows. Interestingly, while behavioural measures were not changed during the predicted time of silent oestrus in the current study, DMI and number of visits to the RIC bins were numerically lower in comparison to 3DB and 3DA oestrus. In addition feeding duration was significantly reduced compared to one day before and one day after oestrus. This finding indicates that cows deemed in silent oestrus may show subtle changes to their behavioural repertoire that are not apparent using commercial oestrous detection regimes. Alternatively, E2 has been shown to suppress feed intake (Ingvarsen and Andersen, 2000) and an increase in E2 concentration at silent oestrus may be sufficient to reduce feed intake but not sufficiently adequate to increase oestrous activity. On the day of oestrus, the higher physical activity and restlessness in dairy cows may replace feeding behaviour (DMI, feeding duration and number of visits to feed; Hurnik *et al.*, 1975; Kiddy, 1977). In the present study, there was a negative relationship between the number of steps and DMI. In comparison to non-oestrus cows, during oestrus, cows spent more time walking and consequently less time resting and eating (Hurnik *et al.*, 1975). Conversely, Lukas *et al.* (2008) found that cows consumed more feed on the day of oestrus, while De Silva *et al.* (1981) reported no change in feed intake on the day oestrus.

4.4.4. First, second and third or more oestrus PP

The percentage of behavioural oestrus at first, second and third or more oestrus detected by the camera was 55.2%, 72.7% and 80%, respectively. Ranasinghe *et al.* (2010) reported similar findings of 55.2 vs 44.8%, 76.2 vs 23.8% and 78.7 vs 21.3% of behavioural and silent oestrus at first, second and third oestrus in commercial dairy Holstein Friesian herd in Japan using milk P4 concentration. A study conducted by Isobe *et al.* (2004) based on milk P4 assay found that the incidence of silent oestrus in 32 Holstein-Friesian in Japan were 83, 46 and 13% at first, second and third oestrus using

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milk P4 profiles and visual observation. Another study, depending on radio-telemetry to continuously record mounting activity, also found that occurrence of silent oestrus at first oestrus (42.1%) was higher than the second oestrus of 12.5% (Shipka, 2000). The rates of behavioural oestrus detection by using pedometers in first, second and third oestrus were 57, 91 and 95%, respectively, but by visual observation, the detection rates of oestrus were 19, 37 and 79% (Firk *et al.*, 2002). In dairy cows, the incidence of silent oestrus in the first oestrus is higher than second and third oestrus depending on oestrogen refractions (Reames *et al.*, 2011). This may be due to the high concentration of E2 in blood during the late gestation that leads to refractoriness of the cow before the first ovulation to E2 (Allrich, 1994).

4.4.4.1. Oestrous activity during first, second and third or more oestrus PP

In the current experiment, a significantly higher number of steps were recorded on the day of oestrus in comparison to 3DB and 3DA oestrus of first, second and third or more oestrus PP as well as an increase in the number of steps with the increase in the number of oestrus PP. These results agree with those reported previously by López-Gatius *et al.* (2005b) which showed that the activity of dairy cows in oestrus increases after day 50 PP and also with those observed in Holstein-Friesian cows in north-eastern Spain by Yániz *et al.* (2006) who found that walking activity during oestrus increased with increasing days in milk. A low number of steps at first oestrus may be due to a NEB in high yielding dairy cows during early lactation, which may lead to low E2 production in the pre-ovulatory follicle (Isobe *et al.*, 2004).

Across first, second and third or more oestrus, lying time and lying bouts were significantly reduced on the day of oestrus compared to 3DB and 3DA oestrus. At third or more oestrus PP on the day of oestrus, cows spent less time lying and had a lower number of lying bouts than second and first oestrus, which may be due to an increase in their activity (López-Gatius *et al.*, 2005b) and restlessness on day of oestrus (Roelofs *et al.*, 2005; Jónsson *et al.*, 2011). Supporting this, in the present study, there was also a negative ($P < 0.001$) correlation between the number of steps taken and lying time ($y = 0.0001x + 9.78$; $r^2 = 0.37$) during the day of oestrus.

4.4.4.2. Feeding behaviour during first, second and third or more oestrus PP

The dry matter intake, number of visits to feed and time spent feeding recorded in the present study were significantly reduced by the occurrence of oestrus during first, second and third or more oestrus PP, while there was no effect of oestrous number PP on these variables. Phillips and Schofield (1990) also found that time spent feeding was reduced on the day of oestrus in 20 Friesian cows housed in a straw yard was significantly reduced at the day of oestrus. Reith and Hoy (2012) also reported that feed intake, time spent

feeding and duration of rumination were reduced on the day of oestrus in dairy cows by 10.3, 20.8 and 17.0%, respectively. The reduction in these variables on the day of oestrus may be due to high physical activity during oestrus (Hurnik *et al.*, 1975), as shown in the present study by the negative relationship between the number of steps taken and DMI ($y = -0.0014 + 22.46; P < 0.001; r^2 = 0.46$) during the day of oestrus.

4.4.4.3. Expression of behavioural oestrus signs during first, second and third or more oestrus PP

Regarding 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP, the results of the present study show that cows were more restless during 2nd and $\geq 3^{\text{rd}}$ oestrus compared to 1st oestrus PP. These results agree with those reported previously by Hurnik *et al.* (1975) who observed free-stall housed Holstein cows for 80 days PP and found that the behavioural activity increases with increasing the number of oestrus PP. The results of the current study revealed that a higher proportion of cows during $\geq 3^{\text{rd}}$ oestrus stood to be mounted compared to 2nd and 1st oestrus PP. The results of the present study agree with these of the Palmer *et al.* (2012) in housed lactating cows. However, in dairy cows, the 1st oestrus PP often takes place without clear signs of oestrous behaviour (Kyle *et al.*, 1992). This low expression of behaviour is thought to be a result of high concentrations of E2 from the foetal origin at the late stage of gestation, which induces 'refractoriness' of the hypothalamus to E2 at the first PP oestrus (Boer *et al.*, 2010). Alternatively, the lower express of oestrous signs before the first ovulation PP may depend on circulating E2 concentrations, (Allrich, 1994). Low LH pulse frequency during the early PP is associated with reduced follicular development (Forde *et al.*, 2011). The dominant follicles that develop may not reduced sufficient oestrus to stimulate the typical oestrous signs (Boer *et al.*, 2010).

4.4.4.4. The frequency of behavioural oestrus signs during first, second and third or more oestrus PP

The results of the present study revealed that no differences in the secondary signs of oestrous behaviour, frequency of sniffing vulva, resting the chin on the back of another cow and mounting or attempt to mount were observed in cows during 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP. The results of the present study agree with those of Roelofs *et al.* (2005a) in a study where cows were observed at 3 h intervals for signs of behavioural oestrus in lactating dairy cows. Walker *et al.* (1996) and Van Eerdenburg *et al.* (1996) also found similar results in a study conducted on dairy cows. The present study shows that the frequency of butting another part of other cows and sniffing udder were significantly higher in $\geq 3^{\text{rd}}$ oestrus compared to 1st and 2nd oestrus PP. In a study conducted by Kerbrat and Disenhaus (2004) found that synchronised dairy express social and agnostic interactions

at the same frequency as present study. The current study found that the total frequency of expression of agonistic interactions was significantly higher in $\geq 3^{\text{rd}}$ oestrus in comparison to 1st and 2nd oestrus PP.

4.4.5. The relationship between variables and the number of steps taken on the day of oestrus

The results of the present study show a positive correlation between the number of cows in oestrus at the same time (SG) and the number of steps taken on the day of oestrus. These results agree with those reported previously by Cutullic *et al.* (2009) who found increased intensity of oestrus expression when there were more cows in oestrus at the same time. The increased step count of cow in the present study also agrees with Yániz *et al.* (2006) who found a 6.1% increase in walking activity during oestrus with additional cows in oestrus.

The positive correlation between DPP and the number of steps taken on the day of oestrus agrees with the results of Firk *et al.* (2002), López-Gatius *et al.* (2005) and Yániz *et al.* (2006) who reported that the rates of oestrus detection increased with an increasing DPP. A low number of steps at first oestrus PP may be due to a negative energy balance in high yielding dairy cows during early lactation, which may lead to low E2 production in the pre-ovulatory follicle (Isobe *et al.*, 2004).

The negative correlation between parity and the number of steps is in agreement with the studies conducted by Nebel *et al.* (1997) and Van Eerdenburg *et al.* (2002) who found less oestrus activity in multiparous compared to nulliparous Holstein cows. The negative correlation between parity and the number of steps taken during oestrus in the present study may be due to increased milk yield in the cows up to parity 4 (Rajala and Gröhn, 1998).

Unsurprisingly, the present study found a negative correlation between LS at oestrus and the number of steps taken on the day of oestrus. A negative correlation between LS at oestrus and the number of steps may be related to the decrease in circulating P4 in a lame cow which reduces the responsiveness to E2 which in turn leads to the reduced expression of oestrus activity (Fabre-Nys and Martin, 1991).

4.4.6. The relationship between variables and oestrous expression (behavioural versus silent oestrus)

The positive correlation between the number of steps taken on the day oestrus and oestrus expression is in agreement with the studies conducted by Palmer *et al.* (2012), Roelofs *et al.* (2005a), López-Gatius *et al.* (2005) and Yániz *et al.* (2006) who found that

behavioural oestrus expression increased with increased DPP in dairy cattle. The higher expression of oestrous activity at later DPP may depend on higher circulating E2 concentrations (Firk *et al.*, 2002).

The positive correlation between increasing the number of cows in oestrus at the same time and behavioural oestrous expression is in agreement with those reported by Roelofs *et al.* (2005a) who also found that the oestrous expression increased with more cows in oestrus at the same time from 1 to 2 to 3 cows in oestrus simultaneously. The results of the present study also agree with those reported previously that oestrous activity increases with increasing number of cows in oestrus in the same time (71%; Arney *et al.*, 1994) and (60.1%; Yániz *et al.*, 2006). This may be due to an increase in walking around sexual partners during oestrous periods as a result of sniffing the anogenital region of fellow herd mates, chin-resting, flehmen, attempt to mount and mounting (Van Vliet and Van Eerdenburg, 1996), which are reported in observational studies of cows in oestrus (Van Eerdenburg *et al.*, 2002).

In the current study, there was a negative relationship between oestrous expression and LS at oestrus. Similarly, Walker *et al.* (2010) and Morris *et al.* (2011) found that lame cows at oestrus showed less oestrous activity compared to non-lame cows. A negative correlation between LS at oestrus and the number of steps may be related to the decrease in circulating P4 in a lame cow which reduces the responsiveness to E2 which in turn leads to the reduced expression of oestrous activity (Fabre-Nys and Martin, 1991).

4.5. Conclusion

During the day of behavioural oestrus, high yielding dairy cows in cubicle housing spend more time walking, less time lying down and a reduced number of lying bouts, but none of these parameters were affected during silent oestrus. In addition, on the day of behavioural oestrus, DM intake, feeding duration and number of visits to feed were reduced. On the day of silent oestrus, only feeding duration was reduced. Technologies that facilitate the on-farm measurement of feeding duration could potentially be used to help farmers detect silent oestrus in their cattle. Where behavioural oestrus is expressed, there is considerable variation in the extent of activity, but the reasons for this remain to be elucidated. It remains to be determined why these differences are seen but one factor worthy of investigation may be circulating E2 concentrations. This study also revealed that the percentage of oestrus observed increases with increasing the number of oestrus PP.

CHAPTER 5a

Factors affecting the expression of activity during oestrus in lactating Holstein Friesian COWS

5a.1. Introduction

One of the main factors key affecting the production and economic efficiency of dairy cows is reproductive efficiency (Diskin and Sreenan, 2000). The poor expression of oestrus is considered one of the main factors contributing to poor reproductive efficiency in dairy cows where AI is used (Butler, 2003). The primary and most definitive sign of oestrus is standing to be mounted by a bull or herd-mate (Van Eerdenburg *et al.*, 2002). However, only 50% of dairy cows are reported to show this behaviour (Van Eerdenburg *et al.*, 2002; Zebari *et al.*, 2018; Chapter 4). Traditionally, on dairy farms, cows were visually observed for oestrus detection three times daily for periods of 20 to 30 min (Van Eerdenburg *et al.*, 2002) but this is particularly difficult on large dairy farms because of short observation periods before feeding and milking (Firk *et al.*, 2002).

However, oestrous expression is greatly reduced in Holstein Friesian cows (Sveberg *et al.*, 2011). Automated methods have been developed to facilitate oestrous detection in the increasing size of cattle herds, and now many dairy farmers use activity monitors as an aid to oestrus detection (Firk *et al.*, 2002). The parameters for oestrus detection by automated oestrus detectors (AOD) include mounting events (Senger, 1994), milk P4 (Saint-Dizier and Chastant-Maillard, 2012), rumination (Reith and Hoy, 2012), milk conductivity and body temperature (Fisher *et al.*, 2008), number of steps, number of lying bouts, lying time, feeding duration and DM intake (Zebari *et al.*, 2018; Chapter 4) and potentially blood E2 (Reames *et al.*, 2011).

Many factors contribute to declining oestrous expression in dairy cattle, which results in poor oestrous detection (Orihuela, 2000). These include management factors (Palmer *et al.*, 2010; Dolecheck, 2015), social interaction (Hurnik *et al.*, 1975), environmental factors (Sankar and Archunan, 2012), milk yield (Lopez *et al.*, 2005), age and parity (Roelofs *et al.*, 2005b; Yániz *et al.*, 2006), nutrition and BCS (Cutullic *et al.*, 2009), days in milk and number of oestrus PP (Mayne *et al.*, 2002). However, there are no recent studies on the effects of such factors on the outputs of activities sensors such as the number of steps, lying time and lying bouts, which indicate one of the secondary signs of oestrus in the dairy cow is restlessness (Dolecheck, 2015).

In order to maximise and improve oestrous detection by AODs, it is important to identify factors that affect these measures of oestrus expression in dairy cattle. Therefore, this study was designed to identify and investigate the effects of the number of oestrus PP, the number of cows in oestrus simultaneously, parity, DPP, milk yield, BCS at oestrus and BCS changes from calving to oestrus, LS at oestrus and LS changes from calving to oestrus and season of the year on the activity of dairy cows during oestrus.

The objectives of this study were to:

- 1- Identify factors affecting the number of steps taken, lying time and lying bouts during the oestrous period.
- 2- Investigate the relationship between different factors affecting oestrous activity and the number of steps taken during the day of oestrus.

5a.2. Materials and methods

The experiment was undertaken between March 2015 and February 2016 at the dairy unit of Harper Adams University, Newport, Shropshire, TF10 8NB, UK.

5a.2.1. Ethical considerations

See section 3.2.

5a.2.2. Experimental animals, housing and management

The lactating Holstein Friesian cows ($n = 92$) used in this study were selected at calving from the commercial dairy herd at Harper Adams University. All of the cows used had previously been fitted with IceQube accelerometers on their back left legs in February 2015. The cows in this herd calve all year around. For the study presented in this chapter lactating cows were recruited at $14 (\pm 4.1; \text{mean} \pm \text{SD})$ days PP (see section 3.1). The cows were submitted for detection of any abnormalities of the reproductive tract by the herd veterinarian. at the start of the study. The average parity of the selected cows was 2.7 ± 0.5 (range 1-5). Cows were kept with the main herd and milked twice a day from approximately 04:30 and 15:30 through a 40 point rotary parlour (Westfalia, GEA Milking System, Germany). The milk yield of the cows was recorded throughout the study at each milking. Their average daily milk yield was 34.6 ± 9.3 kg/d (see section 3.1) during The average body condition score (BCS; Scale 1-5; AHDB Dairy, 2014; see section 3.6.1) and locomotion score (LS; Scale 1-5; as described by Chapinal *et al.*, 2009; see section 3.6.1) of the selected cows were 2.8 ± 0.2 and 2.1 ± 0.5 , respectively the study period.

The cows were housed in a free stall cubicle yard (cubicles 2.7x1.2 m) with sawdust-covered, 3 cm rubber mattresses and grooved concrete passageways. Three times per week the cubicles were bedded with sawdust and lime and passageways were scrapped by an automatic scraper 4 to 5 times per day. The total area available to the cows was approximately 10.8 m² per cow. The cows were fed a total mixed ration (TMR) *ad libitum* (see Table 5a.1a; April 2015 to September 2015 and Table 5a. 1b; October 2015 to March 2016) which was provided daily at approximately 08:30. Nutrients supplied by the ration are shown in Table 5a. 2a; April 2015 to September 2015 and Table 5a. 2b; October 2015 to March 2016. Cows were put out to graze during the day (10:00 to 14:15) in the summer months and continuously housed during winter. Water was also provided *ad libitum* from water troughs at the end of each passageway and at pasture during the summer 2015.

Table 5a. 1a. Dietary composition of the total mixed ration fed from April 2015 to October 2015 (summer).

Ingredient	Summer (kg/head)	kg DM/head
Grazed grass	15.00	2.70
Maize silage	21.70	8.50
Lucerne	6.90	2.70
Chopped wheat straw	0.25	0.22
Protein blend	4.90	4.30
Sweet starch	2.90	2.40
Soya hulls	1.10	0.92
Spey syrup	2.80	1.20
Megalac	0.25	0.25
Limestone flour	0.09	0.09
Dairy minerals	0.12	0.12
Vistacell ultra	0.05	0.04
Acid buff	0.05	0.05
Grade urea	0.07	0.07
Amaferm Provimi	0.10	0.09
Butter extra	0.30	0.30
Water	4.80	0.00
Total	61.38	23.95

(Profeed Nutrition Consultancy, UK, 2015)

Table 5a. 1b. Dietary composition of the total mixed ration fed from October 2015 to March 2016.

Ingredient	kg/head	kg DM/head
Maize silage	26.50	9.50
Lucerne	10.00	3.30
Chopped wheat straw	0.50	0.43
Protein blend	5.50	4.90
Sweet starch	2.50	2.20
Soya hulls	1.50	1.30
Spey syrup	2.60	1.10
Megalac	0.35	0.35
Limestone flour	0.10	0.10
Dairy minerals	0.12	0.12
Vistacell ultra	0.05	0.04
Acid buff	0.08	0.08
Grade urea	0.09	0.09
Amaferm Provimi	0.10	0.09
Butter extra	0.35	0.35
Water	4.20	0.00
Total	54.5	23.95

(Profeed Nutrition Consultancy, UK, 2015)

Table 5a. 2a. Predicted nutrient content of the total mixed ration fed from April 2015 to October 2015 (summer). DM = dry matter, ME = metabolisable energy, CP = crude protein, NDF = neutral detergent fibre.

Nutrient Supplied	
DM (g/kg fresh)	409.0
ME (MJ/kgDM)	12.2
CP (%DM)	17.9
NDF (%DM)	35.2
Fat (%DM)	5.0
Starch and sugar (%DM)	23.5

(Profeed Nutrition Consultancy, UK, 2015)

Table 5a. 2b. Predicted nutrient content of the total mixed ration fed from October 2015 to March 2016. DM = dry matter, ME = metabolisable energy, CP = crude protein, NDF = neutral detergent fibre.

Nutrient Supplied	
DM (g/kg fresh)	455.0
ME (MJ/kgDM)	12.2
CP (%DM)	17.5
NDF (%DM)	32.9
Fat (%DM)	5.0
Starch and sugar (%DM)	21.9

(Profeed Nutrition Consultancy, UK, 2015)

5a.2.3. Milk progesterone assay

Oestrous periods were identified by measuring the concentration of P4 in whole milk. Milk samples (40 mL) were collected from each cow 3 times per week on Monday, Wednesday and Friday afternoon (Walker *et al.*, 2008a). Immediately after sampling one preservative tablet (Broad Spectrum Microtabs II, Advanced Instrument, INC. USA; containing 8 mg Bronopol and 0.30 mg Natamycin) was added to each milk sample. Sample pots were inverted to mix until the tablet was dissolved. The samples were stored in a refrigerator at 4°C until the P4 assay, which was completed within one week of collection. Milk samples were brought to room temperature and mixed well before analysis using an enzyme immunoassay (Ridgeway Science Ltd., Rodmore Mill Farm, Alvington, Gloucestershire, UK), see section 3.4.1.1.

5a.2.4. Data collection and handling

5a.2.4.1. IceQube accelerometers

To monitor cow activity IceQubes (IceRobotics Ltd., Edinburgh, UK) were attached to the back left leg of each cow using a Velcro hook and loop strap (see section 3.5.1).

5a.2.4.2. Definitions

5a.2.4.2.1. Definition of oestrus based on milk P4 profile

A cow was considered to be in oestrus when milk P4 concentrations were <3 ng/mL for two or three days (to allow for milk sampling frequency) before a period when P4 rose to >5 ng/mL for at least 5 days See section 3.6.2.

5a.2.4.2.2. Definition of *behavioural* and *silent* oestrus based on IceQube alert

Each oestrus event identified from the P4 profile was classified as *behavioural* or *silent* oestrus (See section 3.6.3).

5a.2.4.2.3. Number of oestrus post-partum

Each oestrous event that was detected by the milk P4 profile and successfully alerted by the Cow Alert system was included in the first, second and third or more oestrus PP (See section 3.6.5).

5a.2.4.2.4. Number of cows in oestrus simultaneously

This variable takes into account the number of cows in oestrus at the same time; one cow in oestrus (SG 1), two cows in oestrus (SG 2) or three or more cows in oestrus (SG ≥ 3). The sexually active group was defined as the cow or cows that interacted during the day of oestrus.

5a.2.4.2.5. Body condition score (BCS)

The body condition scores (Scale 1-5) of each cow were recorded weekly from calving until pregnancy diagnosis according to AHDB Dairy (2014) see section 3.6.1. Oestrus' were grouped according to the cow's body condition score (BCS; Scale 1-5; AHDB Dairy, 2014) as $BCS \leq 2.5$, $BCS = 2.75$ and $BCS \geq 3$ according to BCS at each oestrous events. These were analysed for the effect of BCS at oestrus on oestrous duration, the number of steps, lying time and lying bouts. Oestrus' were also grouped according to BCS changes between calving and oestrous events as follows: no BCS changes (0), and BCS changes from -0.25 to -0.5, -0.5 to -0.75 and -0.75 to -1. These were used to analyse for the effect of changing in BCS on the oestrous expression.

5a.2.4.2.6. Locomotion score at oestrus

Locomotion scores (Scale 1-5) as described by Chapinal *et al.* (2009) of selected cows were recorded weekly from calving until pregnancy diagnosis, see section 3.6.1. Oestrus' were grouped according to the cow's locomotion score at each oestrus event into one of three groups; LS1, LS2 and LS3. These were statistically used to analyse for the effect of LS at oestrus on oestrous expression. None of the study cows was scored LS4 or LS5. Oestrus' were also grouped according to the LS changes of a cow from calving to oestrous events; $LS \geq -1$, no LS changes and $LS \leq +1$ corresponding to cows LS changes of a cow. These were statistically analysed for the effect of changing of LS on oestrus activity.

5a.2.4.2.7. Parity

Oestrous data were analysed according to the parity of the cow. Only seven cows were in 1st parity and statistically analysed with parity 2 cows. There was no differences ($P = 0.495$), between 1st parity and 2nd parity cows with regards to the number of steps taken on the day of oestrus. Therefore both parity 1 and parity 2 were considered as one group ($\leq 2^{\text{nd}}$ parity cows). Corresponding to cows in their $\leq 2^{\text{nd}}$, 3rd and $\geq 4^{\text{th}}$ parity, cows were grouped into $\leq 2^{\text{nd}}$, 3rd and $\geq 4^{\text{th}}$ parity and in accordance to the parity data were analysed for the effect of parity on oestrus duration, the number of steps, lying time and lying bouts.

5a.2.4.2.8. Season of the year

Time of the year was defined as winter (December, January and February), spring (March, April and May), summer (June, July and August) and autumn (September, October and November). Each oestrus event was described as occurring during one of the 4 seasons to take into account environmental variables such as day length and temperature which could affect the expression of oestrous activity.

5a.2.4.2.9. Day length

Day length was defined as the length of day from sunrise to sunset according to timeanddate.com (timeanddate.com, 2015-2016, Telford, England, UK).

5a.2.4.2.10. Days post-partum (DPP)

The number of days from parturition to oestrous events was defined as DPP, the number of days PP were investigated for the relationship between DPP and activity at oestrus.

5a.2.4.2.11. Milk yield (kg/d)

Cows were milked twice a day throughout the study period (see section 3.1). The milk yield of the cows was recorded at each milking. The milk yield on the day of oestrus was statistically analysed for the relationship between milk yield (kg/d) and oestrous activity.

5a.2.5. Statistical analysis

Statistical analyses were performed using the Genstat statistical software package (Genstat 18th edition, 18.1.14713, VSN International Ltd, UK). All of the data sets analysed were normally distributed. The datasets were analysed using repeated measures ANOVA to compare between groups (*behavioural* and *silent oestrus*) three days before and three days after oestrus and the group x day interaction. A Tukey test was used to compare between different groups on the day of behavioural oestrus. One way ANOVA was used to analyse the number of steps, lying time (h/d) and the number of lying bouts on the day of oestrus to compare between groups SG, BCS at oestrus, LS at oestrus, parity and season of the year. Paired t-tests were used to compare between days before, the day of oestrus and days after oestrus.

Simple linear regression and multiple linear regression were used to determine the relationship between the number of steps on the day of oestrus and SG, parity, day length, season of the year, number of oestrus post-partum, milk yield on the day of oestrus, DPP, BCS at oestrus, BCS changes from parturition to oestrus, BCS at calving, LS at oestrus, LS changes from parturition to oestrus, LS at calving and season.

The relationship between oestrous expression (response variable; behavioural = 1, silent = 0) and the explanatory variables including steps, SG, LS at oestrus, LS change, DPP, parity, milk yield kg/d, BCS at oestrus and BCS change were compared by logistic regression analysis. Additionally, the influence of other explanatory variables including SG, LS at oestrus, parity and DIM on the relationship between steps and oestrous expression 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 and steps. The explanatory variables were tested for co-linearity. Differences were reported as significant at $P < 0.05$ and trends were reported when P was < 0.1 and > 0.05 .

5a.3. Results

5a.3.1. Behavioural versus silent oestrus

Of the 248 oestruses events detected by milk P4 profiles, 138 oestruses were defined as *behavioural* oestruses (55.6%), and 110 were defined as *silent* oestruses (44.4%) (Table 5a. 3). On the day of *behavioural* oestrus the number of steps (3867 ± 243 steps; mean \pm SEM) was increased ($P < 0.001$) compared to the 3DB (1631 ± 127 steps) and 3DA (1711 ± 134 steps) oestrus. However, on the day of silent oestrus, the number of steps (2170 ± 153 steps) were not different ($P = 0.116$) in comparison to the average of 3DB (1682 ± 119 steps) and 3DA (1759 ± 108 steps) oestrus. Furthermore, cows took over twice as many steps during the day of *behavioural* oestrus ($P < 0.001$) compared to *silent* oestrus. There was an interaction ($P < 0.001$) between oestrous activity and the day of behavioural oestrus. In cows showing behavioural oestrus, there was no difference ($P = 0.359$) between the number of steps taken on day -3 and -2, while there was a difference ($P = 0.032$) in the number of steps taken on day -2 and -1. The number of steps also increased ($P < 0.001$) from day 1- to the day of behavioural oestrus. In addition, the number of steps taken subsequently fell ($P < 0.001$) from the day of oestrus to day +1. Furthermore, there was a tendency for the cows to take more steps ($P = 0.068$) on the day of silent oestrus compared to day -3.

Conversely lying time and the number of lying bouts (6.8 ± 0.3 h/d and 7.0 ± 0.3 bouts, respectively) were reduced ($P < 0.001$) on the day of *behavioural* oestrus in comparison to the average of 3DB (10.3 ± 0.5 h/d and 10.4 ± 0.3 bouts, respectively) and 3DA (11.0 ± 0.6 h/d and 10.6 ± 0.4 bouts, respectively) oestrus (Table 5a 3). However, lying time (9.5 ± 0.2 h/d) and the number of lying bouts (10.3 ± 0.3 bouts) were not significantly different on the day of *silent* oestrus in comparison to the average of 3DB (10.3 ± 0.4 h/d and 10.6 ± 0.5 bouts, respectively) and 3DA (10.3 ± 0.5 h/d and 10.7 ± 0.3 bouts, respectively) oestrus. In addition, both lying time and the number of lying bouts were decreased ($P < 0.001$) on the day of *behavioural* oestrus compared to *silent* oestrus. With regards to lying time and the number of lying bouts, there was an interaction ($P < 0.001$) between oestrus and day. Lying time and the number of lying bouts increased on day +1 ($P = 0.001$) compared to the day of behavioural oestrus (day 0). Furthermore, in cows showing behavioural oestrus, there was no difference in the time spent lying ($P = 0.112$) and the number of lying bouts ($P = 0.126$) on days -3, -2 and day -1. Both lying time and the number of lying bouts reduced ($P < 0.001$) from day 1- to the day of oestrus.

Table 5a. 3. Means of the number of steps/d, lying time (h/d) and number of lying bouts/d, from 3 days before, the day of oestrus (0) and 3 days after oestrus, during behavioural ($n = 138$) and silent ($n = 110$) oestrus in lactating Holstein Friesian dairy cows.

Activity	Oe	Day from oestrus							SED	P-value		
		-3	-2	-1	0	1+	2+	3+		Oe Ex	Days	Oe Ex x Days
Steps/d	B	1599	1555	1739	3867	1889	1661	1584	627.0	0.116	<0.001	<0.001
	S	1592	1725	1731	2170	1800	1727	1762				
Lying time (h/d)	B	10.4	10.4	10.0	6.8	11.2	11.0	10.8	0.53	0.838	<0.001	<0.001
	S	10.5	10.3	10.1	9.5	10.5	10.2	10.2				
Lying bouts/d	B	10.3	10.8	10.2	7.0	10.6	10.7	10.6	0.44	0.244	<0.001	<0.001
	S	10.7	10.5	10.5	10.3	10.9	10.7	10.4				

Oe Ex = Oestrous Expression, B = Behavioural oestrus and S = Silent oestrus, 0 = day of oestrus, SED = standard errors of differences

5a.3.2. First, second and third or more oestrus post-partum

From the 248 oestrus events, 89 were first, 81 were second, and 78 were the third or more oestrus PP of these, 27% of first, 69% of second and 76% of third or more oestruses PP were detected by IceQube accelerometer.

The effects of oestrus number PP on the number of steps, lying time and the number of lying bouts are reported in Table 5a.4. On the day of oestrus in the first, second and third or more oestrus PP, cows took more ($P < 0.001$) steps compared to 3DB and 3DA oestrus. However, the number of steps was lower ($P < 0.05$) in the 1st PP oestrus (2975 ± 189.7 steps) compared to 2nd and $\geq 3^{\text{rd}}$ oestrus PP (3615 ± 219 and 3772 ± 237 steps, respectively). There was an interaction ($P < 0.001$) between oestrus number PP and days. Two days preceding and three days following the day of oestrus, cows took more ($P = 0.01$) steps during 1st oestrus PP compared to 2nd oestrus PP.

Cows spent less ($P < 0.001$) time lying on the day of oestrus than other days. Furthermore, on the day of the first oestrus PP cows spent more ($P < 0.05$) time lying than at second and third or more oestrus PP. Moreover, with regard to lying time, there was an interaction ($P < 0.001$) between oestrous number PP and day. Two days preceding and three days following the day of oestrus, cows spent less ($P = 0.041$) time lying during 1st oestrus PP compared to 2nd oestrus PP.

Concerning lying bouts on the day of oestrus, more ($P < 0.001$) were recorded during oestrus compare to 3DB and 3DA oestrus. However, there were no differences ($P = 0.788$) between first (8.7 ± 0.3 bouts), second (8.4 ± 0.4 bouts per day) and third or more (7.9 ± 0.4 bouts per day) oestrus PP. There was no interaction ($P = 0.151$) between day and oestrous number PP.

Table 5a. 4. Means of the number of steps/d, lying time (h/d) and number of lying bouts/d from 3 days before, the day of oestrus (0) and 3 days after oestrus and between 1st ($n = 89$), 2nd ($n = 81$) and $\geq 3^{\text{rd}}$ ($n = 78$) oestrus PP in lactating Holstein Friesian dairy cows.

Activity	Oe No	Days from oestrus							SED	P-value		
		-3	-2	-1	0	1+	2+	3+		Oe No.	Days	Oe No. x Days
Steps/d	1 st Oe	1558	1738	1869	2975	1950	1854	1829	160.6	0.114	<0.001	<0.001
	2 nd Oe	1461	1444	1603	3615	1664	1426	1432				
	$\geq 3^{\text{rd}}$ Oe	1769	1692	1717	3772	1919	1752	1704				
Lying time (h/d)	1 st Oe	10.5	10.3	10.0	8.6	10.2	10.0	10.0	0.37	0.047	<0.001	<0.001
	2 nd Oe	10.8	10.8	10.4	8.2	11.6	11.5	11.3				
	$\geq 3^{\text{rd}}$ Oe	10.1	10.0	10.2	7.7	11.0	10.6	10.3				
Lying bouts/d	1 st Oe	10.8	10.9	10.4	8.7	10.6	10.8	10.4	0.53	0.788	<0.001	0.151
	2 nd Oe	10.4	10.5	10.3	8.4	11.0	10.7	10.6				
	$\geq 3^{\text{rd}}$ Oe	10.2	10.2	10.3	7.9	10.9	10.6	10.5				

Oe = oestrus, SED = standard errors of differences, 1st, 2nd and $\geq 3^{\text{rd}}$ Oe = number of oestrus PP, day 0 = day of oestrus.

5a.3.3. Effect of the number of cows in oestrus simultaneously (Sexual group = SG) on activity

Of the 248 oestruses, 65, 82 and 101 were SG 1, SG 2 and SG ≥ 3 oestruses, respectively. Concerning the effect of SG on step count, the number of steps was higher ($P < 0.001$) on the day of oestrus in comparison to 3DB and 3DA oestrus (Table 5a.5). Analysing these data with regard to the effect of a number of cows in oestrus simultaneously on the number of steps at the day of oestrus, more ($P < 0.001$) steps were recorded (4050 ± 205 steps) when SG ≥ 3 oestruses compare to SG1 (2899 ± 242 steps). Moreover, SG 2 cows took more ($P < 0.001$) steps (3406 ± 201 steps) compared to SG 1 cows. There was an ($P < 0.001$) interaction between SG and time. The number of steps increased ($P = 0.04$) from day -2 to -1 and increased further ($P < 0.001$) from the day -1 to the day of oestrus of SG2 and SG ≥ 3 . The number of steps taken subsequently fell ($P < 0.001$) on day +1 after oestrus in comparison to the day of oestrus and decreased ($P = 0.023$) more on days +2 and +3 compared to day +1 after SG ≥ 3 oestrus.

Regarding the effect of the size of the SG on lying time (h/d) and the number of lying bouts per day, cows spent less ($P < 0.001$) time lying and had fewer lying bouts on the day of oestrus in comparison to 3DB and 3DA oestrus (Table 5a.5). During the day of oestrus lying times was reduced ($P < 0.001$) in SG ≥ 3 (7.8 ± 0.3 h/d) and SG 2 (7.9 ± 0.3 h/d) cows compared to SG 1 (8.7 ± 0.4 h/d) cows. There was no ($P = 0.954$) difference in lying time between SG 2 and SG ≥ 3 oestrus'. There was no effect ($P = 0.336$) of SG on the number of lying bouts on the day of oestrus. There was an interaction ($P < 0.001$) between SG and time with regards to lying time (h/d). On day -2 before oestrus cows that were LS3 spent less ($P = 0.022$) time lying compared to day -1. Lying time was reduced ($P < 0.001$) further from day -1 to the day oestrus and subsequently lying time increased ($P < 0.001$) from the day of oestrus to day +2. However, there was no interaction ($P = 0.182$) between SG and time with regards to lying bouts per day.

Table 5a. 5. Means of the number of steps/d, lying time (h/d) and lying bouts/d, 3 days before, from 3 days before, the day of oestrus (0) and 3 days after oestrus and between one ($n = 65$), two ($n = 82$) or three or more ($n = 101$) cows were in oestrus at the same time in lactating Holstein Friesian dairy cows.

Activity	SG	Days from oestrus							SED	P-value		
		-3	-2	-1	0	1+	2+	3+		SG	Days	SG x Days
Steps/d	1	1486	1762	1786	2899	1791	1627	1656	165.3	0.159	<0.001	<0.001
	2	1624	1596	1726	3406	1840	1743	1696				
	≥3	1641	1578	1713	4050	1894	1674	1640				
Lying time h/d	1	10.0	10.0	10.4	8.7	10.8	10.8	10.7	0.52	0.954	<0.001	<0.001
	2	10.6	10.4	9.8	7.9	11.4	11.2	11.0				
	≥3	10.5	10.7	10.0	7.8	11.2	10.9	10.6				
Lying bouts/d	1	10.6	11.1	11.0	8.8	10.5	10.5	11.5	0.75	0.336	<0.001	0.182
	2	10.0	10.3	9.8	8.3	10.4	10.6	9.9				
	≥3	10.4	11.0	10.4	8.2	11.0	11.0	10.7				

SG = number of cows in oestrus simultaneously (sexual group), SED = standard errors of differences, day 0 = day of oestrus.

5a.3.4. Effect of BCS at oestrus on activity

From all the oestrus events detected using the milk P4 profiles, the effect of BCS at oestrus on the number of steps, lying time and the number of lying bouts are reported in Table 5a.6. On the day of oestrus more ($P < 0.001$) steps were recorded in comparison to 3DB and 3DA oestrus. Regarding the effect of BCS at oestrus on the number steps per day, more ($P < 0.001$) steps (3812 ± 210) were taken by cows that had BCS 2.75 in comparison to BCS ≤ 2.5 and BCS ≥ 3 . There was no interaction ($P = 0.151$) between BCS at oestrus and time.

Concerning the effect of BCS on lying time (h/d), cows with a higher BCS spent less time ($P < 0.001$) lying on the day of oestrus compared to 3DB and 3DA oestrus. On the day of oestrus, cows with BCS ≤ 2.5 spent more ($P < 0.001$) time lying (9.9 ± 0.4 h/d) compared with cows that had BCS 2.75 (7.1 ± 0.2 h/d) and BCS ≥ 3 (7.7 ± 0.4 h/d). There was an interaction ($P < 0.001$) between BCS at oestrus and time with regards to lying time (h/d). On the days preceding and following oestrus, cows that were BCS ≤ 2.5 spent more ($P = 0.016$) time lying compared to cows with a higher BCS.

On the day of oestrus, the number of lying bouts were reduced ($P < 0.001$) in comparison to 3DB and 3DA oestrus. However, there was no effect ($P = 0.444$) of BCS on the number of lying bouts on the day of oestrus. There was also no interaction ($P = 0.137$) between BCS at oestrus and time with regards to the number of lying bouts.

5a.3.5. Locomotion score at oestrus

From all 248 oestrus events detected using the milk P4 profiles, the number of steps taken by cows with LS was higher ($P < 0.001$) on the day of oestrus in comparison to 3DB and 3DA oestrus (Figure 5a.1). Cows which were LS1 at oestrus took more ($P < 0.001$) steps (5354 ± 427 steps) on the day oestrus compared to cows which were LS2 (3350 ± 146 steps) and LS 3 (3202 ± 244 steps). However, there were no differences ($P = 0.224$) between cows which were LS 2 or LS 3, with regard to the number of steps taken on the day of oestrus. There was also an interaction ($P < 0.001$) between LS at oestrus and time. Cows that were LS 3 took fewer ($P = 0.047$) steps on day -3, -2 and -1 before oestrus compared to cows that were LS1 and LS2. In addition, on the day +1 after oestrus cows which were LS 3 took fewer ($P = 0.021$) steps compared to cows which were LS1 and LS2.

Table 5a. 6. Mean of the number of steps/d, lying time (h/d) and number of lying bouts/d from 3 days before, the day of oestrus (0) and 3 days after oestrus and between BCS ≤ 2.5 ($n = 44$), BCS = 2.75 ($n = 130$) or BCS ≥ 3 ($n = 74$) at oestrus in lactating Holstein Friesian dairy cows.

Activity	BCS	Days from oestrus							SED	P-value		
		-3	-2	-1	0	1+	2+	3+		BCS	Days	BCS x Days
Steps/d	≤ 2.5	1680	1699	1758	3259	1815	1712	1664	227.3	0.944	<0.001	0.151
	= 2.75	1629	1575	1704	3812	1920	1681	1746				
	≥ 3	1538	1611	1734	3292	1845	1673	1634				
Lying time h/d	≤ 2.5	11.8	12.3	12.0	9.9	12.1	12.0	12.3	0.55	<0.001	<0.001	<0.001
	= 2.75	10.2	10.1	9.6	7.1	11.0	10.8	10.4				
	≥ 3	10.1	9.9	9.7	7.7	11.3	10.9	10.7				
Lying bouts/d	≤ 2.5	11.0	11.5	11.1	8.9	10.7	11.5	11.3	0.83	0.230	<0.001	0.137
	= 2.75	10.0	10.7	9.7	7.9	10.6	10.7	10.5				
	≥ 3	9.9	10.4	10.1	8.6	10.6	10.3	10.3				

BSC = body condition score, SED = standard errors of differences, ≤ 2.5 = BCS, 2.75 = BCS and ≥ 3 = BCS, day 0 = day of oestrus.

Cows that were LS1 at oestrus spent less ($P < 0.023$) time lying (6.0 ± 0.4 h/d) compared to cows which were LS2 (8.1 ± 0.2 h/d) and LS 3 (8.1 ± 0.4 h/d) on the day of oestrus (Figure 5a.2). There was an interaction ($P = 0.045$) between LS and day with regards to lying time (h/d). On day -2 before oestrus cows which were LS 3 spent more ($P = 0.037$) time lying compared to cows which were LS2 and LS1.

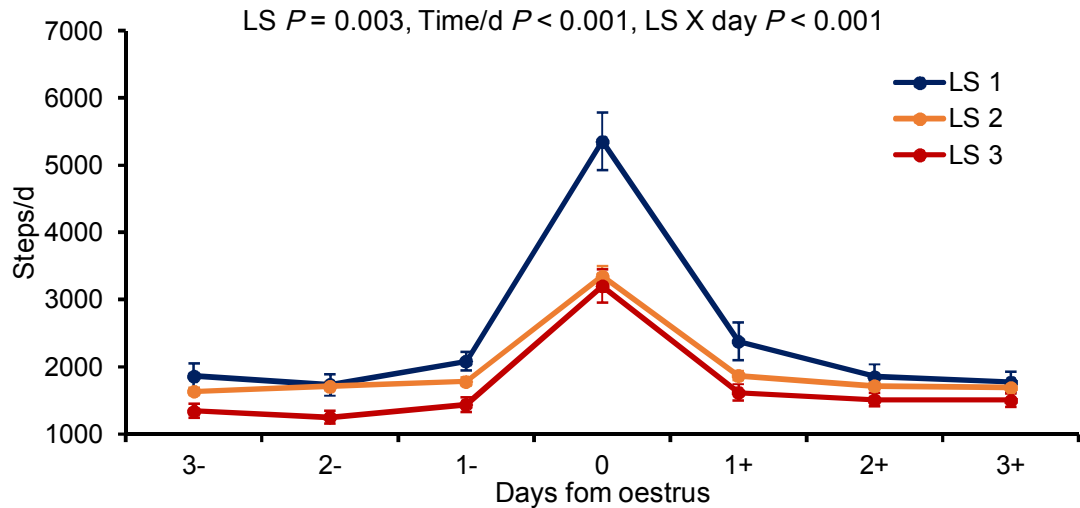


Figure 5a. 1. Mean (\pm sem) number of steps/d from 3 days before, the day of oestrus (0) and 3 days after oestrus and between cows that were LS 1 ($n = 54$), LS 2 ($n = 140$) or LS 3 ($n = 54$) at oestrus (day 0) in lactating Holstein Friesian dairy cows. LS = locomotion score.

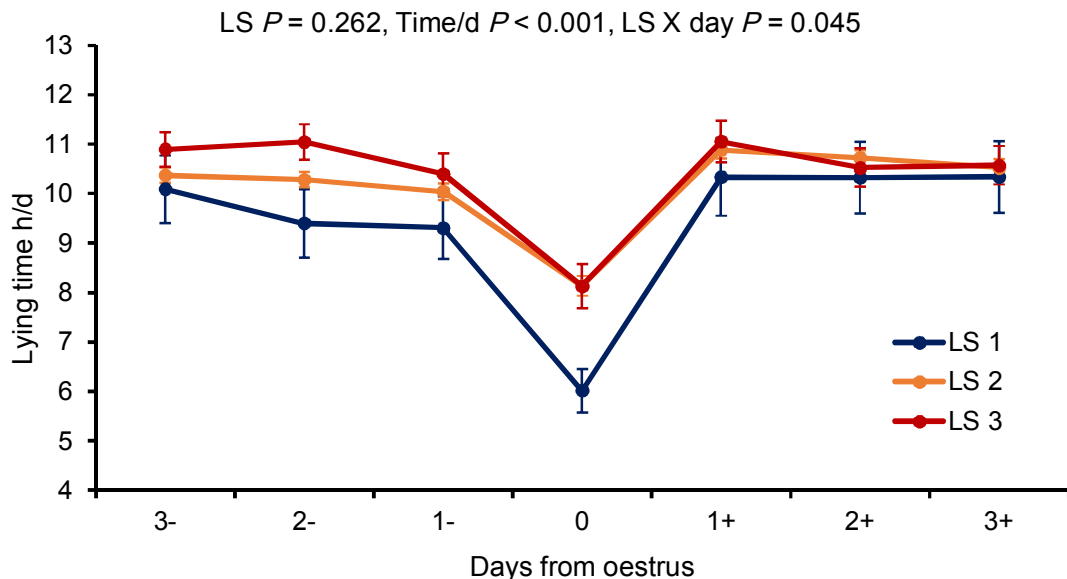


Figure 5a. 2. Mean (\pm sem) of lying time (h/d) from 3 days before, the day of oestrus (0) and 3 days after oestrus and between cows that were LS 1 ($n = 54$), LS 2 ($n = 140$) and LS 3 ($n = 54$) at oestrus (day 0) in lactating Holstein Friesian dairy cows. LS = locomotion score.

There was also an interaction ($P \leq 0.006$) between the LS at oestrus and time with regards to the number of lying bouts. On the day of oestrus, the number of lying bouts were lower ($P \leq 0.028$; 6.6 ± 0.4 bouts) in cows which were LS1 compared with cows that were LS2 (9.0 ± 0.2 bouts) or LS 3 (9.4 ± 0.5 bouts) at oestrus but there were no differences ($P = 0.113$) between LS 2 or LS 3 at oestrus (Figure 5a.3).

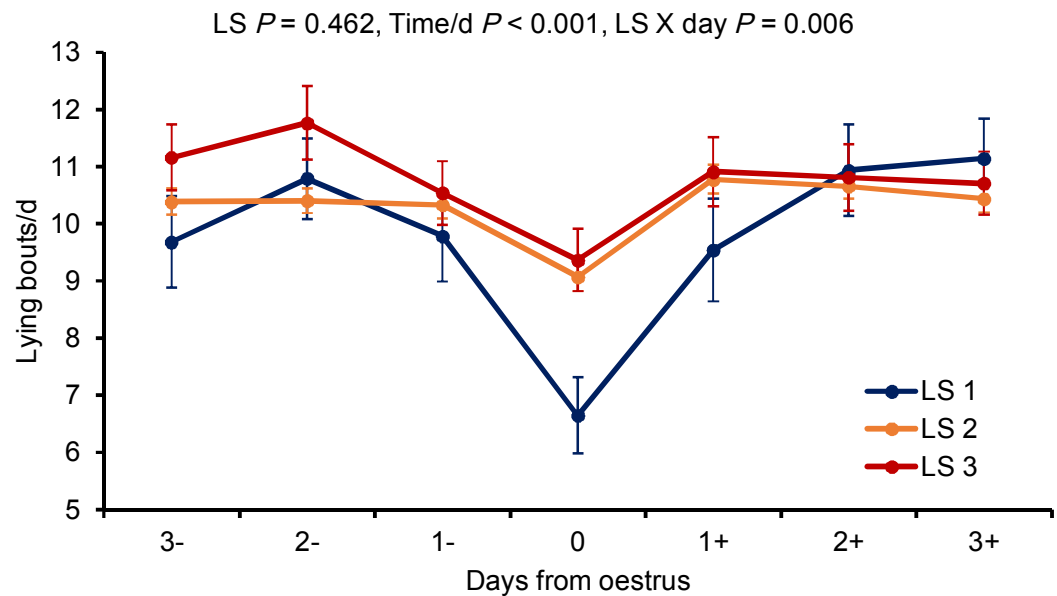


Figure 5a. 3. Mean (\pm sem) number of lying bouts/d from 3 days before, the day of oestrus (0) and 3 days after oestrus and between cows that were LS 1 ($n = 54$), LS 2 ($n = 140$) or LS 3 ($n = 54$) at oestrus (day 0) in lactating Holstein Friesian dairy cows. LS = locomotion score.

5a.3.6. Effect of parity on oestrus activity

Of all 248 oestrus events detected using the milk P4 profiles, the number of oestrus events of parity ≤ 2 , 3 or ≥ 4 cows was 126, 66 and 56, respectively. The effect of parity on the activity of cows measured as the number of steps per day, lying time (h/d) and number of lying bouts per day, are reported in Table 5a.7. On the days preceding and following the day of oestrus parity ≤ 2 cows took more ($P = 0.01$) steps and lay down for longer ($P = 0.043$) than parity 3 cows and parity ≥ 4 cows. Regarding the effect of parity on the number of steps on the day of oestrus, parity ≥ 4 cows took fewer ($P < 0.001$) steps (2967 ± 235.6 steps/d) compared to parity ≤ 2 cows (3845 ± 190.6 steps) and parity 3 cows (3531 ± 210.9 steps). However, there was no difference ($P = 0.104$) between parity ≤ 2 and parity 3 with regards to the number of steps taken on the day of oestrus. There was no interaction ($P = 0.252$) between parity and time with regard to the number of steps per day.

In addition, on the day of oestrus, the lying time of parity ≤ 2 cows (7.4 ± 0.3 h/d) tended to be shorter ($P = 0.073$) compared to parity ≥ 4 (8.6 ± 0.6 h/d), but were not different from parity 3 cows (8.2 ± 0.4 h/d). In addition, on the day of oestrus, no differences ($P = 0.485$) were recorded in the number of lying bouts between parity ≤ 2 (8.8 ± 0.6 bouts), parity 3 (8.2 ± 0.4 bouts) and parity ≥ 4 (8.6 ± 0.6 bouts). There was also no interaction between parity and day with regard to lying time ($P = 0.176$) and lying bouts ($P = 0.526$; see Table 5a.7).

5a.3.7. Effect of season on the oestrus activity

Of the 248 oestruses detected during the study period based on milk progesterone profiles, the number of oestruses recorded during spring, summer, autumn and winter was 40 (16.1%), 74 (29.8%), 81 (32.7%) and 53 (21.4%) oestruses, respectively. From 138 behavioural oestrus, the percentage of behavioural oestrus (out of all oestruses), during spring, summer, autumn and winter were 67.3, 58.1, 51.5 and 49.1, respectively.

The number of steps taken by cows per day during all seasons was increased ($P < 0.001$) on the day of oestrus in comparison to 3DB and 3DA oestrus (Table 5a.8). Cows took more ($P < 0.001$) steps in summer on the day of oestrus (4232 ± 213.6 steps) in comparison to autumn, winter and spring (3787 ± 170.1 , 2793 ± 218.3 and 2993 ± 218.3 steps, respectively). On the day of oestrus, the number of steps taken by cows during autumn was higher ($P < 0.001$) in comparison to winter and spring. There was also an interaction ($P < 0.001$) between the season of the year and days with regards to the number of steps taken by cows. On the days preceding and following the day of oestrus, cows took fewer ($P = 0.001$) steps during winter compared to the other seasons.

The occurrence of oestrus affected ($P < 0.001$) lying duration during spring (8.5 ± 0.5 h/d), summer (7.9 ± 0.3 h/d), autumn (7.9 ± 0.3 h/d) and winter (8.2 ± 0.5 h/d) on the day of oestrus in comparison to 3DB and 3DA oestrus (Table 5a.8). Mean lying time (h/d) was not influenced ($P = 0.661$) by the season of the year, although there was an interaction ($P < 0.001$) between season of the year and day with regards to lying time (h/d). On the days following the day of oestrus, cows spent more ($P = 0.021$) time lying during winter compared to summer.

Table 5a. 7. Mean of the number of steps/d, lying time (h/d) and number of lying bouts/d from 3 days before, the day of oestrus (0) and 3 days after oestrus, between $\leq 2^{\text{nd}}$ ($n = 126$), 3^{rd} ($n = 66$) and $\geq 4^{\text{th}}$ ($n = 56$) parity cows in lactating Holstein Friesian dairy cows.

Activity	Parity	Day from oestrus							SED	P-value		
		-3	-2	-1	0	1+	2+	3+		Parity	Days	Parity x Days
Steps/d	$\leq 2^{\text{nd}}$	1751	1757	1897	3845	2047	1912	1871	165.2	<0.001	<0.001	0.252
	3^{rd}	1432	1558	1592	3531	1724	1455	1472				
	$\geq 4^{\text{th}}$	1441	1431	1542	2967	1553	1450	1421				
Lying time h/d	$\leq 2^{\text{nd}}$	10.7	10.7	10.3	7.4	11.2	11.2	11.1	0.38	0.070	<0.001	0.176
	3^{rd}	10.1	10.0	9.8	8.2	11.0	10.8	10.2				
	$\geq 4^{\text{th}}$	10.0	10.3	9.9	8.6	11.5	10.7	10.8				
Lying bouts/d	$\leq 2^{\text{nd}}$	10.7	11.4	10.5	8.8	10.8	11.2	11.3	0.53	0.292	<0.001	0.226
	3^{rd}	10.0	10.5	10.0	8.2	10.4	10.1	9.7				
	$\geq 4^{\text{th}}$	9.9	9.9	9.8	8.6	10.4	10.1	10.1				

SED = standard errors of differences, $\leq 2^{\text{nd}}$ = 1^{st} and 2^{nd} parity cows, 3^{rd} = 3^{rd} parity cows and $\geq 4^{\text{th}}$ = 4^{th} + parity cows, day 0 = day of oestrus.

The number of lying bouts on the day of oestrus was not affected ($P = 0.138$) by the season of the year (spring - 9.0 ± 0.4 bouts, summer - 8.7 ± 0.3 bouts, autumn - 8.4 ± 0.3 bouts and winter periods - 8.9 ± 0.6 bouts; Table 5a.8). There was no interaction ($P = 0.169$) between the season of the year and days with regards to the number of lying bouts per day.

5a.3.8. The relationships between explanatory variables and the number of steps taken on the day of oestrus

A summary of the relationships between factors affecting oestrus activity and the number of steps taken on the day of oestrus are presented in Table 5a.9. The results show positive ($P < 0.001$) correlations between SG, day length, season, number of oestrus PP and DPP and the number of steps on the day of oestrus. The results show negative ($P < 0.001$) correlations between parity and milk yield (kg/d) and the number of steps. In addition, there was a negative ($P < 0.001$) correlation between LS at oestrus and the number of steps, and BCS change from parturition to oestrus was positively ($P = 0.01$) correlated with the number of steps per day. However, there was no linear relationship between BCS at calving ($P = 0.126$), LS at calving ($P = 0.133$), BCS at oestrus ($P = 0.153$) and LS changes from parturition to oestrus ($P = 0.756$) and the number of steps taken on the day of oestrus in dairy cows.

Step-wise multiple regression analysis indicated that on the day of oestrus, factors affecting oestrus activity that minimised the residual mean square of the number of steps taken on the day of oestrus are shown in Table 5a.10. The number of cows in oestrus at the same time (SG) was chosen as a first explanatory variable to be added to the model as it had the lowest residual value in relation to the number of steps per day in the following equations. For oestrus activity, the statistically significant ($P < 0.05$) explanatory variables were SG (1, 2 and 3+), parity (≤ 2 , 3 and ≥ 4), day length, oestrus PP, milk yield, DPP, BCS changes and LS at oestrus. The addition of other explanatory variables did not reduce ($P > 0.05$) the residual mean squares in the dependent variables, steps on the day of oestrus.

Table 5a. 8. Mean of the number of steps/d, lying time (h/d) and number of lying bouts/d from 3 days before, the day of oestrus (0) and 3 days after oestrus and between different season spring ($n = 40$), summer ($n = 74$), autumn ($n = 81$) and winter ($n = 53$) in lactating Holstein Friesian dairy cows.

Activity	S	Days from oestrus							SED	P-value		
		-3	-2	-1	0	1+	2+	3+		Season	Days	S x Days
Steps/d	sp	1456	1632	1692	2993	1918	1733	1726	211.1	0.010	<0.001	<0.001
	su	1712	1776	1800	4232	2086	1850	1902				
	au	1812	1782	1879	3787	1915	1799	1705				
	wi	1011	1025	1338	2793	1202	1091	1063				
Lying time h/d	sp	10.8	10.4	10.2	8.5	9.9	10.2	10.4	0.51	0.661	<0.001	<0.001
	su	10.4	10.3	10.2	7.9	10.4	10.5	10.7				
	au	10.4	10.4	10.0	7.9	10.6	10.8	10.9				
	wi	10.5	10.4	9.9	8.2	10.8	11.1	11.0				
Lying bouts/d	sp	11.0	10.8	11.0	9.0	11.2	10.9	10.8	0.72	0.138	<0.001	0.169
	su	10.6	10.5	10.3	8.7	10.2	10.3	10.3				
	au	10.3	10.3	9.8	8.4	10.8	10.6	10.5				
	wi	10.5	10.7	10.8	8.9	11.3	11.0	10.9				

SED = standard errors of differences, s = season, sp = spring, su = summer, au = autumn and wi = winter, day 0 = day of oestrus.

Table 5a. 9. The correlations between explanatory variables and the number of steps taken during the day of oestrus (measured using IceQubes) in lactating Holstein Friesian dairy cows.

Factors	Regression	Constant	SE	P-value	r²
SG 1,2 or 3+	1312.4	1443.1	1393	<0.001	0.43
Parity ≤2,3 and ≥4	7990.2	-1355.9	1563	<0.001	0.38
Day-length	359.4	325.0	1508	<0.001	0.32
Season	1948.1	1613.4	1611	<0.001	0.22
Oestrus PP	2149.7	980.4	1682	<0.001	0.17
Milk yield kg/d	8590	-112.3	1582	<0.001	0.16
DPP	2651.8	25.1	1705	<0.001	0.15
LS at oestrus	5856.7	-700.1	1774	<0.001	0.13
BCS change	4799.4	1235.9	1767	0.014	0.04
BCS at calving	7735.6	-1081.9	1783	0.126	0.03
LS at calving	5336.3	-451.5	1800	0.133	0.02
BCS at oestrus	7698	-1209.7	1810	0.153	0.01
LS changes	4348.4	80.5	1814	0.756	0.001

SG = Sexual groups, Oestrus PP = number of oestrus post-partum, DPP = days post-partum, BCS = body condition score, LS = locomotion score and SE = standard error of observation.

Table 5a. 10. Relationships between the number of steps on the day of oestrus and number of cows in oestrus simultaneously (SG; 1,2 and 3+), parity (≤ 2 , 3 and ≥ 4), day length, number of oestrus PP, milk yield (kg/d), DPP, body condition score changes (BCS changes) and locomotion score at oestrus (LS at Oe) in lactating Holstein Friesian dairy cows.

Response variate	Explanatory variates									r^2	SE	P-value
	Constant	SG.	Parity	Day Length	Oe. PP	Milk yield kg/d	DPP	LS at oestrus	BSC change			
Steps	1312 (SE=320)	1443 (SE=143)								0.43	1314	<0.001
Steps	3730 (SE=580)	1177 (SE=143)	-691 (SE=142)							0.51	1217	<0.001
Steps	2107 (SE=736)	970 (SE=151)	-548 (SE=144)	136.3 (SE=40.3)						0.55	1172	<0.001
Steps	1602 (SE=754)	918 (SE=150)	-528 (SE=141)	119.7 (SE=40.2)	341 (SE=144)					0.56	1152	<0.001
Steps	3155 (SE=1135)	861 (SE=152)	-483 (SE=142)	100.1 (SE=41.3)	328 (SE=143)	-34 (SE=18.7)				0.57	1142	<0.001
Steps	2058 (SE=1115)	781 (SE=146)	-554 (SE=136)	129.8 (SE=40)	90 (SE=149)	-23.3 (SE=17.9)	18.6 (SE=4.7)			0.61	1086	<0.001
Steps	2021 (SE=1120)	796 (SE=149)	-521 (SE=149)	127.9 (SE=40.2)	87 (SE=149)	-22.8 (SE=18)	18.3 (SE=4.7)	-196 (SE=354)		0.62	1080	<0.001
Steps	2021 (SE=1120)	796 (SE=149)	-521 (SE=149)	127.9 (SE=40.2)	87 (SE=149)	-22.8 (SE=18)	18.3 (SE=4.7)	196 (SE=598)	167 (SE=354)	0.63	1075	<0.001

SE = Standard error of observation, PP = post-partum and DPP = day post-partum, SG = Sexual group (number of cows in oestrus simultaneously)

5a.3.9. The relationship between explanatory variables and oestrous expression (behavioural versus silent oestrus)

Step-wise logistic regression analysis indicated that on the day of oestrus, the number of steps taken was positively ($P < 0.001$) associated with the oestrous expression. In addition, oestrous expression was also positively associated with SG ($P < 0.001$), DPP ($P < 0.001$) and BCS change ($P = 0.033$). However, oestrous expression was negatively associated with LS at oestrus ($P < 0.001$), parity ($P < 0.001$) and milk yield ($P < 0.001$) (see Table 5a.11). When SG, LS at oestrus, LS change, DPP, parity, milk yield, BCS at oestrus and BCS change ($P = 0.09$) were added to the model that already included steps and the constant, only the SG, LS at oestrus, parity, DPP and milk yield significantly reduced the deviance further.

The regression equation for this model is:

$$\text{Logit (p)} = 0.00016 (\text{steps}) + 2.088 (\text{SG}) + 0.295 (\text{LS}) - 1.82 (\text{parity}) + 0.102 (\text{DPP}) - 0.171 (\text{Milk yield}); P < 0.001$$

Table 5a. 11. Regression coefficients (and s.e.) for the explanatory variables (plus constant) assessed by stepwise logistic regression analysis of oestrous expression in lactating Holstein Friesian dairy cows.

Variable	Estimate	Odds ratios	s.e.	Constant	P-value
Steps/d	0.0011	1.001	0.00014	-2.994	<0.001
SG 1, 2 or 3+	2.309	10.07	0.263	-4.541	<0.001
LS at oestrus	-1.876	0.153	0.240	3.882	<0.001
LS at change	0.095	1.100	0.224	0.265	0.150
DPP	0.0826	1.086	0.011	-3.638	<0.001
Parity ≤ 2 , 3 and ≥ 4	-0.849	0.157	0.218	5.671	<0.001
Milk yield kg/d	-0.219	0.803	0.031	9.370	<0.001
BCS at oestrus	0.294	0.746	0.613	-1.070	0.132
BCS change	0.313	0.408	1.367	0.380	0.033

SG = number of cows in oestrus in the same time, DPP = days postpartum, LS = locomotion score, BCS = body condition and s.e. = standard error of observation

5a.4. Discussion

5a.4.1. Behavioural versus silent oestrus

Of the 248 oestruses, 138 behavioural oestruses (55.6%) and 110 silent oestruses (44.4%) were recorded. The incidence of behavioural oestrus was within the range 51 to 87% found in a study conducted by Roelofs *et al.* (2005a) using pedometers for the detection of oestrus and similar to a study conducted by Palmer *et al.* (2010) who recorded 52% oestrous detection. However, the finding of the present study was lower than reported that by Fricke *et al.* (2014) in oestrous-synchronised Holsteins (70%) using an activity tag (Heatime; SCR Engineers Ltd, Netanya, Israel) attached to the neck. The higher detection rate reported by Fricke *et al.* (2014) may reflect more cows in oestrus at the same time, which increased oestrous activity in the present study. The low percentage of oestrous detection in high yielding dairy cows might be due to their negative energy balance during early lactation which results in lower production of E2 by the pre-ovulatory follicle (Isobe *et al.*, 2004). This also leads to a decrease in the sensitivity of the hypothalamus to E2 as a result of NEBAL which leads to a high incidence of silent oestrus (Isobe *et al.*, 2004). Low BCS and NEBAL are strongly associated with a low level of blood glucose, insulin and IGF-1 post-partum which can reduce the production of E2 by the dominant follicle (Butler, 2003).

Increased activity and restlessness are considered important external indicators of oestrus occurrence (Firk *et al.*, 2002). In the current study, the number of steps was significantly higher on the day of behavioural oestrus (4367 steps), while no significant increase was found during silent oestrus (2170 steps) compared to non-oestrus days. Roelofs *et al.* (2005a) recorded a lower number of steps (2080 steps) on the day of oestrus in Holstein-Friesian cows also housed in free-stalls. Walking activity may be affected by environmental conditions, type of housing and management conditions (López-Gatius *et al.*, 2005b; Yániz *et al.*, 2006). In addition the reported difference in the step count of oestrous cows may be due to differences in the activity recording device (Hockey *et al.*, 2010).

In the present study, cows spent approximately 7 h lying down in 7 lying bouts per day on the day of behavioural oestrus. This finding is in agreement with Esslemont and Bryant (1976) who monitored cows by continuous observation for 25 days and found that cows in oestrus spent less time lying and more time standing than non-oestrus cows. In a more recent study of oestrous-synchronised cows, Dolecheck *et al.* (2015) also found a significant decrease in lying time (10 h/d) and fewer lying bouts (8.4 bouts/d) during oestrus. An increase in the number of steps and a reduction in lying time and lying bouts

on the day of oestrus are due to an increase in restlessness and activity (Jónsson *et al.*, 2011; Roelofs *et al.*, 2005a).

5a.4.2. First, second and third or more oestrus post-partum

From the 248 oestruses detected based on the milk P4 profile, the incidence of silent oestrus at the 1st, 2nd and $\geq 3^{\text{rd}}$ and oestrus PP was 83%, 31% and 24% respectively. Likewise, Isobe *et al.* (2004) previously found a decrease in the incidence of silent oestrus from first, second and third oestrus PP (83% to 46% and 13%, respectively). However, other studies have found a lower incidence of silent oestrus at the 1st oestrus PP (42.1%; Shipka, 2000 and 44.8%; Ranasinghe *et al.*, 2010). Firk *et al.* (2002) reported that the rates of behavioural oestrous detection using a pedometer in the first, second and third oestrus PP were 57, 91 and 95%, respectively, but using visual observation, the detection rates of oestrus were lower (19, 37 and 79%, respectively). The high incidence of silent oestrus before the first ovulation PP may depend on circulating E2 concentrations, due to the high concentration of E2 in the blood during late gestation leading to refractoriness to E2 before the first ovulation (Allrich, 1994).

Similar to the observation reported in Chapter 4, in the current experiment, more steps were recorded on the day of oestrus in comparison to the 3DB and 3DA oestrus as well as an increase in the number of steps with increasing rise in the number of oestrus's PP. These results also agree with those reported previously by López-Gatius *et al.* (2005) and Yániz *et al.* (2006) who showed that the activity of dairy cows in oestrus increases with the number of oestrus's PP. Associated with increased activity from the first to second and third or more oestrus, lying time and lying bouts were significantly reduced on the day of oestrus compared to other days of the study. On the day of the third or more oestrus PP, cows spent less time lying and had fewer lying bouts than at the second and first oestrus PP, which may be due to an increase in activity or restlessness on the day of oestrus (López-Gatius *et al.*, 2005; Roelofs *et al.*, 2005b; Jónsson *et al.*, 2011). Fewer steps at the first oestrus PP may be due to a negative energy balance in high yielding dairy cows during early lactation, which may lead to low LH pulse frequency (Allrich, 1994). The low levels of E2 produced by the pre-ovulatory follicle may be insufficient to stimulate oestrous activity (Isobe *et al.*, 2004).

5a.4.3. Effect of SG on the oestrous activity

In the present study, the number of steps was significantly higher on the day of oestrus when there was more than one cow in oestrus at the same time. These results agree with those reported by Roelofs *et al.* (2005a) who also found that the total number of steps taken during the day of oestrus increased with more cows in oestrus at the same time

from 1 to 2 to 3 cows in oestrus simultaneously (3313 ± 1577 , 3904 ± 2116 and 5415 ± 2747 steps, respectively). The results of the present study also agree with those reported previously that oestrus activity increases with increasing number of cows in oestrus in the same time (71%; Arney *et al.*, 1994) and (60.1%; Yániz *et al.*, 2006). Other studies have also shown that the number of mounts increases from 11 to 36 and 53 when one, two and three cows were in oestrus simultaneously, respectively (Hurnik *et al.*, 1975).

A reduction in lying times and the number of lying bouts were recorded during the day of oestrus in comparison to other days. However, the results of the present study recorded 1-2 h less lying time per day than a study of synchronised lactating Holstein Friesian cows used by Dolecheck *et al.* (2015) which recorded 9.9 ± 0.4 h/d of lying time on the day of oestrus. This is unexpected because in the present study fewer lying bouts were found in SG 3+ and SG 2 in comparison to SG 1 on the day of oestrus. The increase in the activity and reduction in lying time and the number of lying bouts when more cows were in oestrus at the same time in the present study may be due to increased sexual stimulation by other animals (Roelofs *et al.*, 2005a). This leads to an increase in walking around sexual partners during oestrous periods as a result of sniffing the anogenital region of fellow herd mates, chin-resting, flehmen, and attempts to mount and mounting (Van Vliet and Van Eerdenburg, 1996), which are reported in observational studies of cows in oestrus (Van Eerdenburg *et al.*, 2002).

5a.4.4. Effect of BCS at oestrus on activity

Regarding the effect of BCS at oestrus on the number of steps per day, the present study found significantly more steps were taken by cows that had BCS 2.75 in comparison to $BCS \leq 2.5$ and $BCS \geq 3$. Studies conducted on lactating Holstein cows by Madureira *et al.* (2015) and Aungier *et al.* (2012) found that the oestrous activity of thinner cows was approximately 85% lower than those with a higher BCS. These results agree with those reported previously by López-Gatius *et al.* (2005) which showed increased activity of dairy cows during the day of oestrus. Furthermore, Buckley *et al.* (2003) and Chagas *et al.* (2007) agreed that BCS in early lactation is negatively correlated with days to first oestrus PP, and positively associated with the possibility of oestrus detection because of an increase in behavioural activity. Other studies have shown that one of the major factors related to physical activity is BCS at oestrus (Madureira *et al.*, 2015).

In the present study, concerning the effect of BCS at oestrus on lying time (h/d) and the number of lying bouts, thinner cows spent more time lying down throughout the study period. Cows spent less lying time and had fewer lying bouts on the day of oestrus in comparison to the other days. The result of the present study agrees with Dolecheck *et al.* (2015) who reported a similar reduction in lying time (4.08 h/d) and the number of lying

bouts also decreased (8.4 bouts/d) during the day of oestrus compared to non-oestrus cows (lying time of 9.9 h/d and lying bouts of 17.28 bout/d). A study conducted by Livshin *et al.* (2005) reported a significant drop in lying time on the day of oestrus (3.5 h/d) compared to non-oestrus cows (8.8 h/d). Løvendahl and Chagunda (2010) reported that low early postpartum BCS had a negative correlation with oestrus behaviour activity in dairy cows. However, a study conducted on lactating cows housed in a free-stall barn by Bewley *et al.* (2010) found that cow BCS (thin: BCS <2.75, moderate: 2.75 ≥BCS <3.25 and heavy: BCS ≥3.25) had no significant impact on an average daily lying time. The mechanism by which negative energy balance in high yielding dairy cows reduces oestrogen-dependent oestrous activity behaviour is unclear (Madureira *et al.*, 2015).

5a.4.5. Locomotion score (LS) at oestrus

The results of the present study revealed that on the day of oestrus, cows which were LS1 took more steps compared to cows which were LS2 and LS3. Similarly, Roelofs *et al.* (2010) also reported that lame cows spent less time walking during the day of oestrus. A study conducted by Walker *et al.* (2008) found that cows that were LS ≥2 spent less time walking than non-lame during the day of behavioural oestrus. Walker *et al.* (2008) also found that the expression of oestrous behaviour in lame cows (LS ≥2) was reduced by approximately 36%. In contrast to the present study, Hassall *et al.* (1993) reported that non-lame and lame cows did not differ in the amount of time spent walking at pasture. The difference between this study and the current study is that in the present study, cows were monitored on the day of oestrus and compared to 3DB and 3DA oestrus. This indicated that lame cows and non-lame cows walk the same amount of the time, while on the day of oestrus, lame cows walked less than non-lame cows. Lameness is a chronically painful and stressful condition often associated with poor reproductive performance and causes a reduction in behavioural oestrus expression in high yielding dairy cows (Collick *et al.*, 1989). The findings of the present study are further supported by a study conducted by Walker *et al.* (2010) who found that non-lame cows recorded more total oestrous behaviour points than lame cows (2260 ± 307 points vs 1417 ± 206 points).

The results of the current study found that cows which were LS 1 at oestrus spent over 2 hours less lying on the day of oestrus compared to both cows that were LS 2 and LS 3. Walker *et al.* (2008) also showed that Holstein Friesian cows that were LS ≥2 spent more time lying than non-lame cows on the day of behavioural oestrus compared to cows that were LS 1. Ito *et al.* (2010) reported that cows that were severely lame (LS = 4) spent approximately 12.8 h/d compared to non-lame (LS ≤2) cows at 11.2 h/d during non-oestrus days. Blackie *et al.* (2011) found that lame cows spent more time lying (13 h/d)

compared to non-lame dairy cows (10.9 h/d). The lying times for cows during these studies were longer than in the present study.

In the present study, on the day of oestrus, the number of lying bouts were reduced by 25 - 30% in LS 2 and LS 3 cows. However, the number of lying bouts per day either side of oestrus was not different in lame and non-lame cows, in agreement with Ito *et al.* (2010) who showed that the frequency of lying bouts in cows that were severely lame (LS = 4; 8.0 bouts/d) was not different from non-lame cows (8.1 bouts/d) during non-oestrus days. Taken together these findings indicate longer lying bouts in cows that are more lame.

The effects of LS on oestrus activity in the present study may be due to the reduction in P4 concentration before oestrus without affecting E2 concentration (Walker *et al.*, 2008). Higher P4 concentrations increase the number of E2 receptors in the mediobasal hypothalamus during the luteal phase, and this leads to an increase in the sensitivity to E2 and increased intensity of behavioural oestrus (Blache *et al.*, 1994).

5a.4.6. Effect of parity on oestrus activity

In the present study, parity ≤ 2 cows were more active than parity ≥ 4 during the day of oestrus while not significantly different to parity 3 cows.

Similarly, a study conducted by Yániz *et al.* (2006) found no significant differences in activity between cows of parity 2 and parity 3. Parity ≤ 2 cows were more active either side of oestrus than parity 3 or ≥ 4 cows. However, Yániz *et al.* (2006) recorded higher activity at parity 4. Another study conducted on lactating dairy cows by López-Gatius *et al.* (2005) reported that with an increase in parity, walking activity was decreased by about 21.4% on the day of oestrus. Conversely, there are several published studies shown that oestrus activity increases with increased parity (Arney *et al.*, 1994; Dolecheck *et al.*, 2015). However, Roelofs *et al.* (2005b) found a maximum score of primary and secondary signs of behavioural oestrus in primiparous cows compared to multiparous cows.

Regarding the effect of parity number on lying time and lying bouts, the present study showed no differences in lying time on the day of oestrus between parity ≤ 2 and 3, while cows lay down significantly longer each day at parity ≥ 4 compared to parity ≤ 2 . The number of lying bouts was approximately the same across different parities. In synchronised dairy cows on the day of oestrus, Dolecheck *et al.* (2015) found that parity had a significant effect on lying time and the number of lying bouts. It has been reported that milk yield per lactation was higher in later parities, with maximum milk yield at the fourth parity (Garnsworthy *et al.*, 2008). Cow activity at oestrus may be negatively influenced by milk yield (Lopez *et al.*, 2004) and changes in energy balance and altering metabolic profile with each successive lactation (Coffey *et al.*, 2004; Wathes *et al.*, 2007).

In addition, cows may suffer more negative energy balance (Macmillan *et al.*, 1996) because of higher milk yield in later lactations (Garnsworthy *et al.*, 2008), which may reduce oestrous activity.

5a.4.7. Effect of season on the oestrous activity

Concerning the effect of season on activity in the present study, as far as a number of steps are concerned on the day of oestrus, a higher number of steps were recorded during summer in comparison to autumn, winter and spring. These results agree with those reported by López-Gatius *et al.* (2005) who found that walking activity on the day of oestrus in summer (384%) and winter (369%) was significantly higher compared to other seasons in dairy cattle. However, the results of the present study are inconsistent with those reported by Sankar and Archunan (2012) which found that oestrous behaviour, recorded as the number of mounts during oestrus, was higher in winter (61.5 ± 0.4 mounts) compared to summer (49.5 ± 0.3 mount) and spring (39.7 ± 0.4 mount). A higher number of steps during the summer compared with the autumn, winter and spring in the present study may be associated with longer day length in summer. However, it is believed that the expression of behavioural oestrus in cattle can either be negatively influenced or completely inhibited by heat stress (Landaeta-Hernández *et al.*, 2002). The effect of different seasons of the year on oestrous activity could also be due to the effects of environmental on the cows and herd mates willingness to express signs of oestrus on pasture rather than concrete floors, rather than the influence of temperature on the physiological status of the cows in oestrus (Sankar and Archunan, 2012). Globally, some studies have reported that the oestrous activity of dairy cattle during oestrus was higher in the hotter, summer season (Peralta *et al.*, 2005; Uzal and Ugurlu, 2010). While others reported the opposite in the colder, winter season, oestrous activity was higher in comparison to the other seasons of the year on the day of oestrus (Nebel *et al.*, 1997; Sankar and Archunan, 2012). However, reports in this area, which is related to the effect of cold and hot temperature on oestrous expression in dairy cattle are mostly from studies outside of the UK. The studies that found positive and negative relationship between oestrus activity and time of the year or season were conducted in countries that have a large fluctuation in temperature between different seasons.

The results of the current study revealed that the incidence of oestrus was affected by the mean duration of lying. On the day of oestrus, lying time was reduced significantly compared to the 3DB and 3DA oestrus. However, the result of the present study found that on the day of oestrus in different seasons did not influence the mean duration of lying. However, this contradicts with the findings of Reich *et al.* (2010) who found that cows spent more time lying during the winter (12.1 ± 0.4 h/d) compared to the summer ($9.9 \pm$

0.6 h/d). These differences may have been due to heat stress as the temperature-humidity index >68 because dairy cows are known to spend less lying down when experiencing heat stress (Shultz, 1984; Cook *et al.*, 2007).

While Uzal and Ugurlu (2010) found that lying bouts were increased by approximately 47.1% in summer in comparison to autumn in dairy cattle, the number of lying bouts per day was similar across the four seasons in the present study.

5a.4.8. Factors that affect the number of steps taken on the day of oestrus

The factors associated with activity during oestrus have been studied less than those affecting fertility in lactating dairy cows (Cutullic *et al.*, 2009). The results of the present study show a positive correlation between increasing the number of cows in oestrus at the same time (SG) and the number of steps on the day of oestrus. These results agree with those reported previously by Roelofs *et al.* (2005a) and Cutullic *et al.* (2009) that found that oestrous expression in dairy cows increases with increasing the number of cows in oestrus simultaneously. The results of the present study also agree with those reported by Yániz *et al.* (2006) which shows that walking activity during oestrus increases by about 6.1% with additional a cow in oestrus.

In the present study, a positive correlation between day length, season and the number of steps was recorded. These results agree with those reported by López-Gatius *et al.* (2005) who found that walking activity on the day of oestrus was significantly higher in summer compared to winter in dairy cattle. The higher number of steps taken during the autumn and summer compared with spring and winter may be associated with longer day length. Alternatively the effect of the different seasons on oestrous activity could be due to the environmental and herd mates willingness to express oestrous activity at pasture, rather than the influence of temperature on the physiological status of the cows in oestrus (Sankar and Archunan, 2012).

The positive correlation between the number of oestruses PP and the number of steps taken on the day of oestrus agrees with the findings of Firk *et al.* (2002). They found that the rates of oestrous detection increased with an increasing number of oestruses PP, presumably because of more intense oestrous expression. The incidence of behavioural oestrus increased from first to second and third oestrus PP (57, 91 and 95%, respectively). Our results also agree with Ranasinghe *et al.* (2010) found increasing incidence of behavioural oestrus (55.2%, 76.2% and 78.7%) at first, second and third oestrus PP, respectively.

The results of the present study found a positive correlation between oestrus activity and DPP. These results agree with those reported previously by López-Gatius *et al.* (2005)

and Yániz *et al.* (2006) who found that walking activity on the day of oestrus increased with increased days PP in dairy cattle. The low number of steps reported at first oestrus PP may be due to a negative energy balance in high yielding dairy cows during early lactation, which is associated with frequent LH pulse (Butler, 2003) and low E2 production by the pre-ovulatory follicle (Isobe *et al.*, 2004).

In the present study, a negative correlation between parity and the number of steps was recorded. These results agree with studies conducted by Nebel *et al.* (1997) and Van Eerdenburg *et al.* (2002) who found less oestrous activity in multiparous compared to nulliparous Holstein cows. However, the results of the present study disagree with those reported by Lopez *et al.* (2004) who found that oestrous activity was not affected by parity in dairy cows. The negative correlation between parity and the number of steps taken during oestrus in the present study may be due to increased milk yield in the cows up to parity 4 (Rajala and Gröhn, 1998). In the current study, a negative correlation between milk yield (kg/d) and the number of steps was found. Similarly, Villa-Godoy *et al.* (1990) showed a highly negative correlation between milk yield and observed behavioural signs of oestrus in dairy cows as did Lopez *et al.* (2004). In the latter study found a negative correlation between milk yield and oestrous activity in dairy cows ($r = -0.54$) and multiparous cows ($r = -0.48$).

The negative correlation between LS at oestrus and the number of steps taken on the day of oestrus agrees with Walker *et al.* (2010) and Morris *et al.* (2011) who found that lame cows at oestrus were less active compared to non-lame cows. A negative correlation between LS at oestrus and the number of steps may be related to the decrease in circulating P4 in a lame cow which reduces the responsiveness to E2 which in turn leads to the reduced expression of oestrus activity (Fabre-Nys and Martin, 1991). However, the positive correlation found between BCS changes from calving to the onset of oestrus and the number of steps in this study agree with those reported by Villa-Godoy *et al.* (1990) who found that BCS changes were highly correlated with behavioural signs of oestrus in dairy cows. The physical pain associated with lameness may also reduce walking activity during oestrus (Walker *et al.*, 2008b). However, there was no correlation between BCS at calving, LS at calving and LS changes and the number of steps taken on the day of oestrus in dairy cows.

5a.4.9. The relationship between explanatory variables and oestrous expression (behavioural versus silent oestrus)

The results of the present study found a positive relationship between the number of steps taken on the day oestrus and oestrous expression. These results agree with previous studies conducted by Firk *et al.* (2002) and Ranasinghe *et al.* (2010) who reported that the

incidence of behavioural oestrus increased with increase oestrous activity. These results also concurred with those found in Chapter 4 (section 4.3.5). The current study found also a positive relationship between oestrous expression and number of cows in oestrus in the same time (SG). These results agree with those reported previously by Yániz *et al.* (2006) which found that walking expression increases by about 6.1% with increased number of cows simultaneously in oestrus. The results of the present study also agree with those found in Chapter 4 (section 4.3.5).

The results of the current study found a positive relationship between oestrous expression and DPP. These results agree with those reported Chapter 4 (section 4.3.5). A low oestrous expression at early DPP may be due to a negative energy balance in high yielding dairy cows during early lactation, which may lead to low E2 production in the pre-ovulatory follicle (Isobe *et al.*, 2004).

In the present study, a negative relationship between LS at oestrus and oestrous expression was recorded. These results agree with those reported in Chapter 4 (section 4.3.5). A negative relationship between oestrous expression and LS at oestrus may be related to the decrease in circulating P4 in a lame cow which reduces the responsiveness to E2 which in turn leads to the reduced oestrous expression in dairy cattle (Fabre-Nys and Martin, 1991).

The results of the present study found that oestrous expression was negatively associated parity. These results agree with studies conducted by Nebel *et al.* (1997) and Van Eerdenburg *et al.* (2002) who found less oestrous expression in multiparous compared to nulliparous dairy cows. The negative relationship between parity and oestrous expression may be due to increased milk yield in the cows up to parity ≥ 4 (Rajala and Gröhn, 1998). In the present study, a negative relationship between milk yield (kg/d) and oestrous expression was found. These results agree with those reported by Lopez *et al.* (2004) who showed a negative relationship between oestrous expression and milk yield kg/d in dairy cows.

5a.5. Conclusion

Cows spend more time walking, less time lying down and had reduced lying times during the day of *behavioural* oestrus in comparison to other days other days of the oestrous cycle, but none of these parameters is affected during *silent* oestrus. This study also revealed that the number of steps taken increases, but lying time and the number of lying bouts decreases, with increasing days PP. On the day of oestrus, more steps were taken and less time spent resting, and fewer lying bouts were recorded when more cows were in oestrus at the same time. On the day of oestrus, cows with BCS ≤ 2.75 spent more time walking compared with cows that had BCS 2.75 and BCS ≥ 3 . On the day of oestrus more steps but lower lying time and fewer lying bouts were recorded in parity ≤ 2 cows. On the day of oestrus, more steps were taken and less time spent lying down with a fewer number of lying bouts in cows that were LS1. This study also found cows spent more time walking during summer compared to other seasons.

CHAPTER 5b

Factors affecting the behaviour of lactating Holstein Friesian cows on the day of oestrus

5b.1. Introduction

To maintain high reproductive performance in dairy herds using AI, accurately detecting the time of oestrus is critically important (Butler, 2003). Based on the increase of activity, standing and mounting signs of oestrus in dairy cows, the time of ovulation and breeding of lactating dairy cows can be expected within a relatively narrow time window (Sveberg *et al.*, 2011). The interval between the first and the last mounting or attempt to mount was defined as the duration of oestrus (Hurnik *et al.*, 1975). In Holstein Friesian cows the duration of oestrous expression is generally short, Sveberg *et al.* (2011) found the duration of standing oestrus was the only 7.1 h. Different oestrous indicators are needed to determine duration of oestrus and length of oestrus depending on the method of oestrus detection and choice of oestrous signs in dairy cows (Sveberg *et al.*, 2011). While López-Gatius *et al.* (2008) defined the duration of oestrus as intervals between an increase in walking activity to >80% above the mean number for the preceding three days followed by a decrease to <80% of the following two days. Key factors that affect the intensity and duration of oestrous activity on the day of oestrus in lactating Holstein Friesian cows have not been properly reported in the previous literature.

In lactating dairy cows the intensity of oestrous expression and oestrous duration can be affected by the number of oestrus post-partum, number of cows in oestrus in the same time (Hurnik *et al.*, 1975), milk yield (Lopes-Gatius *et al.*, 2005), lameness (Walker, 2008), the type of housing (Hackett *et al.*, 1984), BCS (Mayne *et al.*, 2002; Cutullic *et al.*, 2009), age, parity (Roelofs *et al.*, 2005a; Yaniz *et al.*, 2006) and season (Sanker and Archunan, 2012). The results presented in Chapter 5a also demonstrate the effects of the number of oestrus PP, BCS, LS, parity and season as well as SG affecting on oestrous activity on the day of oestrus in lactating Holstein Friesian cows.

However, limited information is available on the effect of such factors on the behaviour and oestrous expression of lactation Holstein Friesian cows on the day of oestrus. In order to improve the oestrous detection rate accurately, it is important to identify factors that affecting oestrous intensity and duration on the day of oestrus in dairy cattle. Therefore, this study was designed to identify and investigate the effects of number of oestrus PP, number of cows in oestrus simultaneously (SG), parity, BCS at oestrus, LS at oestrus, season of the year and day time on the behaviour on the day of oestrus in Holstein Friesian cows.

The objectives of this study were to:

- 1- Investigate factors that affect the oestrous intensity on the day of behavioural oestrus in Holstein Friesian cows.
- 2- Determine factors that affect the duration of behavioural oestrus in Holstein Friesian cows.

5b.2. Materials and methods

The experiment was undertaken as part of the study described in Chapter 5a (section 5a.2).

5b.2.1. Ethical considerations

See section 3.2.

5b.2.2. Experimental animals, housing and management

Lactating Holstein Friesian cows (n = 92) were used for the study from Feb-2015 to Feb-2016 at Harper Adams University dairy unit (see section 5a 2.2.).

5b.2.3. Milk progesterone assay

Oestrous periods were identified by measuring the concentrations of progesterone in whole milk samples collected three times a week (see section 5a.2.3).

5b.2.4. Data collection and handling

5b.2.4.1. IceQube accelerometer

To monitor cow activity IceQubes (IceRobotics Ltd., Edinburgh, UK) were attached to the back left leg of each cow using a Velcro hook and loop strap (see section 5a.2.4.1). The hour of peak oestrous activity was identified the (0) and compared with 12 hours before (-12 h) and 12 hours after (+12 h) the peak of oestrus activity.

5b.2.4.2. Definitions

5b.2.4.2.1. Definition of oestrus based on milk progesterone profile

A cow was defined to be in oestrus according to their milk P4 concentrations as described in section 5a.2.4.2.1.

5b.2.4.2.2. Definition of behavioural and silent oestrus based on IceQube accelerometer

Each oestrous period identified from the progesterone profiles was classified as behavioural or silent oestrus as described in section 5a.2.4.2.2.

5b.2.4.2.3. Duration of oestrus according to IceQubes

The day of oestrus was identified as the day of peak activity during a period of low milk P4. The duration of oestrus behaviour on the day of oestrus was estimated as the number of hours between an increase in activity to >80% and fall in activity below this threshold (López-Gatius *et al.*, 2008; see section 3.6.4).

5b.2.4.2.4. Oestrus number post-partum

Each oestrus that was detected by milk progesterone profile was defined by its number PP as described in section 5a.2.4.2.3.

5b.2.4.2.5. Number of cows in oestrus simultaneously

This variable takes into account the number of cows in oestrus simultaneously; one cow in oestrus (SG 1), two cows in oestrus (SG 2) or three or more cows in oestrus (SG 3+) as described in section 5a.2.4.2.4.

5b.2.4.2.6. Body condition score (BCS)

Oestrus' were grouped into one of three groups BCS ≤ 2.5 , BCS = 2.75 or BCS ≥ 3 according to cows body condition score at that oestrus events (BCS; Scale 1-5; AHDB Dairy, 2014) as described in section 5a.2.4.2.5.

5b.2.4.2.7. Locomotion score at oestrus

Oestrus' were grouped into one of 3 groups (LS1, LS2 or LS3) according to cows locomotion score at that oestrus event (LS; Scale 1-5; as described by Chapinal *et al.*, 2009) as described in section 5a.2.4.2.6.

5b.2.4.2.8. Parity

Oestrous data were analysed according to whether cows were in their $\leq 2^{\text{nd}}$ parity, 3^{rd} parity and $\geq 4^{\text{th}}$ parity as described in section 5a.2.4.2.7.

5b.2.4.2.9. Season of the year

The time of the year when each oestrous event occurred was taken into account as a variable because of day length could physiologically affect activity on the day of oestrus (López-Gatius *et al.*, 2005; see section 5a.2.4.2.8).

5b.2.4.2.10. Time of the day

The time of day when the peak of oestrous activity of oestrus occurred was designated daytime (07:00 a.m. to 19:00 pm) or nighttime (19:00 pm to 07:00 a.m.).

5b.2.5. Statistical analysis

Statistical analyses were performed using the Genstat statistical software package (Genstat 18th edition, 18.1.14713, VSN International Ltd, UK). All of the data sets analysed were normally distributed. The datasets were analysed using repeated measures ANOVA to compare between groups (*behavioural* and *silent oestrus*) 12 hours before and 12 hours after the peak hour (0) of oestrous activity and the group x day interaction. Tukey test were used to compare between different groups during the peak hour of behavioural oestrus. One way ANOVA was used to compare the number of steps (oestrous intensity) during the hour of peak oestrus activity in the following groups:- number of oestrus PP, SG, BCS at oestrus, LS, parity and season of the year. Chi-square test were used to analyse the incidence of the oestrus during day time and night time on the day of behavioural oestrus. One way ANOVAs were also used to compare the duration of oestrus between different groups. Paired t-tests were used to compare between hours before, the peak hour (0) of oestrus and hours after the peak of oestrus.

Simple linear regression and multiple linear regression were used to determine relationships between oestrus duration (h) and SG, milk yield (kg/d), LS at oestrus, parity, season of the year, DPP, BCS at oestrus, BCS changes from parturition to oestrus and LS changes from parturition to oestrus. Simple linear regression and multiple linear regression were also used to determine the relationship between the oestrous duration (h) and SG, milk yield (kg/d), LS at oestrus, parity, season of the year, DPP, BCS at oestrus, BCS changes from parturition to oestrus and LS changes from parturition to oestrus. Differences were reported as significant at $P < 0.05$ and trends were reported when P was between <0.1 and >0.05 .

5b.3. Results

5b.3.1. Behavioural versus silent oestrus

Mean number of steps per hour during the day of oestrus was affected ($P < 0.001$) by oestrous expression. A higher number of steps per hour were recorded during behavioural oestrus (176.4 ± 11.9 steps) compared with silent oestrus (96.6 ± 13.8 steps) (Figure 5b.1). The number of steps was also affected by time ($P < 0.001$). Regarding the peak of oestrous activity, more steps ($P < 0.001$) were taken during the peak hour of behavioural oestrus (599.1 ± 24.8 steps) compared to silent oestrus (169.5 ± 27.8 steps). Furthermore, there was an interaction ($P < 0.001$) between time and oestrous expression with regards to the number of steps per hour. The number of steps increased ($P = 0.084$) - 7 to -6 hours before the peak of oestrous activity and increased further ($P < 0.001$) from 1 hour before to the peak of oestrus on the day of behavioural oestrus. The number of steps taken per hour subsequently fell ($P < 0.001$) from the peak of oestrus (0) to 1 hour after oestrus and decreased ($P = 0.004$) further until 7 hours after the peak oestrus (Figure 5b.1).

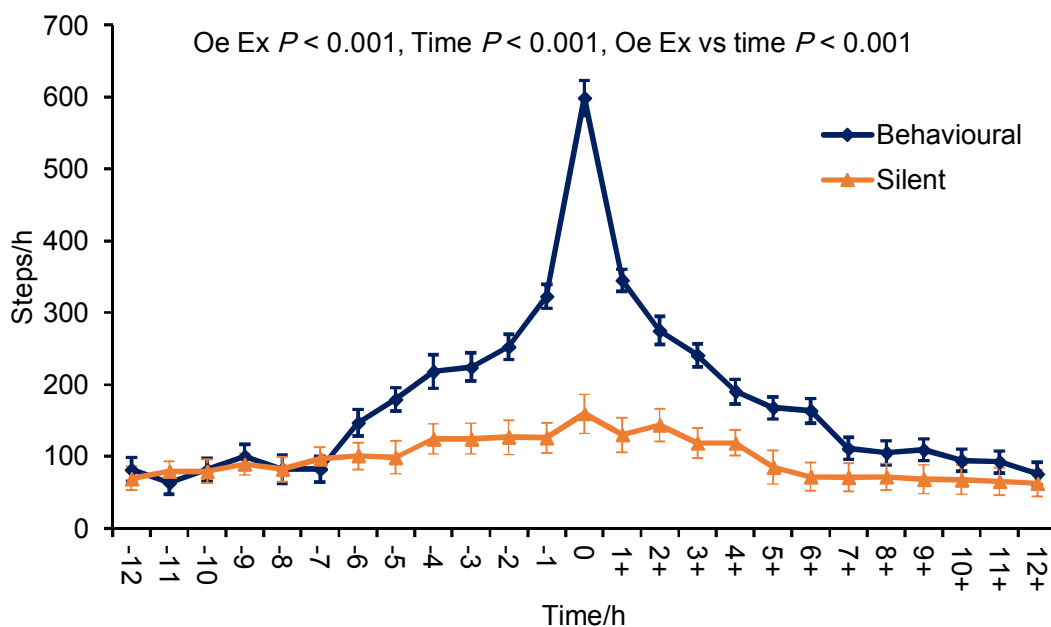


Figure 5b. 1. Mean (\pm sem) number of steps/h from 12 hours before to 12 hours after oestrus during behavioural ($n = 138$) and silent ($n = 110$) oestrus in lactating Holstein Friesian dairy cows. Peak hour of oestrus = 0 h, Oe Ex = oestrous expression.

5b.3.2. First, second and third or more oestrus post-partum

The duration of behavioural oestrus was shorter ($P = 0.051$) at 1st oestrus PP (10.7 ± 0.3 h) compared to 2nd oestrus (11.4 ± 0.4 h) and $\geq 3^{\text{rd}}$ oestrus (11.6 ± 0.4 h) post-partum, while there was no difference ($P = 0.126$) in oestrus duration between 2nd and $\geq 3^{\text{rd}}$ oestrus PP.

Mean number of steps per hour during the day of oestrus were affected ($P = 0.021$) by the number of oestrus PP (Figure 5b.2). More steps per hour were recorded at $\geq 3^{\text{rd}}$ oestrus PP (215 ± 17.1 steps/h) compared to 1st oestrus (169.4 ± 15.1 steps/h) and 2nd oestrus (171.8 ± 18.6 steps/h) PP. There was also an effect ($P < 0.001$) of time on the number of steps per hour on the day of oestrus. There was no interaction ($P = 0.123$) between time/h and the number of oestrus PP (Figure 5b.2). Concerning the peak of oestrous activity, the number of steps per hours were lower ($P = 0.051$) during the peak of the 1st oestrus (471.6 ± 39.3 steps/h) PP compared to 2nd oestrus (604.3 ± 42.1 steps) and $\geq 3^{\text{rd}}$ oestrus (686.2 ± 29.9 steps/h) PP.

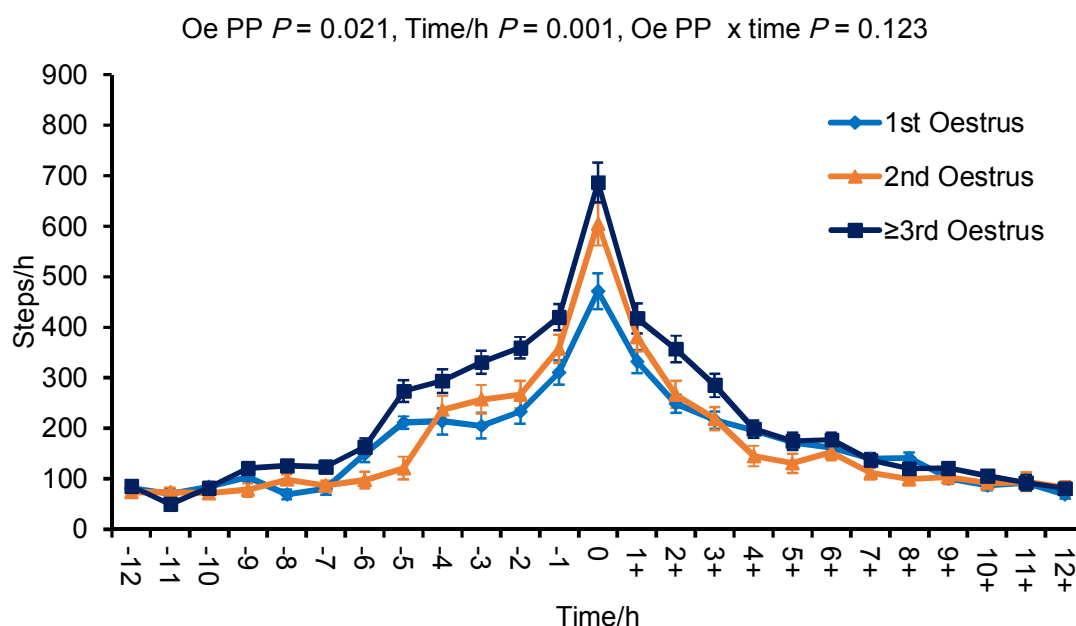


Figure 5b. 2. Mean (\pm sem) number of steps/h during the day of oestrus from 12 hours before to 12 hours after the peak of oestrus during 1st ($n = 24$), 2nd ($n = 57$) and $\geq 3^{\text{rd}}$ ($n = 57$) oestrus PP in lactating Holstein Friesian dairy cows. Oe PP = number of oestrus post-partum, peak hour of oestrus = 0h.

5b.3.3. Effect of the SG on the duration and intensity of oestrus activity

Oestrus was longer (12.5 ± 0.6 h; $P = 0.004$) when SG3+ in oestrus compared to SG1 (10.4 ± 0.5 h), while there was no difference ($P = 0.301$) between SG3+ and SG2 in oestrus (11.6 ± 0.5 h).

The mean number of steps per hour during the day of oestrus was affected ($P < 0.001$) by the number of cows in oestrus simultaneously. More steps per hour were recorded during SG 3+ (221.4 ± 25.7 steps/h) compared to SG 2 (178.8 ± 21.0 steps/h) and SG 1 (116.5 ± 19.9 steps/h). There was an interaction ($P < 0.001$) between time and SG with regards the number of steps per hour (Figure 5b.3). However, the step count was not significantly affected by the size of SG until -7 h before the peak of oestrus and it remained significantly higher in SG 3+ until 9 h after the peak of oestrus. Regarding the peak of oestrus, SG3+ took more steps ($P < 0.001$; 781.0 ± 44.7 steps) during the peak hour of oestrus compared to SG2 (620.4 ± 34.7 steps) and SG1 (315.9 ± 29.9 steps). The number of steps increased ($P < 0.001$) from 7 to 4 hours before the peak of oestrous activity during SG 3+ and increased further ($P < 0.001$) from 1 hour before peak of oestrus (0) on the day of oestrus during SG2 and SG 3+. The number of steps taken subsequently fell ($P < 0.001$) one hour after oestrus in comparison to the peak of oestrus and decreased ($P < 0.001$) further from 1 to 5 hours after the peak oestrus during both SG2 and SG 3+.

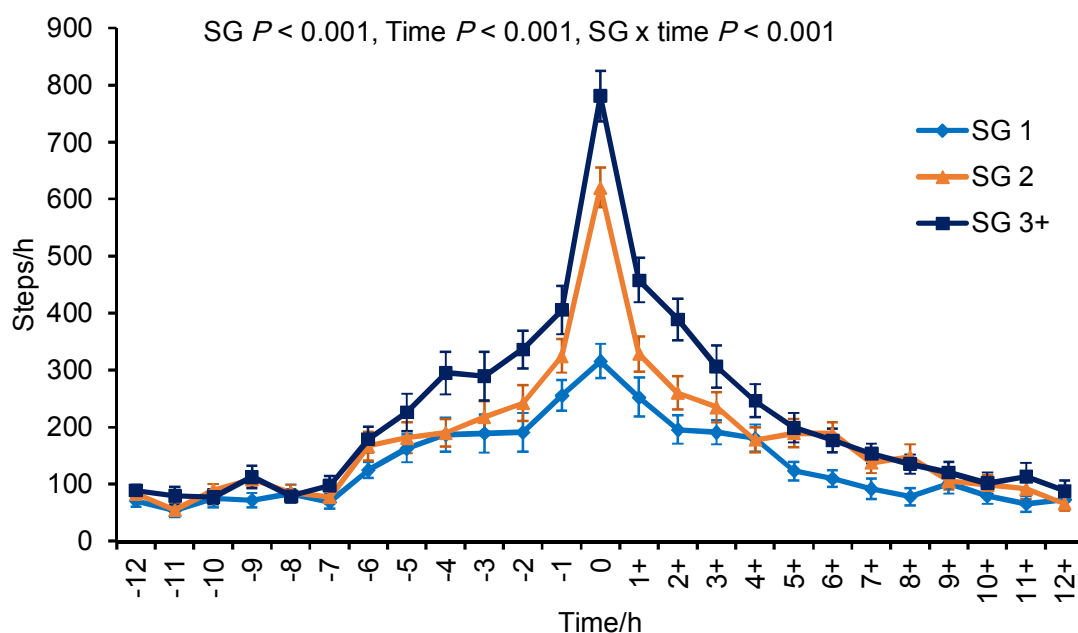


Figure 5b. 3. Mean (\pm sem) number of steps/h during the day of oestrus from 12 hours before to 12 hours after peak of oestrus in lactating Holstein Friesian dairy cows when 1 (SG 1; $n = 36$), 2 (SG 2; $n = 50$) or ≥ 3 (SG3+; $n = 52$) cows were in oestrus simultaneously. SG = number of cows in oestrus simultaneously, peak hour of oestrus = 0h.

5b.3.4. Effect of BCS on the duration and intensity of oestrous activity

The duration of oestrus was not affected ($P = 0.652$) by BCS. The duration of oestrus of a cow which had a BCS ≤ 2.5 , 2.75 or ≥ 3 were 11.4 ± 0.6 h, 12.1 ± 0.5 h and 11.0 ± 0.7 h, respectively.

During the day of oestrus, the mean number of steps per hour was not affected ($P = 0.478$) by different BCS. However, there was an effect of time ($P < 0.001$) on the number of steps per hour. There was also an interaction ($P = 0.04$) between time and BCS with regards the number of steps per hour (Figure 5b.4). Concerning the peak of oestrus activity, more number of steps per hour ($P = 0.002$) were taken (689.3 ± 33.4 steps/h) at the peak of oestrus activity in BCS 2.75 cows compared to BCS ≤ 2.5 (450.7 ± 57.4 steps/h) or BCS ≥ 3 (535.3 ± 57.0 steps/h) cows (Figure 5b.4).

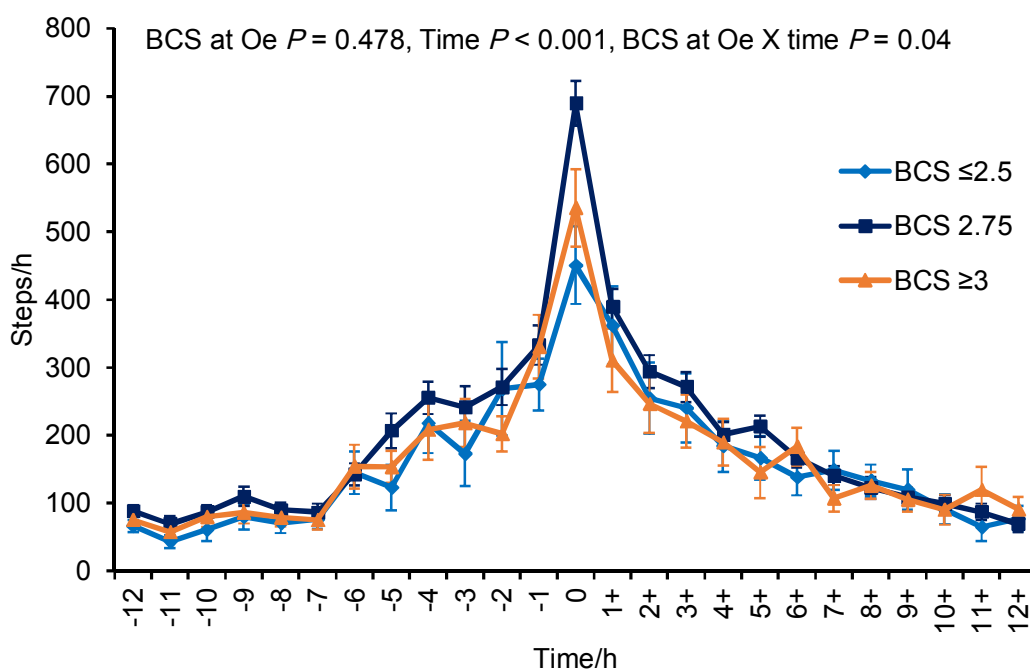


Figure 5b. 4. Mean (\pm sem) number of steps/h during the day of oestrus from 12 hours before to 12 hours after the peak of oestrus in lactating Holstein Friesian dairy cows with different body condition score (BCS), BCS ≤ 2.5 ($n = 22$), BCS = 2.75 ($n = 81$) or BCS ≥ 3 ($n = 35$). Peak hour of oestrus = 0h.

5b.3.5. Effect of LS on the duration and intensity of oestrous activity

Oestrus was longer in cows which were LS1 ($P = 0.05$; 12.3 ± 0.5 h) compared to cows that were LS2 (11.3 ± 0.5 h) or LS3 (10.9 ± 0.6 h), while there were no differences ($P = 0.332$) in oestrous duration between cows which were LS2 or LS3.

On the day of oestrus, the mean number of steps per hours were higher ($P = 0.05$) in the cows which were LS1 (214.4 ± 24.2 steps/h) compare to cows which were LS3 (147.5 ± 24.1 steps/h), while there was no difference ($P = 0.114$) between cows which were LS1 and cows that were LS2 (170.6 ± 18.6 steps/h) Figure (5b.5). Furthermore, time had an effect ($P < 0.001$) on the number of steps per hour. However, there was no interaction ($P = 0.228$) between time and LS with regard the number of steps taken per hour.

In cows that were LS1, the number of steps per hour started to increase significantly from 6 hours before reaching the peak of activity and had decreased to normal level by 6 hours after the peak of oestrus. Concerning the peak of oestrus, no differences ($P = 0.235$) were found in the number steps taken by cows that were LS1 (659.2 ± 30.2 steps/h), LS2 (597.2 ± 36.7 steps/h) or LS3 (586.6 ± 37.1 steps/h) during the peak hour of oestrus.

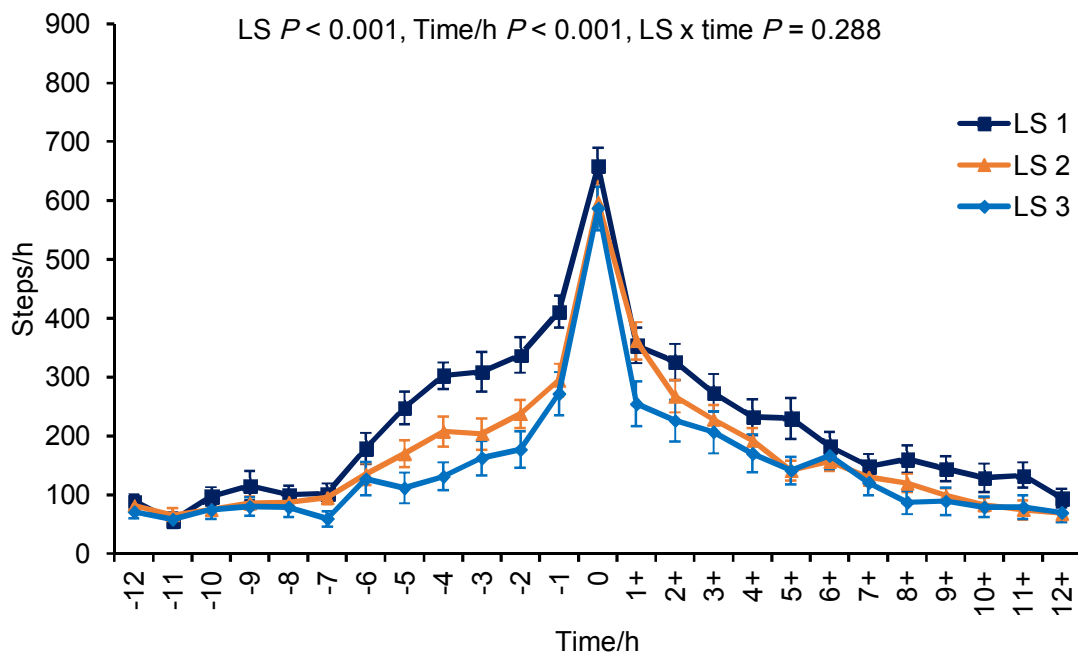


Figure 5b. 5. Mean (\pm sem) number of steps/h during the day of oestrus from 12 hours before to 12 hours after the peak of oestrus in lactating Holstein Friesian dairy with different locomotion score (LS), LS1 ($n = 38$), LS 2 ($n = 73$) or LS 3 ($n = 27$). Peak hour of oestrus = 0h.

5b.3.6. Effect of parity on the duration and intensity of oestrous activity

Oestrous duration was longer ($P < 0.001$) in parity ≤ 2 cows (12.0 ± 0.6 h) and parity 3 cows (11.7 ± 0.6 h) compared to parity ≥ 4 cows (10.7 ± 0.5 h), while there were no differences ($P = 0.183$) in oestrous duration between parity ≤ 2 cows and parity 3 cows.

Mean number of steps per hour on the day of oestrus were lower ($P < 0.001$) in parity ≥ 4 cows (129.5 ± 23.1 steps/h) compared to parity ≤ 2 cows (192.5 ± 20.5 steps/h) and parity 3 cows (189.9 ± 25.4 steps/h), while there was no difference ($P = 0.256$) between parity ≤ 2 cows and parity 3 cows (Figure 5b.6). There was an effect ($P < 0.001$) of time on the number of steps taken per hour. There was also an interaction ($P = 0.023$) between time and parity with regard to the number of steps taken per hour. Concerning the peak of oestrus, more ($P < 0.001$) steps were taken by parity ≤ 2 cows (652.4 ± 36.1 steps/h) and parity 3 cows (637.9 ± 48.8 steps/h) compared to parity ≥ 4 cows (410.1 ± 57.6 steps/h). However, there was no difference ($P = 0.354$) in the number of steps taken between parity ≤ 2 cows and parity 3 cows during the peak hour of oestrus. The number of steps increased ($P = 0.032$) from 7 to 5 hours before the peak of oestrus in parity ≤ 2 and parity 3 cows and increased further ($P < 0.001$) from one hour before peak of oestrus (0) on the day of oestrus. The number of steps taken subsequently fell ($P < 0.001$) to one hour after oestrus in comparison to the peak of oestrus and decreased ($P < 0.001$) further from 1 to 4 hours after the peak of oestrus in parity ≤ 2 and parity 3 cows.

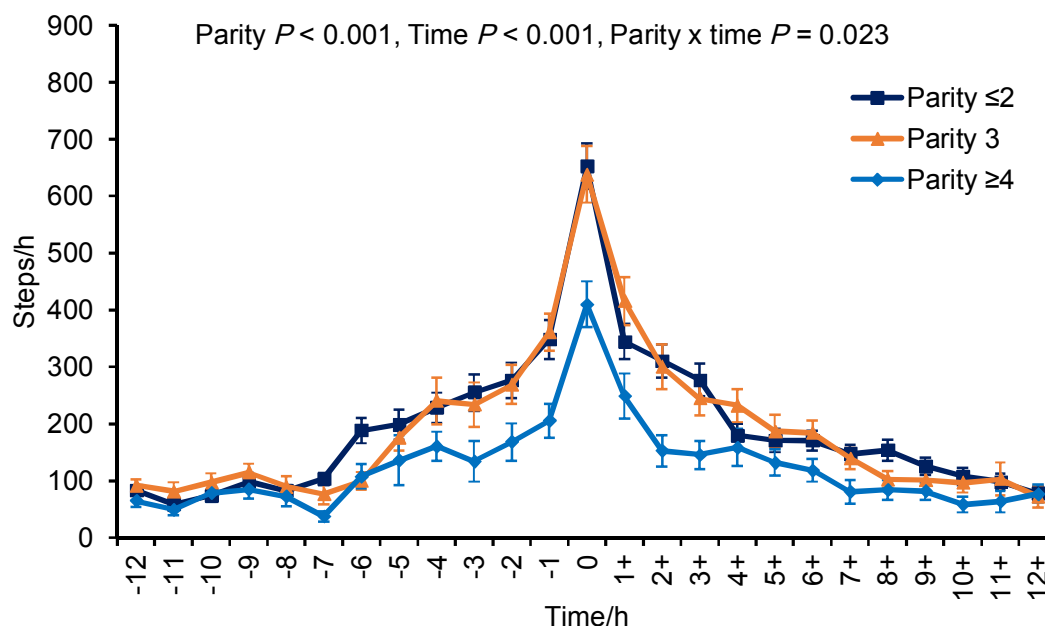


Figure 5b. 6. Mean (\pm sem) number of steps/h during the day of oestrus from 12 hours before to 12 hours after the peak of oestrus in lactating Holstein Friesian dairy cows with different parity, Parity ≤ 2 ($n = 71$), parity = 3 ($n = 39$) or parity ≥ 4 ($n = 28$). Peak hour of oestrus = 0h.

5b.3.7. Effect of the season on the duration and intensity of oestrous activity

Oestrous duration was longer during the summer ($P = 0.039$; 12.4 ± 0.5 h) compared to the winter 10.8 ± 0.5 h and spring 10.7 ± 0.6 h. However, there were no differences ($P = 0.113$) in oestrous duration between summer and autumn (11.9 ± 0.5 h).

During the day of oestrus, the mean number of steps per hour were higher ($P < 0.001$) during summer (168.3 ± 28.2 steps/h) compare to autumn (133.3 ± 26.1 steps/h), winter (123.0 ± 21.1 steps/h) and spring (118.8 ± 20.3 steps/h) (Figure 5b.7). There was an effect ($P < 0.001$) of time on the number of steps taken. Furthermore, there was an interaction ($P < 0.001$) between days and season with the regard to the number of steps per hour. The number of steps increased ($P < 0.01$) from 2 to 1 hour before the peak of oestrous activity during summer and increased further ($P < 0.001$) from 1 hour before to the peak of oestrus (0). The number of steps taken subsequently fell ($P < 0.001$) one hour after the peak of oestrus in comparison to the peak of oestrus (0) and decreased ($P = 0.022$) more from one hour after the peak of oestrus to 5 hours after the oestrus peak during the summer. Concerning the peak of oestrus, more ($P < 0.001$) steps were taken by cows in oestrus during the summer (548.6 ± 28.6 steps/h) on the peak hour of oestrus compared to cows in oestrus during the autumn, winter and spring (359.7 ± 15.7 , 314.7 ± 17.3 and 332.4 ± 18.7 steps/h, respectively). However, there were no differences ($P = 0.186$) in the number of steps taken by cows in autumn, winter or spring during the peak hour of oestrus (Figure 5b.7).

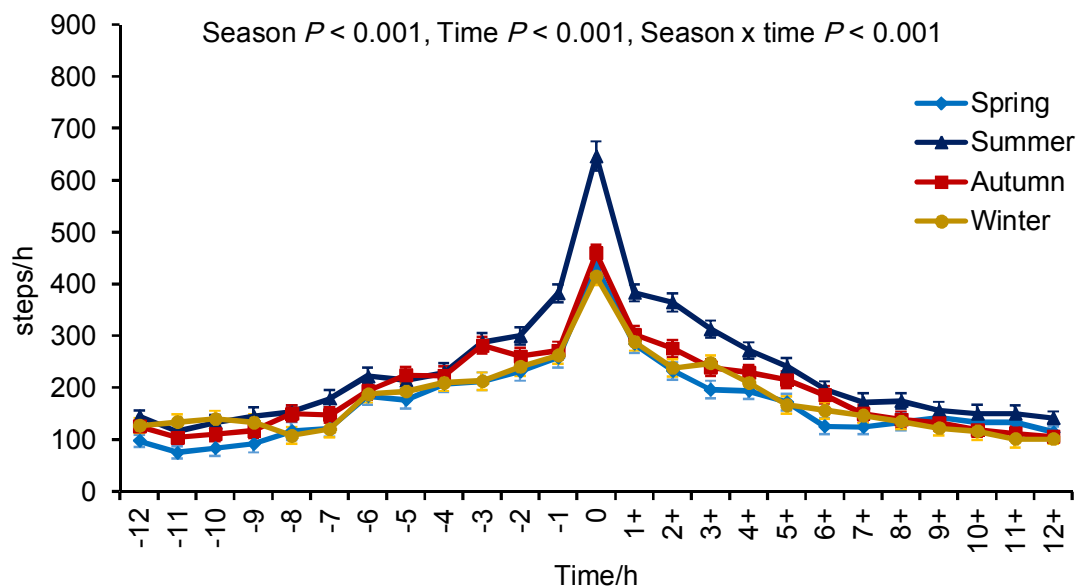


Figure 5b. 7. Mean (\pm sem) number of steps/h during the day of oestrus from 12 hours before to 12 hours after the peak of oestrus in lactating Holstein Friesian dairy cows that were in oestrus during spring ($n = 27$), summer ($n = 43$), autumn ($n = 42$) or winter ($n = 26$). Peak hour of oestrus = 0h.

5b.3.8. The relationships between explanatory variables and the number of steps taken during peak hour of oestrus

A summary of the relationships between factors affecting oestrous intensity and the number of steps taken during peak hour of oestrus are presented in Table 5b.1. The results show positive ($P < 0.001$) correlations between SG, season and DPP and the number of steps taken during peak hour of oestrus. The results show negative ($P < 0.001$) correlations between milk yield (kg/d), LS at oestrus and parity and the number of steps taken during peak hour of oestrus. However, there was no linear relationship between BCS change from parturition to oestrus ($P = 0.101$), BCS at oestrus ($P = 0.230$) and LS changes from parturition to oestrus ($P = 0.343$) and the number of steps taken during the peak hour of oestrus in dairy cows. The results show a high multi colinearity between oestrus PP and DPP ($r^2 = 0.92$) and between parity and age ($r^2 = 0.94$), therefore both oestrus PP and age were excluded from multiple linear regression.

Step-wise multiple regression analysis indicated that on the day of oestrus, factors affecting the number of steps taken during peak hour of oestrus that minimised the residual mean square of the number of steps taken during peak hour of oestrus (Table 5b.2). Number of cows in oestrus at the same time (SG) was chosen as a first independent variable add to the model as it had the lowest residual value in relation to the oestrus duration/h in the following equations. For oestrus intensity, the statistically significant ($P < 0.05$) explanatory were SG (1, 2 and 3+), parity (≤ 2 , 3 and ≥ 4), season, milk yield, LS at oestrus, and DPP. The addition of other explanatory variables did not reduce ($P > 0.05$) the residual mean squares in the dependent variables.

Table 5b. 1. The correlations between explanatory variables and the number of steps taken during peak hour of oestrus using IceQube in lactating Holstein Friesian dairy cows.

Variables	Regression	Constant	SE	P-value	r²
SG 1,2 or 3+	293.1	201.9	246	<0.001	0.50
Milk yield (kg/d)	15.9	-1077	330	<0.001	0.201
LS at oestrus	274.7	-940.7	275	<0.001	0.38
Parity $\leq 2,3$ and ≥ 4	277.2	-1220	259	<0.001	0.45
Season	191.6	106.6	270	<0.001	0.40
DPP	4.16	205.7	332	<0.001	0.22
BCS change	135.5	475.2	346	0.101	0.11
BCS at oestrus	127	-773	348	0.230	0.02
LS change	36.7	425.4	348	0.343	0.001

SG = Sexual groups, DPP = days post-partum, BCS = body condition score, LS = locomotion score and SE = standard error of observation.

Table 5b. 2. Relationships between the number of steps taken during peak hour of oestrus and number of cows in oestrus simultaneously (SG; 1, 2 and 3+), parity, season and DPP in lactating Holstein Friesian dairy cows.

Response Variables	Explanatory variables						r^2	SE	P-value
	Constant	SG.	Parity	Season	LS at oestrus	DPP			
Steps/h	201.9 (SE=42.7)	293.1 (SE=18.7)					0.50	246	<0.001
Steps/h	382 (SE=130)	194.4 (SE=27.5)	-129.7 (SE=27.5)				0.54	236	<0.001
Steps/h	200 (SE=144)	165.5 (SE=29.1)	-0.97.8 (SE=29.5)	54.8 (SE=19.8)			0.55	233	<0.001
Steps/h	277 (SE=160)	152.4 (SE=31.4)	-89 (SE=30.5)	51.1 (SE=20.1)	-33.9 (SE=30.7)		0.56	232	<0.001
Steps/h	178 (SE=160)	142.8 (SE=30.9)	-87.1 (SE=29.9)	45.2 (SE=19.8)	-22 (SE=30.3)	2.06 (SE=0.62)	0.57	1.71	<0.001

SE = Standard error of observation, SG = number of cows in oestrus simultaneously LS = locomotion score and DPP = day post-partum

5b.3.9. The relationships between explanatory variables and oestrous duration/h

A summary of the relationships between factors affecting oestrus activity and oestrus duration/h are presented in Table 5b.3. The results show positive ($P < 0.001$) correlations between SG, season and DPP and oestrus duration/h. The results show negative ($P < 0.001$) correlations between milk yield (kg/d), LS at oestrus and parity and oestrous duration. However, there was no linear relationship between BCS change ($P = 0.114$), BCS at oestrus ($P = 0.185$) and LS changes from parturition to oestrus ($P = 0.605$) and oestrus duration in dairy cows. The results show a high multi colinearity between oestrus PP and DPP ($r^2 = 0.91$) and between parity and age ($r^2 = 0.88$), therefore both oestrus PP and age were excluded from multiple linear regression shown in Table 5b.4.

Step-wise multiple regression analysis indicated that on the day of oestrus, factors affecting oestrous duration/h that minimised the residual mean square of the duration of behavioural oestrus (Table 5b.4). Number of cows in oestrus at the same time (SG) was chosen as a first independent variable add to the model as it has the lowest residual value in relation to the oestrous duration in the following equations. For oestrous duration, the statistically significant ($P < 0.05$) explanatory variables were SG (1, 2 and 3+), milk yield, LS at oestrus, parity (≤ 2 , 3 and ≥ 4), and DPP. The addition of the other explanatory variables did not reduce ($P > 0.05$) the residual mean squares further.

Table 5b. 3. The correlations between explanatory variables and oestrous duration/h in lactating Holstein Friesian dairy cows measured as an increase activity recorded by IceQubes.

Variables	Regression	Constant	SE	P-value	r²
SG 1,2 or 3+	2.79	3.64	2.26	<0.001	0.48
Milk yield (kg/d)	0.343	-22.87	2.39	<0.001	0.32
LS at oestrus	2.25	-13.56	2.80	<0.001	0.29
Parity ≤2,3 and ≥4	2.01	-15.1	2.87	<0.001	0.27
Season	1.75	4.29	2.77	<0.001	0.23
DPP	0.02	11.38	3.12	<0.001	0.22
BCS change	0.21	10.32	3.09	0.114	0.03
BCS at oestrus	1.32	-13.87	3.16	0.185	0.02
LS change	0.12	10.25	3.15	0.605	0.001

SG = Sexual groups, DPP = days post-partum, BCS = body condition score, LS = locomotion score and SE = standard error of observation.

Table 5b. 4. Relationships between oestrus duration/h and number of cows in oestrus simultaneously (SG; 1, 2 and 3+), milk yield (kg/d), locomotion score (LS) at oestrus, parity (≤ 2 , 3 and ≥ 4), day length and DPP in lactating Holstein Friesian dairy cows.

Response variables	Explanatory variables						r^2	SE	P-value
	Constant	SG.	Milk yield kg/d	LS at oestrus	Parity	DPP			
Oestrus duration/h	3.64 (SE=0.62)	2.79 (SE=0.25)					0.48	2.26	<0.001
Oestrus duration/h	13.37 (SE=1.48)	2.03 (SE=0.23)	-0.23 (SE=0.03)				0.62	1.96	<0.001
Oestrus duration/h	12.42 (SE=1.59)	1.89 (SE=0.27)	-0.22 (SE=0.03)	-0.33 (SE=0.03)			0.63	1.92	<0.001
Oestrus duration/h	14.75 (SE=1.83)	1.86 (SE=0.28)	-0.21 (SE=0.02)	-0.27 (SE=0.34)	-0.13 (SE=0.33)		0.66	1.72	<0.001
Oestrus duration/h	15.39 (SE=1.86)	1.84 (SE=0.28)	-0.20 (SE=0.02)	-0.22 (SE=0.32)	-0.06 (SE=0.32)	-0.04 (SE=0.06)	0.67	1.71	<0.001

SE = Standard error of observation, DPP = day post-partum, SG = Sexual group (number of cows in oestrus simultaneously)

5b.3.10. Effect of the day time on oestrous characteristics

From 138 behavioural oestruses that were detected during the study period for all cows, the average duration of behavioural oestrus was 11.5 ± 3.1 h (Table 5b.5). Regarding oestrous duration, there was no difference ($P = 0.310$) between day time and night time. Concerning the incidence of the oestrus during behavioural oestrus, there was no difference ($P = 0.886$) between day time (49.3%) and nighttime (50.7%). There was no difference ($P = 0.610$) in the number of steps taken during oestruses occurring during the day time compared with those that occurred at night time.

Table 5b. 5. The incidence of oestrus and mean (\pm SEM) of the number of steps/d and oestrus duration (hours) during day or night time according to the peak of oestrus in lactating Holstein Friesian dairy cows.

Oestrus characteristics	Day time	Night-time	P-value
The incidence of the peak of oestrus %	49.3	50.7	0.886
Oestrus duration/h	11.7 ± 0.5	11.3 ± 0.4	0.310
Number of steps/d	3947.7 ± 223.8	3789.6 ± 217.8	0.610

5b.4. Discussion

5b.4.1. Behavioural versus silent oestrus

The results of the current study show that the mean number of steps per hour during the day of oestrus were affected by oestrous expression (behavioural vs silent oestrus). More steps per hour were recorded in behavioural oestrus compared to silent oestrus. In a study conducted by Roelofs *et al.* (2005a), the number of steps recorded by pedometers was also higher (841 ± 259 steps/2h) during behavioural oestrus compared to silent oestrus (477 ± 322 steps/2h) in Holstein Friesian cows. Furthermore, the results of the present study found that cows took more steps during the peak hour of behavioural oestrus compared to the 12 h before and the 12 h after oestrus. The present study recorded a higher number of steps per hour than those recorded in a study conducted by Livshin *et al.* (2005) who found that the number of steps was approximately 480 steps/h on the day of behavioural oestrus in dairy cows. The increase in the number of steps per hour on the day of behavioural oestrus is reflective of an increase in restlessness and activity (Roelofs *et al.*, 2005b; Jónsson *et al.*, 2011) but not all cows show this increase in activity.

5b.4.2. First, second and third or more oestrus post-partum

The duration of behavioural oestrus recorded in the present study was 11.2 ± 0.4 h. The results of the present study found that the oestrous duration was shorter at 1st oestrus PP compared to 2nd oestrus and $\geq 3^{\text{rd}}$ oestrus PP. These results (10-11 h) are within the range of oestrus duration (4 to 26 h) found by Silper *et al.* (2015). However, Løvendahl and Chagunda, (2010) recorded shorter duration oestruses (9.2 h) in non-lactating Holstein heifers. Differences in the duration of oestrus based on the number of steps and walking activity in lactating dairy cows, may due many factors, as shown in the present study. In addition housing and management system (Yániz *et al.*, 2006), and the type of pedometers or AOD system are also likely to yield differences in oestrous duration (Roelofs *et al.*, 2005b).

The present study shows that a significantly more steps per hour were recorded during the day of oestrus at the $\geq 3^{\text{rd}}$ oestrus PP compared to 1st or 2nd oestrus PP. Concerning the peak of oestrus, the number of steps was significantly lower during the peak hour of the 1st oestrus PP compared to the peak hour of 2nd oestrus and $\geq 3^{\text{rd}}$ oestrus PP. Similarly, Homer (2013) found that the peak of oestrus activity was higher on the day of 2nd or 3rd oestrus PP compared to the 1st PP in lactating dairy cows. However, these finding disagree with Yániz *et al.* (2006) who found walking activity on the day of oestrus was not significantly affected by the number of oestrus PP in dairy cows. A lower oestrus

activity at 1st oestrus PP in dairy cows may be due to negative energy balance in lactating dairy cows during early lactation, which may lead to lower E2 production from the pre-ovulatory follicle (Isobe *et al.*, 2004). In addition, the lower activity at 1st oestrus PP compared to 2nd and $\geq 3^{\text{rd}}$ oestrus PP in lactating dairy cows may be due to high E2 concentrations in blood during the late gestation period which lead to refractoriness of the cow to E2 before 1st ovulation (Allrich, 1994).

5b.4.3. Effect of the SG on the duration and intensity of oestrous activity

The results of the present study show that the duration of oestrus was longer in SG3+ compared to SG1. These results are within the range of 2.6 to 26.2 h reported by Stevenson *et al.* (1996). The results of the present study agree with those reported by Roelofs *et al.* (2005a) who found that the duration of oestrus (based on the number of steps) when 1 cow, 2 cows or 3 cows were in oestrus at the same time were 9.0 ± 3.8 , 10.0 ± 4.4 and 11.3 ± 4.5 h, respectively. However, the duration of oestrus in the present study was longer than that reported by Hurnik *et al.* (1975) who found that oestrus duration increased from 7.5 to 10.1 h with an increased number of cows from one to three cows in oestrus simultaneously. Diskin (2008) reported that the average duration of standing oestrus in dairy cows was 8.1 h with 9.1 standing events or mounts recorded during standing oestrus. The longer duration of oestrus of SG3+ in the present study is likely to be due to increasing sexual stimulation by other animals (Roelofs *et al.*, 2005a) when more cows in oestrus simultaneously as a result of more walking around sexual partners during oestrous periods (Van Vliet and Van Eerdenburg, 1996) which has been reported in observational studies of cows in oestrus (Van Eerdenburg *et al.*, 2002).

In the present study, a higher number of steps per hour on the day of oestrus and the peak hour of oestrus were recorded when SG3+ in oestrus compared to SG2 and SG1 in oestrus. These results agree with those reported by Roelofs *et al.* (2005) who found that the number of steps (2h time intervals) increased with more cows in oestrus. Several previous studies found that the intensity of oestrus was more when more cows are in oestrus simultaneously; cows received more oestrous points (Van Vliet and Van Eerdenburg, 1996), displayed more mounts and stands (Hurnik *et al.*, 1975), increased walking activity (Yániz *et al.*, 2006) and took more steps (Varner, 1994). During the peak hour of oestrus, cows may experience restlessness due to an increase in the systemic concentrations of E2 hormone produced by the pre-ovulatory follicle which could lead to a reduced lying time and lying bouts with increased activity coincident with the ovulatory LH surge (Walton *et al.*, 1987; Shipka, 2000). An increase in the intensity of oestrus in SG3+ compared to SG1 may be due to an increase in walking around sexual partners during oestrus periods when three cows are in oestrus in the same time as result of sniffing the

anogenital region of fellow herd mates, chin-resting, flehmen, attempting to mount and mounting (Van Vliet and Van Eerdenburg, 1996).

5b.4.4. Effect of BCS on the duration and intensity of oestrous activity

The results of the present study found no difference in oestrous duration between cows with different BCS. This finding agrees with that found by Palmer *et al.* (2010) in a study conducted on Holstein cows. However, the oestrus duration of all of the cows used in the present study, regardless of BCS, were lower than the oestrus duration (13.0 ± 1.4 h) reported by Sveberg *et al.* (2011) of 13 lactating Holstein-Friesian cows with BCS 2.5 housed on an outdoor pad. These differences may be explained by differences in the threshold definitions used in the two studies. However, differences in floor surface may be important here as the cows in the present study were housed in a concrete floored covered yard. In addition, number of cows in oestrus simultaneously, environmental factors and milking system may have important role in (Roelofs *et al.*, 2005a).

The results of the present study show that the highest number of steps per hour during the peak of oestrus were taken by cows with BCS 2.75 (689.3 ± 33.4 steps/h) compared to cows that had BCS ≤ 2.5 and BCS ≥ 3 . These intensity of oestrus was considerably greater than the that reported by Dolecheck (2015) of 258.6 steps/h in housed lactating Holstein cows with a BCS ≥ 2.5 . The difference in oestrous intensity in lactating dairy cows may be due to combination of various other factors that have been shown affect oestrous expression such as flooring type (Phillips and Schofield, 1990), housed vs. pasture (Palmer *et al.*, 2010), spontaneous vs synchronised oestrus (Walton *et al.*, 1987) and milk yield (Lopez *et al.*, 2005).

5b.4.5. Effect of LS on the duration and intensity of oestrous activity

The results of the present study revealed that oestrous duration was over one hour longer in cows that were LS1 compared to cows that were LS2 or LS3, while there was no difference in the oestrous duration between cows that were LS2 or LS3. The results of the present study agree with those of Walker *et al.* (2008b) who found that lameness reduced the duration of oestrus. Walker *et al.* (2008b) also found that lame cows spend less time standing on their feet, due in part to spending less time standing and walking compared with non-lame cows as a result of the pain of lameness.

On the day of oestrus in the current study, the mean number of steps per hour was 1.5 times higher in cows that were LS1 compared to cows which were LS3, while there was no difference between cows that were LS2 and cows which were LS3. This finding also agrees with the results reported by Walker *et al.* (2008a) who used the scoring system of Van Eerdenburg *et al.* (2002) to show that severely lame cows expressed fewer signs of

oestrous intensity (284 ± 128 points) compared to moderately lame and non-lame cows. Using the same scoring system, Dobson *et al.* (2008) also reported that severely lame cows have less intense oestruses (284.5 ± 42.7 points) than moderately (657.9 ± 96.8 points) or normal cows (583.2 ± 64.9 points). However, during the hour of peak oestrous activity, the present study found no difference in the number of steps between cows with different LS. Walker *et al.* (2008b) also found that the peak of oestrous activity was not affected by increasing lameness score in dairy cows. Walker *et al.* (2008b) also showed that despite the pain, lame dairy cows display some signs of sexual behaviour during the peak of oestrus although minimising the frequency of mounting behaviour and sexual attractive to other cows. Reduction in oestrous duration and intensity with increased LS may be due to the effects of the stress of lameness which resulting in reduced LH pulsatility which is necessary to drive E2 production by the dominant follicle; the consequently low E2 leads to reduced oestrous intensity (Dobson *et al.*, 2008). In dairy cows, many physiological, psychological, behavioural, and environmental factors can influence the duration and intensity of oestrus (Orihuela, 2000). For example, some studies reported that stress is known to have a negative impact on reproductive hormones from the hypothalamus-pituitary-ovarian axis (Moberg, 1985; Dobson *et al.*, 2003). Alternatively, chronically stressed lame cows have been shown to have low P4 exposure before oestrus which was associated with a low intensity of sexual behaviour and shorter oestrous duration in dairy cattle (Dobson *et al.*, 2008).

5b.4.6. Effect of parity on the duration and intensity of oestrous activity

In the current study, oestrus was approximately 2 h longer in parity ≤ 2 cows and parity 3 cows compared to parity ≥ 4 cows, while there were no difference in the duration of oestrus between parity ≤ 2 cows and parity 3 cows. Løvendahl and Chagunda (2010) found that oestrous activity lasted just over 1h longer in lactating heifers (9.24h) compared to multiparous cows (8.12h). While Roelofs *et al.* (2005) found that oestrous duration was 3h longer in primiparous compared to multiparous cows. However, Van Vliet and Van Eerdenburg (1996) recorded a shorter oestrus duration in primiparous compared with multiparous cows. Other studies found that there was no significant difference in oestrus duration between primiparous and multiparous cows (Lyimo *et al.*, 2000).

During the day of oestrus, the mean number of steps per hour was significantly lower in parity ≥ 4 cows compared to parity ≤ 2 and parity 3 cows. In the present study, more steps were taken during the peak hour of oestrus by parity ≤ 2 and parity 3 cows compared to parity ≥ 4 cows. The results of the current study agree with those reported by Roelofs *et al.* (2005a) who shows more steps were taken with a more intense oestrus on the day of oestrus by primiparous compared to multiparous cows. López-Gatius *et al.* (2005) also

found that younger cows had significantly greater expression of oestrous activity than older cows on the day of oestrus. Similarly, Yániz *et al.* (2006) found that an increase in parity was related to a decline in walking activity at oestrus in dairy cattle. However, Lyimo *et al.* (2000) found no difference in the intensity of oestrous behaviour between parity in lactating dairy cows. Why these differences in oestrous intensity among different parities is unclear. To our knowledge, no clear information is available on the effect of parity on oestrous duration and oestrus intensity patterns per hour in lactating dairy cows. However, reduced oestrous expression in older cows may be related to increasing milk yield, more negative energy balance or increased chance of poor locomotion in older cows (Morris *et al.*, 2011). This is confirmed by the finding of a negative correlation between milk and parity and oestrous duration in the present study may be due to high milk yield in the cows (Rajala and Gröhn, 1998).

5b.4.7. Effect of the season on the duration and intensity of oestrous activity

In the current study, a cow in oestrus during summer had a longer oestrus compared to oestrus during the winter and spring, while there was no difference in oestrus duration between summer and autumn. Differences between the duration of oestrus activity in high yielding dairy cows based on observation and activity monitoring may be due to the disconnection of secondary signs of oestrus behaviour and standing oestrus (Valenza *et al.*, 2012). Indeed, it has been reported that one of the secondary signs of oestrus, an increased activity that can be detected by pedometer in dairy cows, were significantly increased 1 to 3h before the starting of standing oestrus (Sveberg *et al.*, 2011). In addition, Diskin (2008) reported that both the duration of standing oestrus and intensity of oestrous expression in dairy cattle are affected by a range of environmental factors including underfoot surface type, the sexually active group size and the presence of a bull.

The results of the present study showed that the mean number of steps per hour was significantly more in cows that had oestrus during summer compared to autumn, winter and spring and more steps were taken at the peak of oestrus. These results are consistent with López-Gatius *et al.* (2005) who found that cows spent more time walking during the day of oestrus in the summer compared to the winter season. However, the results of the present study disagree with those reported by Yániz *et al.* (2006) who found that the season of the year did not affect the walking activity in lactating cows. The present results agree with those reported by Homer (2013) who found that oestrus activity in dairy cows was higher in summer compared to autumn and winter. Greater oestrus activity in summer in the present study may be due to longer day length compared to the other seasons. It may also be because of the pasture access that the cows had during the summer, which gives better footing for oestrous behaviour. However, the results of the

present study disagree with those reported by Sankar and Archunan (2012) who found that the oestrous activity was higher in winter (61.5 ± 0.4 mounts) compared to summer (49.5 ± 0.3 mounts) and spring (39.7 ± 0.4 mounts) in dairy cattle. In addition, the other previous studies suggest that the duration and intensity of oestrus in summer are reduced in lactating dairy cows as a result of heat stress (Hansen, 1997; Nebel *et al.*, 1997). The effects of season on oestrus duration and intensity in dairy cows may differ among different studies due to the differences in the country of study, climate, management practices and system (Lim *et al.*, 2007).

5b.4.8. The relationships between explanatory variables and the number of steps taken on peak hour of oestrus

The results of the present study show a positive relationship between increasing the number of cows in SG and the number of steps taken during peak hour of oestrus. The results of the present study agree with those reported by Yániz *et al.* (2006) who found that walking activity during oestrus increases by about 6.1% with each increase in the number of cows in SG. The results of the current study also show a positive relationship between increasing the number of cows in SG and oestrous intensity. López-Gatius *et al.* (2005) also found that the intensity of oestrous activity on the day of oestrus was significantly higher in summer compared to other seasons in dairy cattle. These results agree also with those reported in Chapter 4 (section 4.3.4) and Chapter 5a (section 5a.3.8).

The findings of a positive correlation between DPP and the number of steps taken during peak hour of oestrus agree with those reported by López-Gatius *et al.* (2005) and Yániz *et al.* (2006) who found that oestrous intensity increased with increased days PP in dairy cattle. Similarly, Chapter 5a (section 5a.3.8) found that oestrous intensity increased with increased DPP.

The results of the present study found negative correlations between milk yield (kg/d) and oestrous intensity during the peak hour of oestrus. Similarly, Villa-Godoy *et al.* (1990) found a highly negative correlation between milk yield and oestrous intensity in dairy cows. These results are also concurred with those reported in Chapter 4 (section 4.3.4) and Chapter 5a (section 5a.3.8).

In the present study, a negative correlation between LS at oestrus and oestrous intensity during peak hour of oestrus was found. The results of the present study found also negative correlations between parity and oestrous intensity during the peak hour of

oestrus. Similarly, Chapter 4 (section 4.3.4) and Chapter 5a (section 5a.3.8) found that oestrous intensity had a negative correlations with LS and parity.

5b.4.9. The relationships between explanatory variables and oestrous duration/h

The results of the present study showed positive correlations between SG and oestrous duration/h. The results of the present study agree with those reported by Roelofs *et al.* (2005a) who found the duration of oestrus based on the number of steps was increased with increased number of cows in SG. Similarly, Hurnik *et al.* (1975) found also that oestrous duration increased from 7.5 to 10.1 h with an increased number of cows from one to three cows in oestrus simultaneously. The longer duration of oestrus of SG3+ in the present study may be due to increasing sexual stimulation by other animals (Roelofs *et al.*, 2005a) when more cows are in oestrus at the same time.

The results of the present study found a positive relationship between oestrous duration and DPP. Similarly, Homer (2013) found that oestrous duration was shorter at early DPP compared to the later DPP in lactating dairy cows. A shorter oestrous duration at early day PP in dairy cows may be due to a negative energy balance in lactating dairy cows during early lactation, which may lead to low E2 production in the pre-ovulatory follicle (Isobe *et al.*, 2004). The present study found also a positive relationship between oestrous duration and season. However, the results of the current study showed negative correlations between milk yield and parity and oestrous duration. The negative correlation between milk yield and parity and oestrous duration in the present study may be due to high milk yield in the cows (Rajala and Gröhn, 1998). High yielding dairy cows may suffer an increased negative energy balance which results in decreasing oestrous activity (Macmillan *et al.*, 1996). The present study found also a negative relationship between LS at oestrus and oestrous duration. This results agree with those reported by Walker *et al.* (2008b) who found that lameness reduced the duration of oestrus. Shorter oestrous duration in lame cows may due to stress having a negative impact on reproductive hormones from the hypothalamus-pituitary-ovarian axis (Moberg, 1985; Dobson *et al.*, 2003). An earlier study revealed that in chronically stressed lame cows low P4 exposure before oestrus was associated with a low intensity of sexual behaviour and shorter oestrous duration in dairy cattle (Dobson *et al.*, 2008).

5b.4.10. Oestrus characteristics during day and night time

Interestingly, regarding the timing of the peak of oestrous activity in the current study, the peak was evenly distributed between day and night. Similarly, Aungier *et al.* (2015) found no effect of time of day on the incidence of oestrus in dairy cows. However, Orihuela *et al.* (1983) found that a higher proportion of cows came into oestrus during night time and Dransfield *et al.* (1998) showed numerically more first standing events (28.4%) during the early morning compared to other periods of the day. However, other studies found that the onset of oestrus in dairy cattle mostly occurs during day time (Amyot and Hurnik, 1987; Gwazdauskas *et al.*, 1990). The numerically highest percentage of the peak of oestrus in the night are coherent with the daily pattern of the number of steps. Hurnik *et al.* (1975) found that the highest number of lactating cows in true oestrus is between 6 am, and 8 am, although this study shows that onset of oestrus occurred at a greater frequency from 6 am to 12 am, based on mounting behaviour.

The present study found that the duration of oestrus was not affected by time of day when the peak of oestrus occurred. This is in agreement with the reports of Esslemont and Bryant (1976) and Xu *et al.* (1998) who found no significant variation in the oestrus duration and oestrus activity during different times of the day in lactating dairy cows. However, other studies reported that more frequent oestrus activity occurred during the evening or hours of darkness (Orihuela *et al.*, 1983; Mattoni *et al.*, 1988). Some variation in the incidence, activity and duration of oestrus during a different time of the day may be associated with differences in the management system such as feeding, muck/slurry and milking time, or due to different environmental conditions (Nebel *et al.*, 2000).

The results of the present study found no difference in duration of oestrus at day or night time. However, the oestrous duration in the current study was longer than that found by Sveberg *et al.* (2011) (7.1 h) in dairy cows. In contrast, the oestrous duration of the present study was shorter than that reported by Silper *et al.* (2015) who found that oestrus duration was 14.3 ± 4.1 h in dairy cattle. Differences in oestrous duration in dairy cows based on observation (Amyot and Hurnik, 1987) and activity monitoring (in the present study and Aungier *et al.*, 2015) may be due to the disconnection of the secondary signs of oestrus measured by activity monitors and standing oestrus (Valenza *et al.*, 2012). Indeed, it has been reported that secondary signs of oestrus such as an increase in restlessness in dairy cows were significantly increased 1 to 3 h before the starting of standing oestrus during both day and night time (Sveberg *et al.*, 2011).

The result of the current study found that peak of oestrus activity was not affected by the day time and the night time. Similarly, Sankar and Archunan (2012) reported that oestrous activity was not influenced by the time of the day and also found that mounting activity

was distributed evenly across 24 h of the day in dairy cattle. However, Esslemont and Bryant (1976) found that greater oestrous activity, such as mounting activity happened during the night time compared to daytime in dairy cows. In contrast, De Silva *et al.* (1981) reported that cows that displayed oestrus in morning hours show more oestrous activity (11.4 mounts/h) compared to the evening time (7.6 mounts/h). Environmental factors such as weather (De Rensis and Scaramuzzi, 2003), day length (Phillips and Schofield, 1990), ambient temperature (Wilson *et al.*, 1998) and photoperiod (Garcia *et al.*, 2002) can influence the oestrous duration and intensity in dairy cattle.

5b.5. Conclusion

This study revealed that the duration and intensity of oestrus increases with days PP. The intensity of oestrus is greater, and oestrus lasts longer with increasing number of cows in oestrus simultaneously. Cows which were BCS 2.75 during oestrus spent more time active with greater intensity and longer oestrous duration on the day of oestrus. In addition, the duration of oestrus was also longer when cows had parity ≤ 2 with greater intensity on the day of oestrus. Oestrous duration was longer, and oestrous intensity was higher in LS1 cows. A cow in oestrus during summer had a longer oestrus with greater intensity compared to the other seasons. However, there was no effect of the time of day on the duration or intensity of oestrus.

CHAPTER 6

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

6.1. Introduction

Poor oestrous expression results in considerable economic losses to the dairy industry as a consequence of reduced reproductive performance in herds where artificial insemination is used (Lopez *et al.*, 2002; Yániz *et al.*, 2008). The primary behavioural sign of oestrus is that cows stand to be mounted (Humik *et al.*, 1975; Gordon, 2011). However, several studies have shown that 40-50% of oestruses in dairy cows are silent and cannot be detected by visual observation or automated methods of oestrous detection (Palmer *et al.*, 2010; Zebari *et al.*, 2018; Chapter 4) and other studies have shown that 35-40% of oestruses in cows are silent and cannot be detected by a teaser bull (Holmann *et al.*, 1987).

Oestradiol plays a key role in the regulation of the female reproductive system and particularly in oestrous activity (Dobson, 1978; Mondal *et al.*, 2006). During the follicular phase of the dairy cow's oestrous cycle, developing follicles secrete E2 at increasing concentrations from the end of the luteal phase following a fall in concentrations of P4 (Domènech *et al.*, 2011). Oestradiol induces oestrus through its influence on the hypothalamus (Allrich, 1994). In addition, E2 induces the release of GnRH by the hypothalamus that causes the LH surge which results in ovulation (Kaneko *et al.*, 1991; Gazal *et al.*, 1998). There is a positive correlation between the concentrations of E2 in blood plasma and milk (Gorecki *et al.*, 2004; Walker *et al.*, 2010). In general, it has been reported that most silent oestrous events occur due to a lack of exposure to P4 before a rise in E2 concentration in the blood (Allrich, 1994). After a certain threshold of E2 is reached there will be an LH surge, which usually results in ovulation (Dieleman *et al.*, 1986; Mondal *et al.*, 2006). In dairy cattle, the determination of P4 concentration alone in milk is not enough to accurately predict the time of oestrus because of a considerable variation in the time from the fall in P4 concentration to ovulation (Roelofs *et al.*, 2006). Therefore, the determination of the peak of E2 may indicate more accurately the time of pre-ovulation and could help achieve a high fertilization rate when AI is applied in dairy cows (Cavalieri *et al.*, 1997).

Oestrous expression varies greatly between cows (Chapter 4). In general, it has been reported that most silent oestrus events occur due to a lack of exposure to P4 before a rise in E2 concentration in the blood (Allrich, 1994). For example, using the oestrous behaviour scoring system developed by Van Eerdenburg *et al.* (2002), Zebari *et al.* (2018) reported that the number of points scored during the oestrus period ranged between 0 and 2921 points. Although many factors have been highlighted as being involved with the extent of oestrous expression (see Chapter 5), mechanisms responsible for the variation in the expression of oestrus in dairy cows undergoing spontaneous oestrus remain to be

fully elucidated. However, the variation that exists between cows during oestrus is thought to be induced by E2 (Dieleman *et al.*, 1986; Lyimo *et al.*, 2000). In support of this, frequent measurements of blood E2 concentration in relation to the behavioural signs of oestrus (Lyimo *et al.*, 2000; Roelofs *et al.*, 2004) have shown a positive dose-dependent relationship between oestrous intensity and maximum plasma E2 concentrations.

Changes in milk E2 concentrations during the oestrous period have also been reported (Madureira *et al.* (2015). However, there is a paucity of published information on the relationship between oestrous activity and the concentrations of milk E2 in dairy cows.

Therefore, the objective of this study was to:

- 1- Determine the efficacy of measuring milk E2 concentration for detecting oestrus in lactating Holstein Friesian cows undergoing a spontaneous oestrous cycle.
- 2- Investigate the relationship between the number of steps taken during oestrus and the concentration of milk E2 in lactating Holstein Friesian cows undergoing a spontaneous oestrous cycle.

6.2. 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.

6.2.1. Ethical considerations

See section 3.2.

6.2.2. Experimental animals, housing and management

Thirty-nine multiparous (parity 2.9 ± 0.4 mean \pm SD; range 2 to 4) Holstein Friesian cows 71.1 ± 12.7 days PP were used for the study (see section 3.1). Cows had previously been submitted for an ultrasound assessment of their reproductive tract by the herd veterinarian approximately 35 days post partum. The average body condition scores (BCS; Scale 1-5; AHDB Dairy, 2014) and locomotion scores (LS; Scale 1-5; as described by Chapinal *et al.*, 2009) of the selected cows were 2.8 ± 0.3 (range 2.5 to 3.0) and 2.1 ± 0.4 (range 1 to 3), respectively (see section 3.6.1). Cows were milked twice a day at 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 was 34.2 ± 6.2 kg/d (see section 3.1).

Cows were housed within the main herd of approximately 200 in a free stall house (stocking density was maintained at approximately 105 cubicles per 100 cows; cubicles 2.7x1.2 m) with sawdust-covered, 3 cm rubber mattresses and grooved concrete passageways. Three times per week the cubicles were bedded with sawdust and lime and passageways were scraped by automatic scrapers 4-5 times per day. Cows were fed a total mixed ration (TMR) *ad libitum* (see Table 6.1) which was provided daily at approximately 08:30 h. The nutrient supply of the rations is shown in Table 6.2. Water was also provided *ad libitum* from water troughs at the end of each passageway.

6.2.3. Milk samples

Milk samples (80 mL) were collected from each cow daily at the afternoon milking according to Walker *et al.* (2008). Milk samples were collected from 7 days before the expected day of oestrus and continued until 4 days after the day identified as the day of oestrus by both the GEA Rescounter II and IceQubes (IceRobotics Ltd., Edinburgh, UK). The milk samples were stored in a freezer at approximately -20 °C until assayed for E2 and P4 concentrations. The daily milk samples were analysed for the period from 4 days before, the day of oestrus and 3 days after the day of oestrus for E2 and P4 assay according to Walker *et al.* (2010) see section 3.4.1.

Table 6. 1. Dietary composition of the trial ration.

Ingredient	kg/head	kg DM/head
Maize silage	28.00	8.96
Lucerne	8.00	3.60
Chopped wheat straw	0.30	0.26
Protein blend	5.25	4.66
Sweet starch	2.00	1.76
Soya hulls	2.15	1.94
Spey syrup	1.50	0.63
Megalac	0.15	0.15
Butterfat extra	0.15	0.15
Dairy minerals	0.15	0.15
Acid buff	0.12	0.12
Salt	0.08	0.07
Saccharomyces cerevisiae	0.03	0.02
Water	5.00	0.00
Total	52.88	22.47

(Profeed Nutrition Consultancy, UK, 2018)

Table 6. 2. Predicted nutrient content of the ration. DM = dry matter, ME = metabolisable energy, CP = crude protein, NDF = neutral detergent fibre.

Nutrient Supplied	
DM (g/kg fresh)	425.0
ME (MJ/kgDM)	12.2
CP (%DM)	16.5
NDF (%DM)	36.4
Fat (%DM)	4.8
Starch and sugar (%DM)	18.7

(Profeed Nutrition Consultancy, UK, 2018)

6.2.3.1. Milk progesterone assay

Duplicate milk samples (10 μ L) were brought to room temperature (22 °C) and vortexed thoroughly to ensure homogeneity of the milk sample. Milk samples were analysed for P4 concentrations using a commercial 96 well microtitre plate-based, enzyme-linked immunosorbent assay (ELISA; Ridgeway Science Ltd., Gloucestershire, UK). The absorbance of each well was determined at 570 nm using a microplate reader (BMG LABTECH, Allmendgrün 8, 77799 Ortenberg, Germany). Progesterone concentrations (ng/ml) were estimated from the absorbances using a four parameter logistic standard curves generated by MARS data analysis software (Version: 2.30 R3, Omega, BMG LABTECH, 2007-2012) see section 3.4.1.1. The declared sensitivity of the assay was 0.15 ng/mL. The within and between assay coefficients of variation were 9.5% and 12.6%, respectively.

6.2.3.2. Milk oestradiol assay

Milk samples were brought to room temperature (22 °C) and mixed well to ensure the homogeneity of the sample. Duplicate milk samples (100 μ L) were analysed for E2 concentration using a commercially available plasma and serum E2 ultrasensitive 96 well microtitre plate-based, enzyme-linked immunosorbent assay (ELISA; Oestradiol Ultrasensitive; ALPCO, Salem, NH 03079, US; assay sensitivity <1.399 pg/mL). The microtiter plates were previously coated with polyclonal antibody. Milk E2 concentrations were determined according to the procedure provided by ALPCO with slight modification with an additional standard (1.5 pg/mL) prepared by mixing equal volumes of 0 pg/mL and 3 pg/mL standards. Microplates coated with polyclonal antibody and standards; 0, 1.5, 3, 10, 50 and 200 pg/mL were also brought to room temperature approximately 3 h prior to use. The absorbance of each well was determined at 450 nm using a microplate reader (BIOTEK Instrument Limited, Bedfordshire England, UK). The results were calculated automatically using a 4 parameter logistics curve fit by Gen5 All-In-One microplate reader software (Version: 2.01.14, 2006-2012 BioTek Instruments) see section 3.4.1.2. The within and between assay coefficients of variation were 4.3% and 8.1%, respectively.

2.6.3.3. Validation of E2 assay for milk sample

For milk E2 assay validation, 38 samples of milk (100 μ L) in duplicate were analysed for E2 concentration using a commercially available plasma and serum E2 ultrasensitive 96 well microtitre plate-based enzyme-linked immunosorbent assay (ELISA; Oestradiol Ultrasensitive; ALPCO, Salem, NH 03079, US). The assay sensitivity was <1.399 pg/mL). Milk E2 concentrations were determined according to the procedure provided by ALPCO with slight modification with an additional standard (1.5 pg/mL) prepared by mixing equal

volumes of 0 pg/mL and 3 pg/mL standards. The within and between assay coefficients of variation were 4.3% and 8.1%, respectively. The mean (\pm SEM) concentration of milk E2 in these samples was 8.1 ± 2.3 pg/mL. The range of milk E2 in these samples was 1.8 – 16.9 pg/mL. This was within the range 1.6 to 22.3 pg/mL and 2.1 ± 0.2 pg/mL and 20.9 ± 0.2 pg/mL found by (Lopez *et al.*, 2002) and Madureira *et al.* (2015), respectively.

6.2.4. Milk fat analyses

Milk samples were analysed to quantify total fat using a Milko-Scan Minor analyser (Foss, Denmark) calibrated according to AOAC (2012) for cow's milk. The samples were thawed by placement in a water bath at approximately 35 °C and shaken well to ensure that all of the milk contents were mixed well.

6.2.5. Blood samples

To determine the relationship between milk and blood E2 concentrations, milk samples were collected from 38 Holstein Friesian cows from 78 ± 16.1 days in milk, with a live weight of 664 ± 52.5 kg. The average body condition score (BCS; Scale 1-5; AHDB Dairy, 2014) and locomotion score (LS; Scale 1-5; as described by Chapinal *et al.*, 2009) of the selected cows were 2.9 ± 0.2 and 2.3 ± 0.4 , respectively. Cows being blood sampled as part of another study in compliance with the United Kingdom Animals (Scientific Procedures) Act 1986 (amended 2012) see (section 3.4.2).

6.2.5.1. Serum progesterone assay

Serum samples and standards (0, 1, 2, 5, 10 and 20 ng/mL) were removed from the freezer (-20 °C) and placed into an incubator to bring up to 22°C. Duplicate serum samples were analysed for P4 concentration using a commercial 96 well microtitre plate ELISA (Ridgeway Science Ltd., Gloucestershire, UK). The absorbance of each well was recorded by a microplate reader (BMG LABTECH, Allmendgrün 8, 77799 Ortenberg, Germany). Progesterone concentrations (ng/ml) were determined from the absorbance using a standard curve generated by MARS data analysis software (Version: 2.30 R3, Omega, BMG LABTECH, 2007-2012) see section 3.4.2.1. The sensitivity of the assay was 0.04 ng/mL. The within and between assay coefficient of variation were 6.6% and 12.4%, respectively.

6.2.5.2. Serum oestradiol assay

Serum samples (38 samples) were brought to room temperature (22 °C) approximately 3h before analysis. Serum E2 was determined in duplicate according to the procedure provided by ALPCO without modification using E2 Ultrasensitive ELISA microplate kit

coated with a polyclonal antibody (ALPCO, NH 03079, USA). The E2 standards were 0, 3, 10, 50 and 200 pg/mL (see section 3.4.2.2). The declared sensitivity of the assay was <1.399 pg/mL. The within and between assay coefficient of variation were 6.3% and 9.8%, respectively.

6.2.6. Correlation between concentrations of milk and serum oestradiol, and progesterone

The range of milk and serum E2 concentrations were 1.7 to 12.3 pg/mL and 6.4 to 25.8 pg/mL, respectively. Milk E2 concentrations were correlated ($P < 0.001$) with serum E2 (pg/mL). The following equation describes the relationship:

$$y = 0.19x - 6.55; r^2 = 0.55 \quad (n = 38)$$

The range of milk and serum P4 concentrations were 1.3 to 28.4 ng/mL and 2.6 to 38.8 ng/mL, respectively. Milk P4 concentrations were also correlated ($P < 0.001$) with serum P4 (ng/mL) concentrations. The following equation describes the relationship:

$$y = 1.11x + 0.69; r^2 = 0.63 \quad (n = 38)$$

6.2.7. Cow's activity

6.2.7.1. IceQube accelerometer

To monitor cow activity IceQubes (IceRobotics Ltd., Edinburgh, UK) were attached to the back left leg of each cow using a Velcro hook and loop strap (Dolecheck *et al.*, 2015) see section 3.5.1.

6.2.7.2. GEA pedometer

Cow activity was also measured using GEA pedometers (GEA Farm Technologies, Düsseldorf, Germany). This is the standard activity monitor used on the HAU dairy unit. All cows were equipped with a GEA Rescounter II pedometer on right front leg, according to the manufacturer recommendations (see section 3.5.2).

6.2.8. Definitions

6.2.8.1. Definition of oestrus based on milk progesterone profile

A cow was considered in oestrus when milk P4 concentrations were <3 ng/mL for two to three days before a period when milk P4 rose to >5 ng/mL for at least 5 days (Isobe *et al.*, 2004) see section 3.6.2.

6.2.8.2. Definition of behavioural and silent oestrus based on activity

Each oestrus identified by the milk P4 profile was classified as behavioural or silent oestrus (see section 3.6.3).

6.2.8.3. Duration of oestrus according to IceQube and GEA

The duration of oestrus is defined according to an increase in walking activity and the number of steps to >80% of the mean level for the preceding 3 days (López-Gatius *et al.*, 2008; see section 3.6.4).

6.2.9. Artificial insemination and pregnancy diagnosis

All cows ($n = 28$) which showed behavioural oestrus were artificially inseminated 12h after oestrous detection. Cows that did not return to oestrus within 30 days of insemination ($n = 26$; 92.9%) 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). Cows were designated pregnant (PD+) or non-pregnant (PD-). Cows that had returned to oestrus 18 to 30 days after AI were also considered to be non-pregnant ($n = 2$; 7.1%). Overall, of the 28 cows, 57.1% ($n = 16$) were diagnosed PD+ and 42.9% ($n = 12$) were diagnosed PD- (see section 3.7). There was no difference in pregnancy rate following insemination with the different bulls.

6.2.10. Data-set construction

During the study, 39 cows were detected in their second or third oestrus PP by milk P4 profile. Data from all 39 oestrous events were collected during the study period. The five parameters analysed were the number of steps taken by cows on each day (from IceQube), activity (GEA in AU), milk E2 concentrations (pg/mL), milk P4 concentrations (ng/mL), and oestrous duration per hours. Prior to the statistical analysis, data were summarised to one value per day using Microsoft Excel. A day was defined as the period from midnight to midnight. The day of oestrus was defined as day (0) and compared with four days before (-4, -3, -2 and -1; 4DB) and three days after (+1, +2 and +3; 3DA).

6.2.11. Statistical analysis

Statistical analyses were performed using the Genstat statistical software package (Genstat 18th edition, 18.1.14713, VSN International Ltd, UK). All of the data sets analysed were normally distributed. The datasets (number of steps recorded by IceQubes, oestrous activity measured by GEA Rescounter II and milk yield) were analysed using 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. Factorial one way ANOVA was used to compare behavioural and silent oestrus on the day of oestrus. Paired t-tests were used to compare between the days before, the day of oestrus and the days after oestrus.

Simple linear regression was used to compare the relationship between milk E2 (pg/mL) and milk fat content (g/L). The relationship between oestrous expression (response variable; behavioural = 1, silent = 0) and the explanatory variables including steps, E2 concentrations, milk yield, LS, DPP, parity, BCS and P4 concentrations were compared by logistic regression analysis. Additionally, the influence of other explanatory variables including steps, LS, parity and BCS 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. Simple linear regression and multiple linear regression were used to compare the relationship between the number of steps taken on the day of oestrus and milk E2, steps/d, DPP, LS at oestrus, milk yield kg/d, parity, BCS at oestrus and milk P4. Simple linear regression and multiple linear regression were also used to compare the relationship between oestrous duration/h and milk E2, steps/d, DPP, LS at oestrus, milk yield kg/d, parity, BCS at oestrus and milk P4. Relationship between pregnancy outcome (response variable; pregnant=1, non-pregnant = 0) and the explanatory variables including E2 concentrations, the number of steps, duration of oestrus/h and parity were also compared by logistic regression analysis. Additionally, the influence of other explanatory variables including P4 on day 0, P4 on day +3, milk yield, oestrous duration, duration of oestrus cycles, bulls, LS, BCS and days in milk (DIM) 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. Logarithmic regression analysis was also used to determine the relationship between the duration of behavioural oestrus and milk E2 pg/mL concentration. Differences were reported as significant at $P < 0.05$ and tendencies were reported when P was between 0.1 and >0.05 .

6.3. Results

6.3.1. Behavioural versus silent oestrus

6.3.1.1. Oestrous activity

Of the 39 oestruses detected by milk P4 profile, the percentage of behavioural and silent oestrus were 71.8% and 28.2% respectively. The duration of oestrus recorded by IceQube accelerometer and GEA ResCounter II pedometer were 12.3 ± 0.6 h and 12.8 ± 0.7 h, respectively (Table 6.3). On the day of behavioural oestrus the number of steps (2831 ± 224.2 steps; mean \pm SEM) and GEA activity (1149 ± 23.8 arbitrary unit; AU) were higher ($P < 0.001$) compared to 4DB and 3DA, while on the day of silent oestrus the number of steps (1140 ± 157.1 steps) and GEA activity (407 ± 41.7 AU) were similar in comparison to 4DB (1006 ± 149.1 steps) and 3DA (972.6 ± 134.6 steps) oestrus. There was an interaction ($P < 0.001$) between oestrous expression and time with regards the number of steps.

6.3.1.2. Milk oestradiol and progesterone profile

Oestradiol concentration increased ($P < 0.001$) from 4DB oestrus (1.9 ± 0.5 pg/mL) to 8.2 ± 1.1 pg/mL (mean \pm SEM) on the day of behavioural oestrus subsequently fell ($P < 0.001$) to 2.6 ± 0.6 pg/mL 3DA oestrus (Figure 6.1). Regarding silent oestrus, the concentrations of E2 on the day of predicted silent oestrus (2.4 ± 0.3 pg/mL) were also increased ($P < 0.001$) compared to 4DB (1.4 ± 0.2 pg/mL) oestrus and 3DA (1.6 ± 0.2 pg/mL) oestrus. The concentrations of E2 in milk were higher ($P < 0.001$) on the day of behavioural oestrus compared to silent oestrus. In cows showing behavioural oestrus, there was no difference ($P = 0.125$) in the milk concentration of E2 on day -2 and day -1. Milk E2 levels increased ($P < 0.001$) between day -1 and day 0 (day of oestrus). Milk E2 levels subsequently fell to 4.9 ± 0.9 pg/mL on day +1 ($P = 0.028$) and 1.9 ± 0.5 pg/mL on day +2 ($P = 0.008$) after oestrus in comparison to the day of behavioural oestrus (day 0). In contrast during silent oestrus, E2 levels remained at basal levels during the four days before oestrus. On the day of silent oestrus, E2 was 1.6 fold higher than pro-oestrus, and E2 concentrations returned to basal levels one day after oestrus. There was also no interaction ($P = 0.020$) between oestrous expression and time with regards the concentration of E2.

Concerning milk P4 concentrations, they were high during the 4DB (9.2 ± 1.4 ng/mL) oestrus and remained elevated until one day before oestrus (Figure 6.2). The concentration of P4 fell ($P < 0.001$) on the day of behavioural oestrus (1.1 ± 0.3 ng/mL) compared with mean levels of 4DB (9.2 ± 1.4 ng/mL) and 3DA (9.2 ± 1.2 ng/mL) oestrus.

On the day of silent oestrus, the concentration of P4 (1.8 ± 1.4 ng/mL) was also reduced ($P < 0.001$) compared 4DB (6.6 ± 1.4 ng/mL) and 3DA (4.6 ± 0.9 ng/mL). There was an interaction ($P = 0.008$) between oestrous expression and time.

6.3.1.3. Milk yield

Regarding the effect of oestrous expression on milk production, milk yield was reduced ($P < 0.001$) on the day of behavioural oestrus (31.2 ± 1.4 kg/d) compared to 4DB (34.8 ± 1.4 kg/d) and 3DA (34.6 ± 1.4 kg/d) while on the day of silent oestrus milk yield (35.6 ± 1.5 kg/d) was similar ($P = 0.258$) in comparison to 4DB (36.3 ± 1.2 kg/d) and 3DA (35.8 ± 1.4 kg/d) (Table 6.3). When comparing behavioural and silent oestrus, no differences ($P = 0.120$) in milk yield were found.

On the day of behavioural oestrus, milk yields (kg/d) were negatively ($P < 0.001$) correlated with the duration of behavioural oestrus recorded by IceQube accelerometer ($y = -1.08x + 45.23$; $r^2 = 0.30$; Figure 6.3). There was also a negative ($P < 0.001$) correlation ($y = -0.75x + 37.51$; $r^2 = 0.41$) between milk yield (kg/d) and the concentration of E2 (pg/ml) in milk (Figure 6.4). There was no correlation ($P = 0.102$; $r^2 = 0.06$) between milk E2 (pg/mL) and milk fat content (g/L).

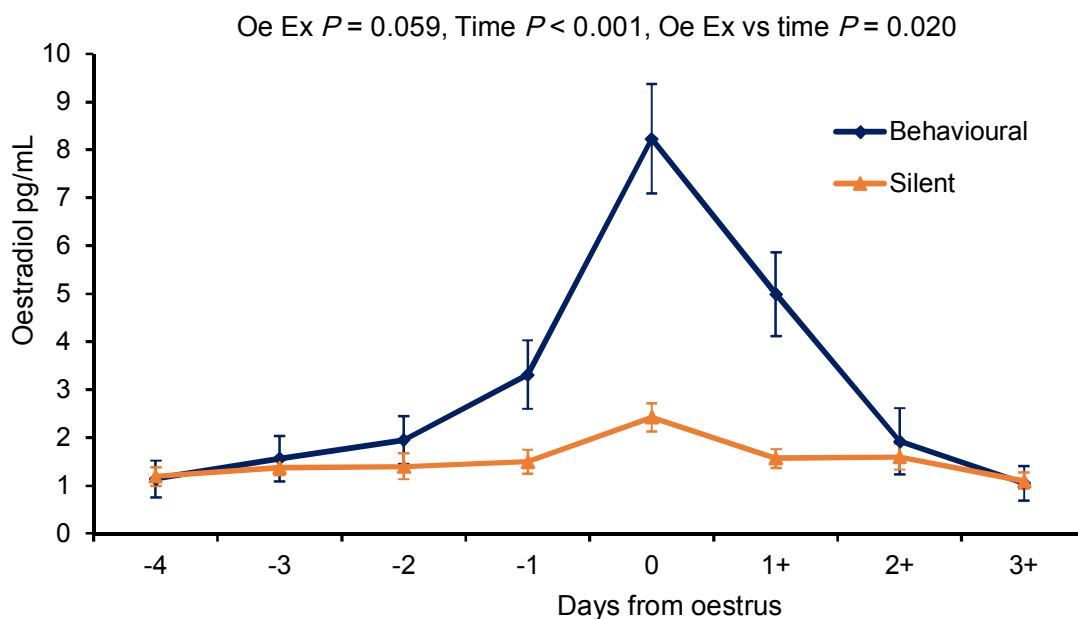


Figure 6. 1. Mean (\pm SEM) milk concentration of E2 ng/mL, 4 days before, on the day of oestrus (0) and 3 days after and during behavioural ($n = 28$) and silent ($n = 11$) oestrus in lactating Holstein Friesian dairy cows. Oe EX = Oestrus Expression, 0 = day of oestrus.

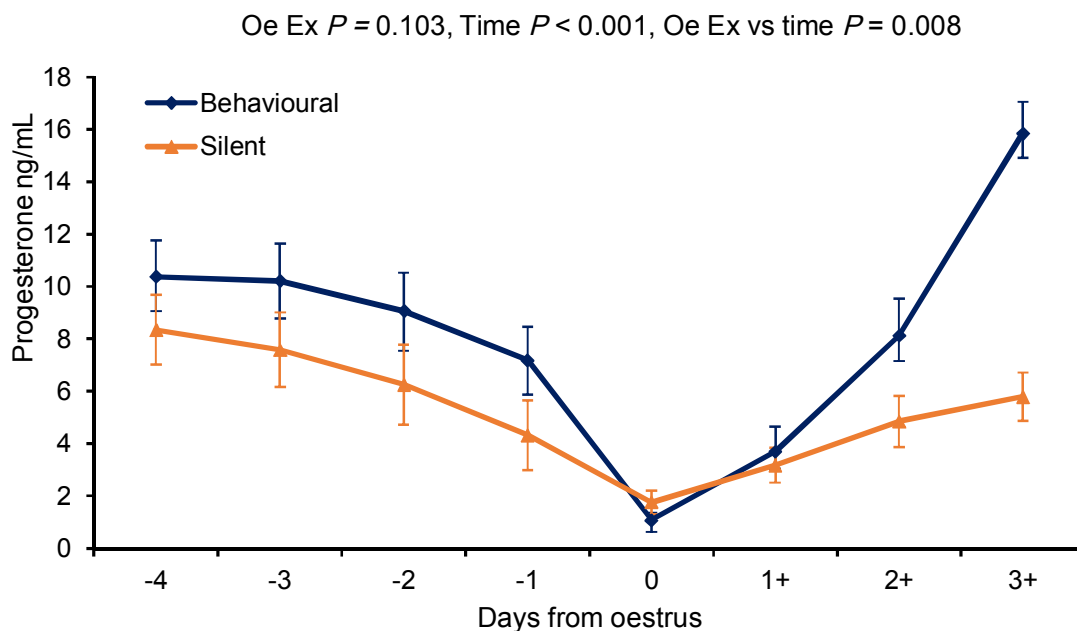


Figure 6. 2. Mean (\pm SEM) milk concentration of P4 ng/mL, 4 days before, on the day of oestrus (0) and 3 days after and during behavioural ($n = 28$) and silent ($n = 11$) oestrus in lactating Holstein Friesian dairy cows. Oe EX = Oestrus Expression, 0 = day of oestrus.

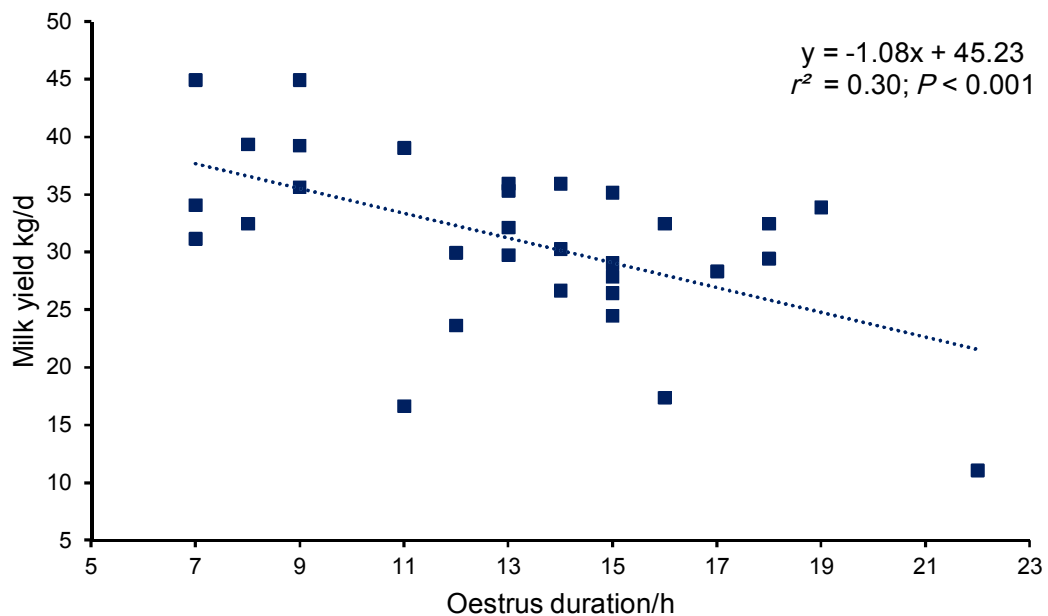


Figure 6. 3. The relationship between milk yield (kg/d) and the duration of behavioural oestrus recorded by IceQube accelerometer in lactating Holstein Friesian dairy cows.

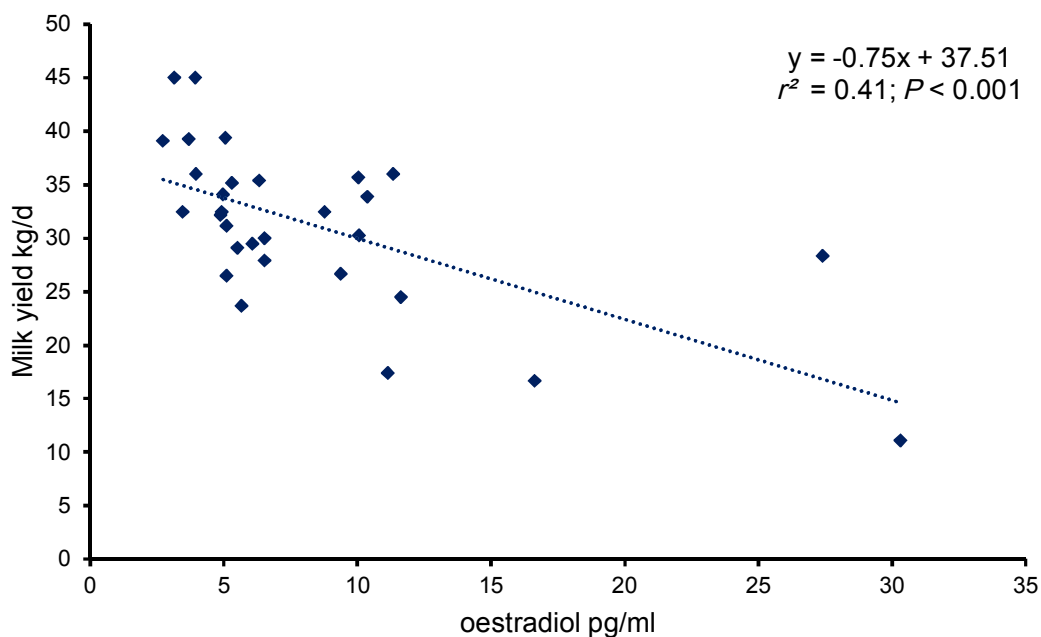


Figure 6. 4. The relationship between milk yield (kg/d) and the concentration of E2 (pg/mL) in milk on the day of oestrus in lactating Holstein Friesian dairy cows.

6.3.2. The relationship between milk oestradiol and progesterone concentrations and the number of steps taken during behavioural oestrus

Of the 28 behavioural oestruses, the concentrations of E2 in milk were increased by 313.6% and 210.2% in comparison to 4DB and 3DA oestrus, respectively. Similarly, with increasing the concentrations of E2, the number of steps were also increased by 168.9%, and 161.8% compared to 4DB and 3DA oestrus, respectively (Figure 6.5). There was a positive ($P < 0.001$) relationship between the concentrations of E2 (pg/mL) in milk on the day of oestrus and the number of steps taken ($y = 184.3x + 1187.9$; $r^2 = 0.73$) on the day of behavioural oestrus. In addition, there was a negative ($P = 0.003$) relationship between the concentrations of P4 (ng/mL) in milk on the day of oestrus and the number of steps taken during the day of behavioural oestrus ($y = -399.9x + 2974.8$; $r^2 = 0.41$).

There was a positive ($P = 0.03$) correlation between the concentrations of E2 (pg/mL) in milk on the day of oestrus and the number of steps taken during the day of silent oestrus ($y = 401.9x + 169.3$; $r^2 = 0.55$) and there was a negative ($P = 0.05$) relationship between the concentrations of P4 (ng/mL) in milk and the number of steps taken ($y = -243.3 + 1567.2$; $r^2 = 0.28$) on the day of silent oestrus. The number of steps increased with increased E2 concentrations and decreased P4 concentration (Figure 6.6).

Table 6. 3. Mean of the number of steps/d, GEA activity (AU) and milk yield (kg/d), from 4 days before, to 3 days after oestrus (day 0) and during behavioural ($n = 28$) or silent ($n = 11$) oestrus in lactating Holstein Friesian dairy cows.

Activity	Oe Ex	Days from oestrus								SED	P-value		
		-4	-3	-2	-1	0	1+	2+	3+		Oe Ex	Days	Oe Ex x time
Steps/d	B	999	1016	1015	1182	2831	1252	998	994	224.2	0.102	<0.001	<0.001
	S	999	985	1066	975	1140	988	940	990				
GEA activity/AU	B	369	365	369	406	1149	412	364	386	203.0	0.091	0.008	0.231
	S	303	314	320	327	407	325	302	314				
Milk yield (kg)	B	34.7	34.7	34.8	35.1	31.2	34.7	34.6	34.6	3.1	0.573	<0.001	0.451
	S	36.3	36.3	36.4	36.3	35.6	35.9	35.7	35.7				

Oe Ex = Oestrus Expression, B = Behavioural oestrus and S = Silent oestrus, 0 = day of oestrus, SED = standard errors of differences

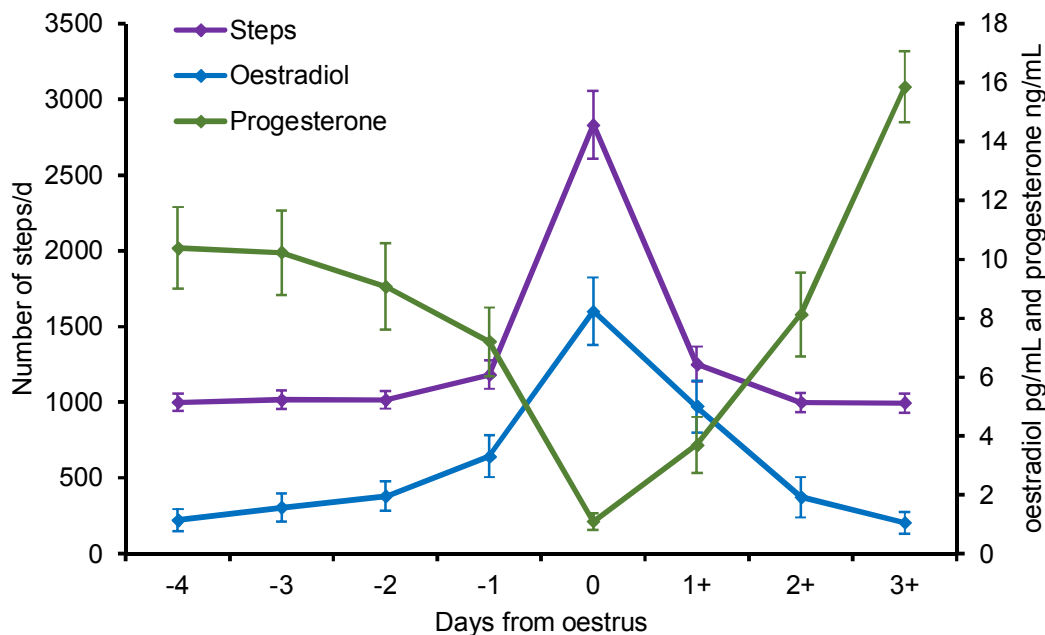


Figure 6. 5. Mean (\pm SEM) of the number of steps/d, milk concentration of E2 (pg/mL) and P4 (ng/mL), from 4 days before to 3 days after behavioural oestrus ($n = 28$) in lactating Holstein Friesian dairy cows. 0 = the predicted day of oestrus.

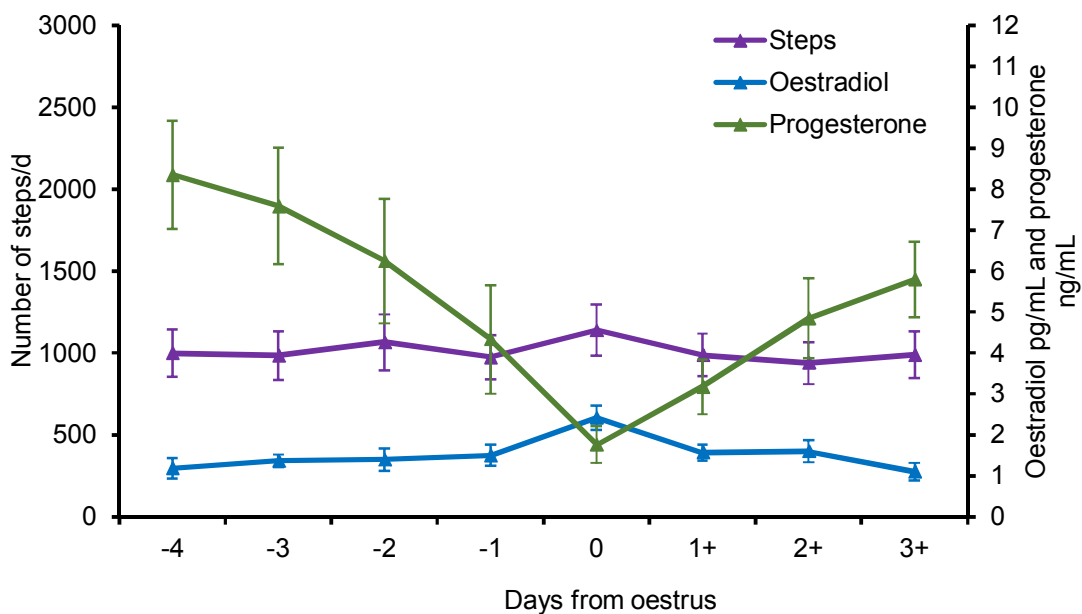


Figure 6. 6. Mean (\pm SEM) of the number of steps per day, milk concentration of E2 (pg/mL) and P4 (ng/mL), from 4 days before to 3 days after silent oestrus ($n = 11$) in lactating Holstein Friesian dairy cows. 0 = the predicted day of oestrus.

6.3.3. The relationship between explanatory variables and oestrous expression (behavioural versus silent oestrus)

Step-wise logistic regression analysis indicated that on the day of oestrus, the number of steps taken was positively ($P < 0.001$) associated with oestrous expression (Table 6.4). In addition, oestrous expression was also positively associated with milk E2 concentration ($P < 0.001$) and DPP ($P = 0.016$). However, oestrous expression was negatively associated with milk yield ($P < 0.001$), LS at oestrus ($P = 0.019$) and parity ($P = 0.017$). When milk P4 concentrations on the day of oestrus and BCS at oestrus ($P = 0.09$) were added to the model that already included steps as a constant, only the E2, milk yield, parity and LS at oestrus significantly reduced the deviance further.

The regression equation for this model is:

$$\text{Logit (p)} = -0.5 + 1.42 (\text{E2}) + 0.0007 (\text{steps}) - 0.123 (\text{milk yield}) - 0.33 (\text{parity}) - 0.27 (\text{LS}); P < 0.001$$

Table 6. 4. Regression coefficients, odds ratios (and s.e.) for the explanatory variables (plus constant) assessed by stepwise logistic regression analysis of behavioural and silent oestrus in lactating Holstein Friesian dairy cows.

Variable	Estimate	Odds ratios	s.e.	Constant	P-value
Steps/d	0.00014	1.001	0.0005	-1.796	<0.001
E2	2.015	7.504	0.771	-7.07	<0.001
Milk yield	-0.381	0.683	0.131	15.13	<0.001
LS at oestrus	-1.210	0.298	0.562	3.59	0.019
DPP	0.0493	1.05	0.023	-2.14	0.016
Parity	-0.520	0.594	0.227	2.838	0.017
BCS at oestrus	0.32	1.374	1.61	0.20	0.844
P4	-0.151	0.860	0.223	1.261	0.509

E2 = oestradiol, DPP = days postpartum, LS = locomotion score, BCS = body condition at oestrus, P4 = progesterone and s.e. = standard error of observation

6.3.4. The relationships between explanatory variables and the number of steps taken on the day of oestrus

A summary of the relationships between explanatory variables and the number of steps taken during the day of oestrus are presented in Table 6.5. The results show positive ($P < 0.001$) correlations between E2 and DPP and the number of steps on the day of oestrus. The results show negative ($P < 0.001$) correlations between LS at oestrus and milk yield (kg/d) and the number of steps. In addition, there was a negative ($P = 0.027$) correlation between parity and the number of steps per day. However, there was no linear relationship between BCS at oestrus ($P = 0.431$) and milk P4 ($P = 0.408$) and the number of steps taken on the day of oestrus.

Step-wise multiple regression analysis indicating factors affecting oestrous activity that minimised the residual mean square of the number of steps taken are shown in Table 6.6. Milk E2 was chosen as a first independent variable to add to the model as it had the lowest residual value in relation to the number of steps. For oestrous activity, the statistically significant ($P < 0.05$) explanatory variables were milk E2, DPP, LS at oestrus, milk yield and parity ($\leq 2, 3$ and ≥ 4). The addition of other explanatory variables did not reduce ($P > 0.05$) the residual mean squares in the dependent variables further.

Table 6. 5. The relationships between factors and the number of steps taken during the day of oestrus using IceQube in lactating Holstein Friesian dairy cows.

Factors	Regression	Constant	SE	P-value	r^2
Milk E2	169	1295	828	<0.001	0.61
DPP	49.49	971	859	<0.001	0.58
LS at oestrus	-1152	4758	984	<0.001	0.45
Milk yield kg/d	-93.5	5762	1197	0.004	0.12
Parity $\leq 2,3$ and ≥ 4	-272	3320	1252	0.027	0.13
BCS at oestrus	749	435	1327	0.431	0.01
Milk P4	-118	2629	1326	0.408	0.01

E2 = oestradiol, DPP = days post-partum, LS = locomotion score, BCS = body condition score, P4 = progesterone and SE = standard error of observation.

Table 6. 6. Relationships between the number of steps on the day of oestrus and milk E2, DPP, LS at oestrus, milk yield (kg/d) and parity (≤ 2 , 3 and ≥ 4) in lactating Holstein Friesian dairy cows.

Response variables	Explanatory variables						r^2	SE	P-value
	Constant	Milk E2	DPP	LS at oestrus	Milk yield kg/d	Parity			
Steps	1295 (SE=203)	169 (SE=21.9)					0.61	828	<0.001
Steps	245 (SE=443)	106.4 (SE=25)	28.4 (SE=7.5)				0.71	711	<0.001
Steps	1001 (SE=732)	90 (SE=25.2)	23.1 (SE=7.6)	-386 (SE=40.3)			0.74	680	<0.001
Steps	1639 (SE=1209)	89 (SE=25.5)	20.99 (SE=8.3)	-389 (SE=186)	-13.6 (SE=20.4)		0.75	670	<0.001
Steps	1594 (SE=1307)	89.2 (SE=25.9)	21.2 (SE=8.7)	-391 (SE=190)	-13.4 (SE=20.1)	-7.4 (SE=18.7)	0.76	665	<0.001

SE = Standard error of observation, E2 = oestradiol, DPP = day post-partum and LS = locomotion score

6.3.5. The relationships between explanatory variables and oestrous duration/h

A summary of the relationships between explanatory variables and oestrous duration/h are presented in Table 6.7. The results show positive ($P < 0.001$) correlations between milk E2 concentrations, number of steps taken on the day of oestrus and DPP and oestrous duration/h. The results show negative ($P < 0.001$) correlations between LS at oestrus, milk yield (kg/d) and parity and oestrous duration. However, there was no linear relationship between BCS at oestrus ($P = 0.197$) and milk P4 concentration ($P = 0.478$) and oestrous duration in dairy cows. The results show a high multi-collinearity between parity and age ($r^2 = 0.97$), therefore cows age was excluded from the multiple linear regression.

Step-wise multiple regression analysis indicated that on the day of oestrus, many factors affecting oestrous duration that minimised the residual mean square of the model including the constant and the duration/h of behavioural oestrus (Table 6.8). Milk E2 concentrations was chosen as a first independent variable add to the model as it has the lowest residual value in relation to the oestrus duration. For oestrous duration, the significant ($P < 0.05$) explanatory variables were milk E2, DPP, parity ($\leq 2, 3$ and ≥ 4) LS at oestrus and milk yield. The addition of other explanatory variables (BCS at oestrus and milk P4) did not reduce ($P > 0.05$) the residual mean squares further.

Table 6. 7. The correlations between explanatory variables and the duration of oestrus measured using IceQube in lactating Holstein Friesian dairy cows.

Factors	Regression	Constant	SE	P-value	r²
Milk E2	0.48	8.88	2.18	<0.001	0.67
Steps/d	0.002	6.75	2.52	<0.001	0.54
DPP	0.15	2.97	1.99	<0.001	0.68
LS at oestrus	-3.88	20.52	1.97	<0.001	0.61
Milk yield kg/d	-0.41	1.57	3.01	<0.001	0.35
Parity ≤2, 3 and ≥4	-3.64	23.11	2.10	<0.001	0.67
BCS at oestrus	4.99	0.7	3.66	0.197	0.02
Milk P4	0.31	12.66	3.74	0.478	0.01

E2 = oestradiol, DPP = days post-partum, LS = locomotion score, BCS = body condition score, P4 = progesterone and SE = standard error of observation.

Table 6. 8. Relationships between oestrous duration (h) and milk E2, DPP, parity (≤ 2 , 3 and ≥ 4), LS at oestrus and milk yield (kg/d) in lactating Holstein Friesian dairy cows.

Response variables	Explanatory variables						r^2	SE	P-value
	Constant	Milk E2	DPP	Parity	LS at oestrus	Milk yield kg/d			
Duration/h	8.88 (SE=0.67)	0.48 (SE=0.06)					0.65	2.18	<0.001
Duration/h	4.13 (SE=0.95)	0.27 (SE=0.06)	0.096 (SE=0.02)				0.83	1.21	<0.001
Duration/h	11.81 (SE=1.50)	0.22 (SE=25.2)	0.04 (SE=7.6)	-1.73 (SE=0.31)			0.92	1.04	<0.001
Duration/h	12.46 (SE=1.49)	0.21 (SE=0.04)	0.06 (SE=0.01)	-1.21 (SE=0.12)	-0.84 (SE=0.46)		0.93	1.00	<0.001
Duration/h	10.96 (SE=2.02)	0.19 (SE=0.04)	0.05 (SE=0.01)	-1.08 (SE=0.43)	-0.95 (SE=0.47)	-0.05 (SE=0.04)	0.94	0.99	<0.001

SE = Standard error of observation, E2 = oestradiol, DPP = day post-partum and LS = locomotion score

6.3.6. PD+ versus PD-

Of the 28 behavioural oestruses which were followed by AI, 57.1% were diagnosed pregnant (PD+) and 42.9% were diagnosed non-pregnant (PD-). Step-wise logistic regression analysis indicated that on the day of behavioural oestrus, both E2 concentrations and the number of steps taken on the day of behavioural oestrus were positively ($P < 0.001$) associated with the pregnancy outcome following AI (Table 6.7). In addition, pregnancy outcome following AI was also negatively ($P = 0.018$) associated with milk P4 concentrations on the day of oestrus and positively ($P = 0.041$) associated with milk P4 concentrations on day +3. However, the pregnancy outcome following AI was negatively ($P = 0.004$) associated with parity. When milk P4 concentrations on the day of oestrus and day +3 and milk yield ($P = 0.09$) were added to the model that included a constant and E2, only the number of steps taken on the day of behavioural oestrus, oestrous duration and parity significantly reduced the deviance further.

The regression equation for this model is:

$$\text{Logit (p)} = -7.10 + 1.167 (\text{E2}) + 0.00126 (\text{Steps}) + 0.739 (\text{oestrous duration/h}) - 1.271 (\text{Parity}); P < 0.001$$

Table 6. 9. Regression coefficients (and s.e.) for the explanatory variables (plus constant) assessed by stepwise logistic regression analysis of pregnancy outcome following AI at behavioural oestrus in lactating Holstein Friesian dairy cows.

Variables	Estimate	Odd ratios	s.e.	Constant	P-value
Oestradiol pg/mL	1.288	3.624	0.541	-7.98	<0.001
Steps/d	0.0015	1.002	0.00056	-3.97	<0.001
Oestrous duration/h	0.307	1.357	0.145	-3.61	0.013
Parity	-1.326	0.265	0.504	4.04	0.004
Progesterone on day (0)	-1.085	0.338	0.898	1.092	0.018
Progesterone on day (+3)	0.1201	0.346	0.0630	-1.68	0.041
Milk yield kg/d	0.164	1.178	0.111	-5.02	0.094
Duration of oestrous cycle	0.214	1.238	0.202	-4.60	0.273
Bull 1	0	0.250	1.41	0	0.127
2	-1.39	2.500	1.80	0	0.442
3	0.92	1.002	1.64	0	0.577
4	9.2	1.016	43.3	0	0.831
5	-0.15	0.875	1.52	0	0.919
6	9.2	1.016	43.3	0	0.831
Locomotion score	0.773	2.167	1.016	-1.39	0.139
Body condition score	1.95	7.060	1.84	-5.19	0.278
Days in milk	0.0303	1.031	0.0302	-1.95	0.307

s.e. = standard error of observation

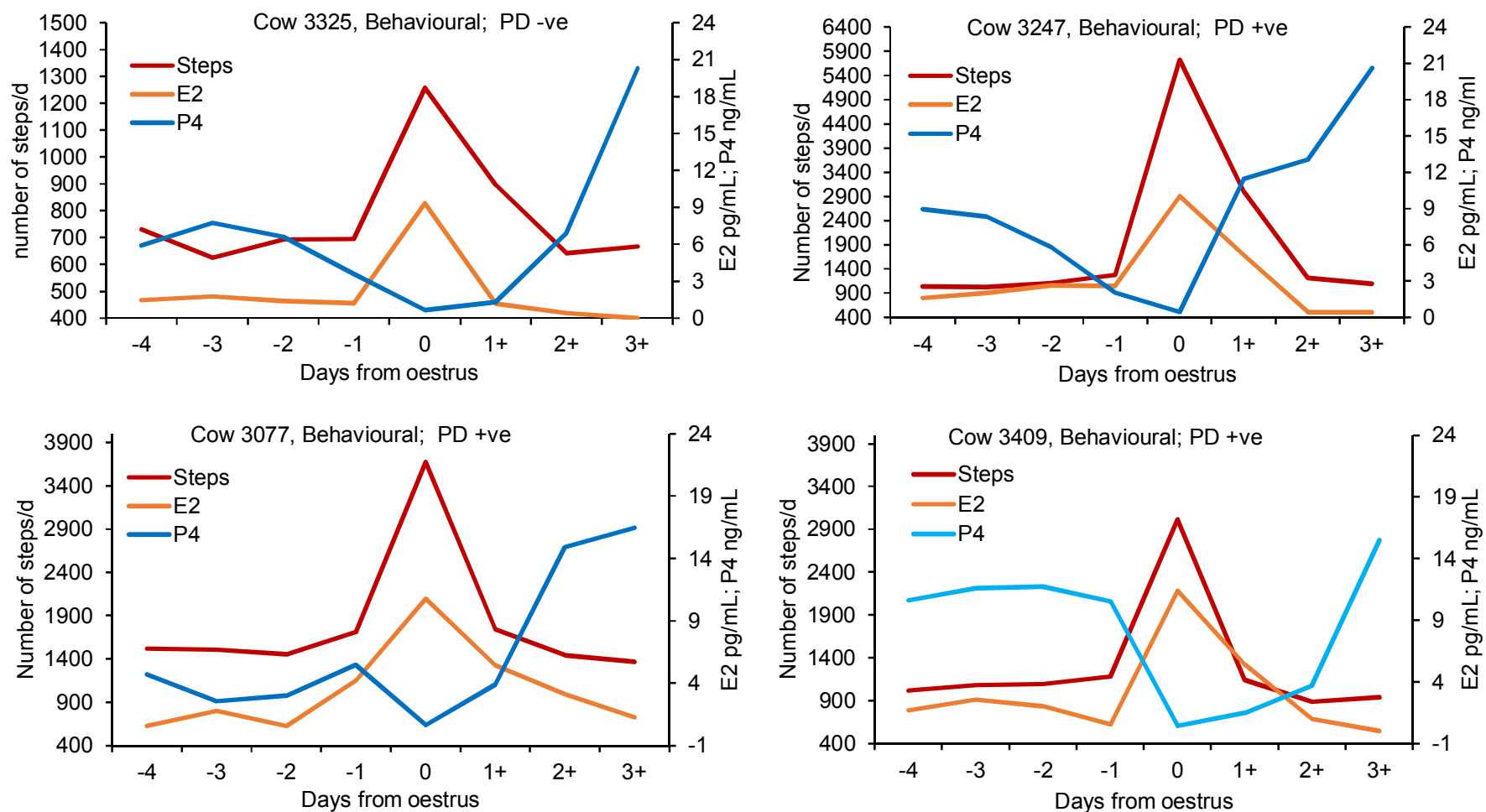


Figure 6. 7. Milk E2 (pg/mL), P4 (ng/mL) profile and the number of steps taken of *behavioural oestrus* (plotted against days, 4DB, day of oestrus (0) and 3DA oestrus in lactating Holstein Friesian dairy cows. Cows 3325 oestrus results in PD- and cows 3247, 3307 and 3409 oestrus results in PD+.

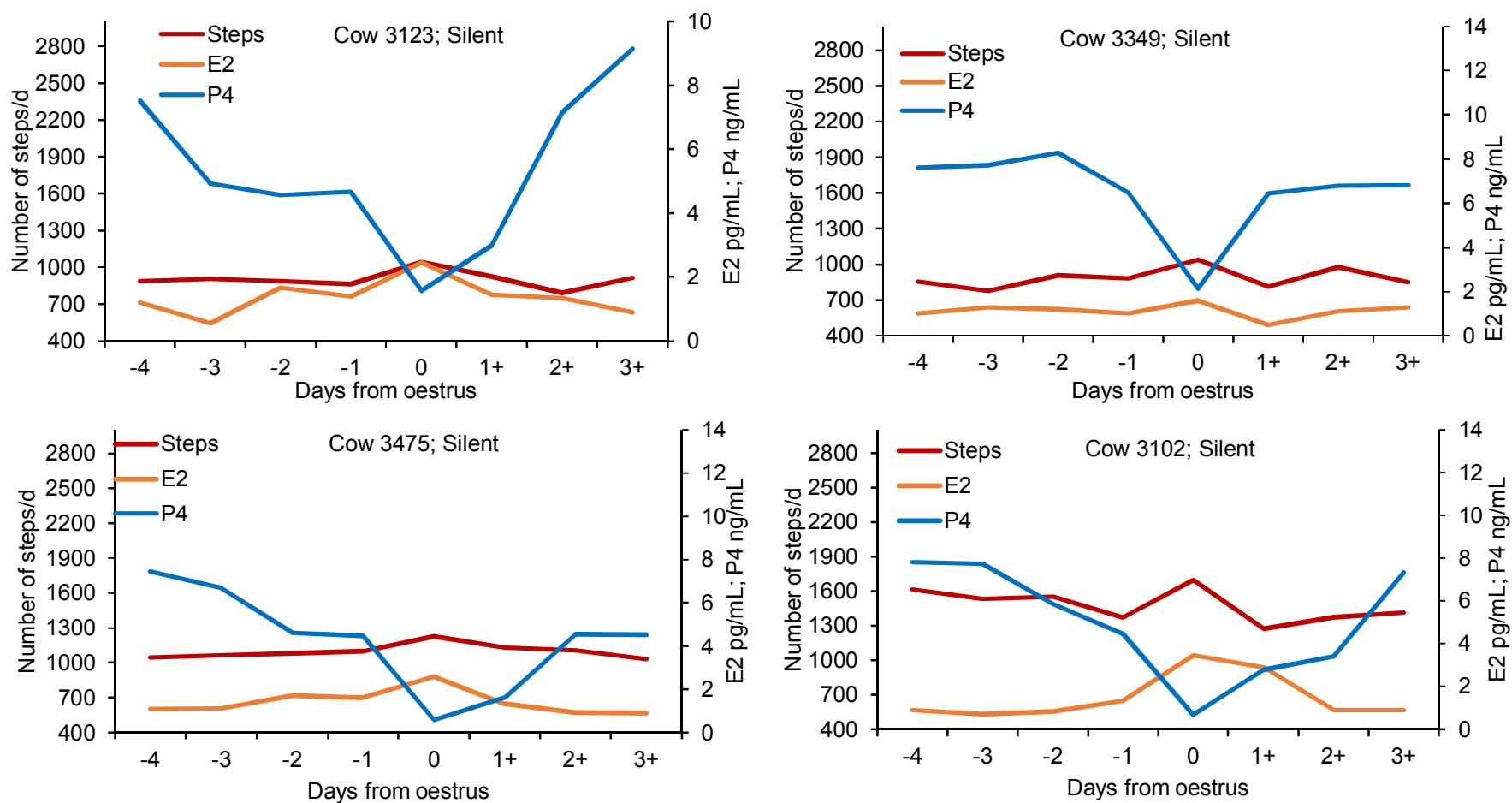


Figure 6. 8. Milk E2 (pg/mL), P4 (ng/mL) profile and the number of steps taken of *silent oestrus* plotted against days, 4DB, day of oestrus (0) and 3DA oestrus of lactating Holstein Friesian dairy cows 3123, 3319, 3475 and 3102.

6.3.7. The length of the oestrous cycle and duration of oestrus

The average length of oestrous cycles was 22.5 ± 1.9 days (range 19 – 28 days). The average duration of behavioural oestrus based on the number of steps recorded by IceQube accelerometer was 13.1 ± 0.7 h (range 7 - 22 h), and the duration of oestrus based on the activity recorded by GEA pedometer was 13.4 ± 0.6 h (range 7 - 23 h). The number of steps taken were correlated ($y = 280.5x - 833.2$; $r^2 = 0.75$; $P < 0.001$) with the oestrous duration. A positive correlation ($y = 1.26x + 15.30$; $r^2 = 0.42$; $P < 0.001$) existed between the length of the oestrous cycle and the duration of behavioural oestrus recorded by the IceQube accelerometers (Figure 6.9).

The concentrations of E2 in milk were positively ($P < 0.001$) correlated with the duration of behavioural oestrus recorded by IceQube accelerometer ($y = 5.39 \ln(x) + 2.73$; $r^2 = 0.67$; Figure 6.10).

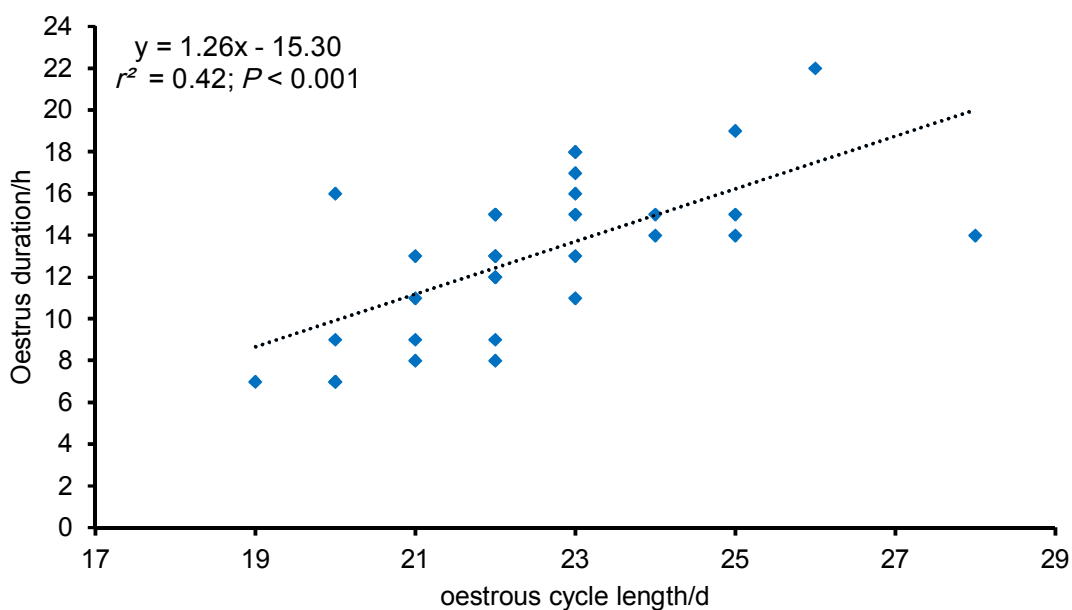


Figure 6. 9. The relationship between the length of the oestrous cycle (days) and the duration of behavioural oestrus (h) recorded by IceQube accelerometers in lactating Holstein Friesian dairy cows.

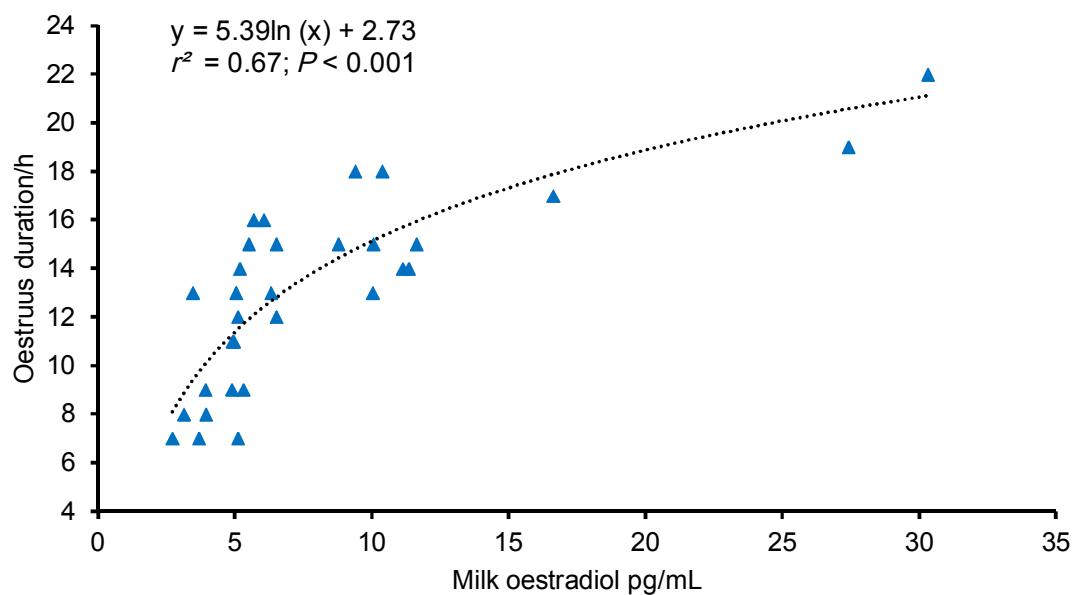


Figure 6. 10. The relationship between the concentration of milk E2 (pg/mL) and the duration of behavioural oestrus (h) recorded by IceQube accelerometers in lactating Holstein Friesian dairy cows.

6.4. Discussion

6.4.1. Behavioural versus silent oestrus

6.4.1.1. Number of steps and GEA activity

From 39 oestrus events identified from milk P4 profiles during this study, 71.8% were associated with behavioural oestrus detected by behavioural activity monitors. This was within the range 51% to 87% found by (Roelofs *et al.*, 2005) using pedometers for oestrous detection. Furthermore, Fricke *et al.* (2014) reported similar findings of 70% vs 30% of behavioural and silent oestrus in oestrus-synchronised Holstein cows using neck mounted activity monitors (Heatime, SCR Engineer Ltd, Netanya, Israel). However, Ranasinghe *et al.* (2010) reported 76.2% and 78.7% behavioural oestruses at second and third oestrus identified by milk P4 in a commercial Holstein Friesian dairy herd in Japan. Firk *et al.* (2002) reported much higher rates of behavioural oestrous detection (91% and 95%) at second and third oestrus using pedometers. In dairy cows, the incidence of silent oestrus is thought to depend on refractoriness to E2 (Allrich, 1994). In a previous study Chapter 4 (Zebari *et al.*, 2018) showed reduced incidence of silent oestrus with increasing post partum interval which may be due to a return to positive energy balance. In the current study, the cows were over 70 days PP at the start of the study so the lower percentage of silent oestruses in the current study may be due to a return to positive energy balance. Luteinizing hormone pulse frequency also increases during the PP period, this will stimulate a higher production of E2 by the preovulatory follicle (Isobe *et al.*, 2004). This is confirmed by the finding of a positive correlation between the number of steps taken on the day of oestrus and milk E2 in the present study.

On the day of behavioural oestrus, more steps and GEA activity were recorded compared to 4DB and 3DA, while no increase was found during silent oestrus. Roelofs *et al.* (2005) recorded a lower number of 2080 steps on the day of oestrus in Holstein Friesian cows housed in free-stalls using pedometers (Nedap Agri B. V., Groenlo, Netherlands). This result also was higher than the finding by Sakaguchi *et al.* (2007) of around 950 steps during oestrus in dairy Holstein heifers kept in a paddock in Japan. Dairy cow activity during oestrus may be affected by environmental conditions, type of housing and management conditions (Lopez-Gatius *et al.*, 2005; Yániz *et al.*, 2006) as well as to the accuracy of the device (Hockey *et al.*, 2010). The increase in the number of steps per day 1DB and on the day of oestrus mirrors the increase in milk E2 concentrations.

6.4.1.2. Milk E2 and P4 profile

In the current study, an increase in the concentration of E2 in milk was recorded on both the day of behavioural and silent oestrus. Similarly, Domènech *et al.* (2011) reported that prior to ovulation the concentration of E2 in milk was increased in dairy cattle. The result of the current study agrees with those of Madureira *et al.* (2015) who found plasma E2 concentration to be higher (8.9 ± 0.2 pg/mL) in cows that show greater activity compared to cows that show lower activity (8.1 ± 0.2 pg/mL) on the day of oestrus in Holstein cow. The milk E2 concentrations reported in the current study on the day of behavioural oestrus were higher than those reported by Meisterling and Dailey (1987) and Gyawu and Pope (1990) in defatted milk (4.5 and 5.9 pg/mL, respectively). This difference may be partly explained by the partitioning of the steroid E2 to the fat fraction of the milk since Abeyawardene *et al.* (1984) reported that 52% of the E2 content in milk is distributed in the lipid fraction. Thus full-fatted milk has an E2 concentration twice that of defatted milk. Another study conducted on 23 Holstein cows by Lopez *et al.* (2002) used a radio-immunoassay for determination of the E2 concentrations in milk and plasma and also found lower concentrations of E2 (5 pg/mL) in milk compared to plasma. However, this finding was lower than those reported by Gorecki *et al.* (2004) of 14.2 pg/mL of E2 in dairy goat whole milk. Abeyawardene *et al.* (1984) reported that milk lipid content is an important factor determining the E2 concentration in milk because approximately 52% of the E2 content in milk is distributed in the lipid. The results of the present study revealed the concentration of E2 in milk on the day of silent oestrus were lower compared to during behavioural oestrus. In lactating dairy cows, lower concentrations of E2 on the day of silent oestrus may be related to the amount of E2 secreted by the developing ovulatory follicle (Beam and Butler, 1999) since follicle size and diameter have been positively related to the peak concentration of E2 (Perry *et al.*, 2014).

As expected, the results of the current study found that the concentration of milk P4 was significantly reduced on both the day of behavioural and silent oestrus in comparison to 4DB and 3DA. Domènech *et al.* (2011) found that the concentration of P4 in milk was reduced in non-pregnant dairy cows. The reduction of P4 in the milk of the present study was similar to those reported by Meisterling and Dailey (1987) who found that P4 in milk decreases around 89% during visually observed oestrus and 86% during non-visually observed oestrus in dairy cattle. Firk *et al.* (2002) also showed that the P4 profile in milk was dropped by approximately 30% on the day of oestrus. Furthermore, Kerbrat and Disenhaus (2004) found a reduction in P4 of approximately 80% in Holstein cows. In addition, Walker *et al.* (2008) found a significant reduction in milk P4 concentration on the day of oestrus in non-lame, moderately lame and severely lame Holstein Friesian cows on the day of observed oestrus to 12-3 days before and 6 days after oestrus. Desaulniers *et*

al. (1989) reported that the concentrations of milk P4 was 1.2 ng/mL around the time of oestrus in Holstein heifers. Isobe *et al.* (2004) also found that milk P4 concentration dropped to <5 ng/mL during silent oestrus in free-stall housed HolsteinFriesian cows. In another study conducted by Roelofs *et al.* (2006) found also a lower in P4 concentration in milk of <2 ng/mL in Holstein Frisian dairy cows. Cerri *et al.* (2011) also reported the low concentration of P4 of approximately 1.7 ± 0.1 ng/mL in lactating Holstein cow. In the lactating dairy cow, the blood concentration of P4 was decreased around the time of oestrus and ovulation as a result of a pattern of follicular wave development (Wiltbank *et al.*, 2006) and the release of PGF₂ α from endometrial tissue (Shaham-Albalancy *et al.*, 2001).

6.4.1.3. Milk yield

On the day of behavioural oestrus, milk production was significantly reduced by 11.5% and 10.9% in comparison to 4DB and 3DA respectively, while no reduction was found during silent oestrus. Similarly, previous studies reported that milk production was reduced by approximately 9% to 10% on the day of behavioural oestrus in Holstein cows (Gwazdauskas *et al.*, 1983). Furthermore, Humik *et al.* (1975) found also that milk yield was reduced approximately 9% on the day of oestrus in Holstein cows when two and three cows in oestrus simultaneously, while when one cow in oestrus no significant changes were found. The result of the current study shows a negative effect of oestrus on milk yield. There was also a negative correlation between milk yield and the concentrations of E2 in milk. Harrison *et al.* (1989) also reported that milk yield was reduced on the day of visually observed oestrus and also found a negative relationship between milk production and day of visually observed oestrus. Conversely, a study that used an observation score system twice daily for 30 min for the detection of behavioural oestrus by Van Eerdenburg *et al.* (2002) found no relationship between milk yield and behavioural oestrus expression in dairy cows. Madureira *et al.* (2015) also found no correlation ($r^2 = 0.02$; $P < 0.10$) between milk yield and the concentration of plasma E2 in Holstein cow. A decrease of milk E2 concentrations with an increase in milk yield may be due to E2 being diluted by the higher levels of milk production (Lopez *et al.*, 2004). In addition, since dairy cows in oestrus have previously been shown to have a lower dry matter intake (Chapter 4), the lower milk yield may be an effect of this. The difference in the results of different studies regarding the relationship between milk yield and oestrous expression in dairy cows may be due to the period of milk yield data were collected, different sample size, differences in the level of milk yield and the method used for detecting oestrus (Lopez *et al.*, 2004).

6.4.2. The relationship between E2 and P4 profile with the number of steps

The results of the present study found that increased concentration of E2 in milk were associated with an increased number of steps on the day of behavioural oestrus, in comparison to 4DB and 3DA. Regarding silent E2 in the current study, the E2 concentration of in milk was increased 75.8% and 62.9%, and the number of steps was also increased 13.3% and 17.2% in comparison to 4DB and 3DA, respectively. Similarly, in a study conducted on synchronised Holstein Friesian cows by Walker *et al.* (2008) found that the concentrations of E2 in defatted milk measured by RIA were increased significantly in non-lame, moderate lame and severely lame cows, in comparison to 6-3 days before oestrus and 2 days after oestrus in synchronised dairy cows. The result of the present study also agrees with the suggestion reported by van Eerdenburg (2008) who found that at the time of increasing concentrations of E2, the number of steps taken by cows was increased, and the expression of oestrous behaviour was also increased in dairy cattle. The results of the present study agree with those of Aungier *et al.* (2015) which showed a positive ($P = 0.04$; $r^2 = 0.21$) correlation between the peak serum E2 concentration and the duration of oestrus-related behaviours. Similarly, several studies found a positive relationship between blood E2 concentrations (Lyimo *et al.*, 2000; Madureira *et al.*, 2015) and activity in dairy cattle. However, other studies have shown no relationship between circulating E2 concentration and oestrous behaviour, suggesting that the threshold values of E2 needed to induce oestrous behaviour may be different between individual cows (Walton *et al.*, 1987; Coe and Allrich, 1989).

During the day of oestrus, the P4 concentration in milk was negatively correlated with the number of steps taken. These results are consistent with those of Rosenberg *et al.* (1990) who found that higher concentrations of P4 were positively related to the delayed onset of behavioural oestrus in the dairy cow.

6.4.3. The duration of the oestrous cycle and behavioural oestrus

The normal length of the oestrous cycles in dairy cows is considered to be between 18 to 24 days (Bleach *et al.*, 2004). In the current study, the average length of the oestrous cycle was 22.5 ± 1.9 days. This result was 0.5 days shorter than those reported by Savio *et al.* (1990) of 23.1 days in Friesian dairy cows. However, the result of the current study was 1.5 days longer than the length reported by Hall *et al.* (1959) of an average of the oestrous cycle length of 21 days in dairy cows. Another study conducted at the University of Missouri by Kirby *et al.* (1997) reported oestrous cycles more than 2 days longer than the 24 to 28 days in the present study. The difference between the length of the oestrous cycle in dairy cows may be due to the difference in the lifespan of the corpus luteum (CL),

and the principal is prostaglandin F-2 α of endometrial origin as a luteolytic factor in cattle (Thatcher *et al.*, 1984).

The average duration of oestrus recorded by IceQube accelerometers and GEA Rescounters II was 12.3 h and 12.8 h, respectively. Roelofs *et al.* (2005) reported similar duration of oestrus (12.3 h), while a study conducted on 12-month-old Holstein heifers by Silper *et al.* (2015) reported a longer oestrus duration of (14.3 h). However, the duration of behavioural oestrous activity in the current study was approximately 3 h shorter than that reported by Valenza *et al.* (2012) who used a different activity monitoring system in lactating Holstein cows. The difference between the duration of oestrus activity in dairy cows may be due to the disconnection of the secondary signs of oestrus behaviour detected by activity monitors (restlessness) and standing (true) oestrus (Valenza *et al.*, 2012; Roelofs *et al.*, 2005).

6.4.4. The relationship between explanatory variables and oestrus expression (behavioural versus silent oestrus)

The results of the present study found that the number of steps taken on the day oestrus were positively associated with the oestrous expression. This results agree with those reported in Chapter 4 (section 4.3.5) and Chapter 5a (section 5a. 3.9). In the present study, there was a positive relationship between oestrous expression and milk E2 concentration. The results of the present study agree with those reported by Aungier *et al.* (2015) who showed a positive correlation between the peak serum E2 concentration and oestrous activity in lactating dairy cows. Similarly, several studies found a positive relationship between blood E2 concentrations and activity in dairy cattle (Lyimo *et al.*, 2000; Madureira *et al.*, 2015).

The results of the present study found that days PP were positively associated with the oestrous expression. However, in the present study, there was a negative relationship between oestrous expression and milk yield, LS at oestrus and parity. These results concur with those found in Chapter 4 (section 4.3.5) and Chapter 5a (section 5a. 3.9).

6.4.5. The relationships between explanatory variables and the number of steps taken on the day of oestrus

The results of the present study showed positive correlations between E2 and the number of steps on the day of oestrus. The result of the present study agree with those reported by van Eerdenburg (2008) who found that at the time of increasing concentrations of E2, the number of steps taken by cows was increased. In the present study, a positive relationship between DPP and the number of steps on the day of oestrus was found. However, the results of the current study found a negative correlations between LS at

oestrus, milk yield and parity and the number of steps. These results concur with those found in Chapter 4 (section 4.3.4) and Chapter 5a (section 5a. 3.8).

6.4.6. The relationships between explanatory variables and oestrous duration/h

In the present study, a positive correlation was found between milk E2 concentrations and oestrous duration. Similarly, Aungier *et al.* (2015) found a positive correlation between peak serum E2 concentration and the duration of behavioural oestrus in dairy cattle. The results of the present study also found that the number of steps taken on the day of oestrus and DPP were positively associated with oestrous duration. However, in the present study, a negative correlations between LS at oestrus, milk yield (kg/d) and parity and oestrous duration were found. This results agreed with those found in Chapter 5b (section 5b.3.9).

6.4.7. PD+ and PD-

Oestrus was longer and more intense in PD+ cows compared to PD- cows. This finding is in agreement with those of López-Gatius *et al.* (2005) and Ribeiro *et al.* (2012). It may be that when the cow is more active at oestrus, oestrous detection is more accurate which may lead to them being AI'd at a more precise time increasing pregnancy rates (Cruppe, 2011). Also recent study conducted on lactating Holstein cows by Madureira *et al.* (2015) found that cows that were more active during synchronised oestrus, had a higher pregnancy rate (37.4%) compared with those that were less active during oestrus (pregnancy rate 23.9%). An examination of the relationship between milk E2 concentrations, the number of steps taken on the day of behavioural oestrus with PD following AI by linear logistic regression analysis revealed a positive relationship between E2 concentrations, the number of steps taken and PD following AI. This appears to be the first study to report a relationship between milk E2, the number of steps with PD following AI in dairy cows undergoing spontaneous oestrous cycles. In addition, the results of the current study showed that milk P4 concentrations on the day of oestrus were negatively correlated with PD following AI. The results of the current study showed that oestrous duration were positively correlated with PD following AI. Cows that show longer periods of behavioural activity on the day of oestrus would be expected to show improve in fertility (Madureira *et al.*, 2015). In addition, Bisinotto *et al.* (2013) reported that fertility rate increases and early embryonic development increases when circulating P4 increased faster early in the subsequent cycle. This may be due to changes in the receptor profile of the endometrium (Lonergan, 2011). Furthermore, the positive correlation between increasing activity on the day of behavioural oestrus and pregnancy rate may be because the stronger signs of oestrus allowing AI to be more accurately timed in relation to

ovulation, or because higher E2 levels make the uterine environment more suitable for the developing embryo (Cruppe, 2011).

Milk P4 concentration at +3 DA oestrushave a positive association with PD following AI. The importance of P4 during early pregnancy in cows is well documented (Mann and Lamming, 2001). Several studies report an association between low maternal P4 concentrations in both the milk and plasma of inseminated cows and pregnancy failure (Mann *et al.*, 1995; Butler *et al.*, 1996). Other studies show both improved pregnancy rates and increased embryo development after P4 supplementation in the early days after ovulation (Garrett *et al.*, 1988; Mann and Lamming, 1999). The results of the present study agree with those reported by Mann *et al.* (2006) who found that an increase in P4 between day 4 and 5 postovulation result in a consistent increase in pregnancy rates in dairy cows. The positive relationship between an increase in pregnancy rate with increased P4 concentration following early ovulation may be due to the effects of P4 on the maternal uterine environment that lead enhanced to embryo development and pregnancy (Mann and Lamming, 2001).

The results of the present study found a negative relationship between cow parity and PD following AI. These results agree with those reported by Chebel *et al.* (2004), who found that younger cows had a significantly higher conception rate (27.4%) compared to older cows (24.1%). Another study conducted by Pursley *et al.* (1998) found that pregnancy rates were higher for cows in parity two (43%) compared to cows in parity three or more (26%). This might be partially explained by the higher incidence of post-parturient diseases in older cows (14.9%) compared to younger cows (6.2%) (Chebel *et al.*, 2004). Therefore, it is possible that older cows experienced lower PD+ because they were at a higher risk of peri-parturient problems known to affect fertility in dairy cows (Chebel *et al.*, 2004). In addition, older cows had a higher milk yield than younger cows, which increased the energy demands for milk synthesis, therefore affecting their energy status (Butler and Smith, 1989). It has been reported that a consistent increase in average milk production per cow is associated with a decrease in conception rates (Butler and Smith, 1989).

7.5. Conclusion

On the day of behavioural oestrus, the number of steps was increased, and there was an increase in milk E2 concentration. However, on the day of silent oestrus, only milk E2 was increased with no change in activity. Oestrous activity and duration were both positively correlated with milk E2 concentrations. On the day of oestrus, E2 concentrations were higher in cows that became pregnant following AI and these cows had higher oestrous activity levels.

CHAPTER 7

Fatty acid profile of milk for determining reproductive status in lactating Holstein Friesian cows

7.1. Introduction

Oestrus is a behavioural characteristic that ensures the cow is mated close to the time of ovulation (Roelofs *et al.*, 2010). In dairy herds using AI, detection of oestrus in a high percentage of lactating dairy cows is essential to maximise reproductive performance (Forde *et al.*, 2011). However, one of the predominant reproductive dysfunctions causing poor fertility in dairy cows is silent oestrus when an ovulation occurs without an associated behavioural oestrus (Yániz *et al.*, 2008). It has been reported that standing oestrous behaviour is detected in only 50% of oestrous cows (Lyimo *et al.*, 2000). Further to this, studies (Palmer *et al.*, 2010; Zebari *et al.*, 2018; Chapter 4) reported that only 50 to 60% of cows express behavioural signs of oestrus, with the remaining 40 to 50% having ovulations without expression of behavioural oestrus (silent oestrus) which cannot obviously be detected by observation or automated methods of oestrous detection.

Chemical communication has an important role (Sankar and Archunan, 2004) in mammalian sexual behaviour and reproductive processes. Oestrous cows produce olfactory chemical factors which attract the bull (Rekwot *et al.*, 2001). The bull responds to these pheromones (Rekwot *et al.*, 2001) or chemical factors which are released from one individual and are sensed by other individuals of the same species as a result of specific receptors for these chemicals. As a result of sensing these signals, specific endocrine and behavioural reactions are induced in another individual of the same species (Vyas *et al.*, 2012). Oestrous-specific pheromones have been detected in the urine (Ramesh Kumar *et al.*, 2000), faeces (Wiegerinck *et al.*, 2011) and vaginal secretions (Rivard and Klemm, 1989; Rekwot *et al.*, 2001) of cows. Bulls express the Flehmen response to vaginal secretions from oestrous cows that have been applied to cows in dioestrus (Sankar and Archunan, 2004).

In addition, oestrous and non-oestrous cows have been differentiated by bulls through the detection of pheromones in urine (Vyas *et al.*, 2012). Various molecules have been proposed as chemical indicators of oestrus (Sankar and Archunan, 2008). During oestrus, fatty acids (FA) such as tridecanoic, myristic and pentadecanoic acids have been found in greater concentrations in cows urine than at other stages of the oestrous cycle (Kumar and Archunan, 2006). Furthermore, Gnanamuthu and Rameshkumar (2014) reported that valeric, caproic, myristic, gadoleic and pelargonic acids were present in cow faeces during oestrus but not during pro-oestrus and dioestrus. Mozūraitis *et al.* (2017) reported that the concentrations of acetic acid, propanoic acid, butanoic acid and pentanoic acid were significantly higher in faecal samples of cows in oestrus compared with non-oestrus cows. The appearance of these FA at higher concentrations during oestrus may be due to the

changing concentrations of circulating steroid hormones and may be involved in attracting the opposite sex (Kumar and Archunan, 2006).

Milk is a readily available medium with the potential for oestrous detection. In a study where the day of AI was considered to be the day of oestrus, Toledo-Alvarado *et al.* (2018) found that specific milk fatty acid profiles changed during the oestrous phase compared to other phases of the oestrous cycle. However, it was unclear whether the cows used in this study were oestrous synchronised or naturally oestrous cycling. Although the milk from cows in oestrous has been shown to attract bulls (Sankar and Archunan, 2004), there are no known published studies relating concentrations of milk FA to oestrous activity in cows undergoing spontaneous oestrous cycles.

The present study was designed to:

- 1- Determine the quantitative differences in milk fatty acid profile in dairy cows during oestrus and day 14 after oestrus (di-oestrus).
- 2- Investigate whether the milk fatty acid profile could be used to determine the reproductive status of dairy cows.
- 3- Investigate whether the milk fatty acid profile could be used as an alternative method of oestrous detection in dairy cows.
- 4- Investigate the relationship between milk FA and the oestrous activity in dairy cows.

7.2. Materials and methods

The experiment was undertaken between August and October 2017 at the Dairy Unit of Harper Adams University, Newport, Shropshire, TF10 8NB, UK.

7.2.1. Ethical considerations

See section 3.2.

7.2.2. Experimental animals, housing and management

Multiparous (parity 2.8 ± 0.1 mean \pm SD; range 2 to 4), lactating Holstein Friesian cows ($n = 32$) 60.9 ± 17.7 days into their lactation period were used for the study (see section 3.1). The cows were submitted for detection of any abnormalities of their reproductive tract by the herd veterinarian. The average LS (Scale 1-5; as described by Chapinal *et al.*, 2009) of the selected cows was 2.5 ± 0.5 (range 2 to 3) see section 3.6.1. The cows were producing 34.4 ± 6.6 kg per day milk (see section 3.1) with a mean BCS (Scale 1-5; AHDB Dairy, 2014) of 2.9 ± 0.3 (range 2.5 to 3.0) at the start of the study (see section 3.6.1). Cows were housed with the main herd in a free stall cubicle house (cubicles 2.7 x 1.2 m, with 3 cm thick rubber mattresses, 105 cubicles per 100 cows). The cubicles were bedded with sawdust and lime three times per week and passageways were scraped using an automatic device 4 to 5 times per day. Study cows were milked twice a day at approximately 05:00 and 16:30 through a 40-point internal rotary milking parlour (Westfalia, GEA Milking System, Germany).

Cows were fed a total mixed ration (TMR) *ad libitum* (Table 7.1) provided daily at approximately 07:30 h. Nutrients supplied in the ration are shown in Table 7.2. Water was also provided *ad libitum* from water troughs at the end of each passageway area.

7.2.3. Determination of the day of oestrus and duration of oestrus

Cows were monitored for signs of spontaneous oestrus using two automated methods throughout the duration of the experiment. These were an IceQube (IceRobotics Ltd., Edinburgh, UK) attached to the back left leg of each cow (see section 3.6.1) and a GEA Rescounter II pedometer (GEA Farm Technologies, Düsseldorf, Germany) attached on the right front leg (see section 3.6.2).

The 'Oestrus' milk samples were collected on the day of behavioural oestrus. Oestrus was identified using the Cow Alert IceQube system (IceRobotics Ltd., Edinburgh, UK) and GEA Rescounter II (GEA Farm Technologies, Düsseldorf, Germany). The oestrus was identified from the increase in physical activity (see section 3.6.3). The intervals between

the two basal thresholds of physical activity that were indicative of non-oestrus animals were considered to be the period of oestrous duration (see section 3.6.4).

Table 7. 1. Dietary composition of the total mixed ration fed to the cows throughout the study period.

Ingredient	kg/head	kg DM/head
Maize silage	26.00	8.84
Grass silage	3.00	1.56
Lucerne	7.00	3.15
Chopped wheat straw	0.35	0.30
Protein syrup	4.00	0.96
Protein blend	5.25	4.66
Sweet starch	2.25	1.98
Soya hulls	2.00	1.80
Megalac	0.20	0.20
Dairy minerals	0.15	0.15
Acid buff	0.10	0.10
Salt	0.10	0.09
Saccharomyces cerevisiae	0.03	0.02
Water	5.00	0.00
Total	52.88	22.47

(Profeed Nutrition Consultancy, UK, 2017)

Table 7. 2. Predicted nutrient content of the total mixed ration. DM = dry matter, ME = metabolisable energy, CP = crude protein, NDF = neutral detergent fibre.

Nutrient Supplied	
DM (g/kg fresh)	430.0
ME (MJ/kgDM)	12.1
CP (%DM)	16.7
NDF (%DM)	36.1
Fat (%DM)	4.4
Starch and sugar (%DM)	20.9

(Profeed Nutrition Consultancy, UK, 2017)

7.2.4. Collection of milk samples

When oestrus was identified by both the GEA Rescounter II and IceQubes, a milk sample (80 mL) was collected between 1 and 12 hours after the onset of oestrus (termed the “oestrous” sample). On day 14 of the subsequent oestrous cycle, a second milk sample (80 mL) was collected (termed the day 14 “post oestrus” sample). The samples were split into two aliquots of 40 mL and stored in a freezer at -20 °C until analysis for short and long chain FA profiles using gas chromatography (GC – subsequently described in section 7.2.5 and 7.2.6) and milk composition using a Milko-Scan Minor analyser (Foss, Denmark) calibrated according to AOAC (2012).

7.2.5. Short chain (Volatile) fatty acid determination using gas chromatography (GC)

7.2.5.1. Preparation of standard solutions

Volatile fatty acid (VFA) standards (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids as well as 2-methylvaleric acid; Sigma-Aldrich Company Ltd., Dorset, UK) were weighed (250 mg) and placed in 50 mL tubes and dissolved in approximately 50 mL of distilled water.

7.2.5.2. The relative response factors determination of volatile fatty acids to 2-methylvaleric acid as internal standard

Short chain FA standards (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids) as well as 2-methylvaleric acid (Sigma-Aldrich Company Ltd., Dorset, UK) were weighed (250 mg) into 50 mL tubes and dissolved with approximately 50 mL of distilled water. The volatile FA (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids) were mixed and 0.5 mL of this mixture was added to 0.5 mL of 2-methylvaleric acid solution to be used as the internal standard (IS; Yang and Choong, 2001). This mixture (0.1 µL) was subsequently injected into a Hewlett Packard HP6890 GC (Agilent Technologies Inc. Germany) equipped with a flame ionisation detector and utilising a capillary column (30.0 m x 250 µm x 0.25 µm) supplied by Greyhound Chromatography and Allied Chemicals (Merseyside, UK). The initial temperature of the oven (30 °C) was held for 2 min, before increasing by 8 °C/m to 110 °C. This temperature was then held for 4 min, then the temperature was increased by 5 °C/min until 170 °C and further increased by 4 °C/min to 250 °C. Finally the programmed temperature was held at 250 °C for 15 min. Nitrogen gas was used as the carrier at a flow rate of 2.7 mL/min with an initial pressure of the column head of 11.72 psi and at post-run pressure of 13.92 psi. The temperature of the detector and injector was 250 °C.

The relative response factor (RRF_{VFA}) was calculated by dividing the peak area ratio of each VFA to 2-methylvaleric acid by the ratio of their weight according to Yang and Choong (2001) using the following equation:

$$RRF_{VFA} = (A_{VFA}/A_{IS}) \div (W_{VFA}/W_{IS})$$

$$= (A_{VFA}/W_{VFA}) \div (A_{IS}/W_{IS})$$

Where A = peak area and W = weight in milligrams, IS = internal standard, VFA = volatile fatty acid

7.2.5.3. Quantitative determination of VFA by GC

Milk samples were thawed at room temperature, and one millilitre of each milk sample was transferred to a GC vial and 50 μ L of the 2-methylvaleric acid (0.5% w/v) aqueous solution was added then mixed thoroughly. Milk samples were shaken periodically on the GC to avoid clotting. Then, 0.1 μ L of the mixture was injected into the GC. Between the injection of each milk sample, 0.1 μ L of 5% oxalic acid and 0.1 μ L of methanol were injected into the GC on approximately eight occasions to flush the GC. The concentration of each VFA (mg/mL) was determined using the procedure of Yang and Choong (2001) by the following equation:

$$VFA \text{ (mg/mL)} = (A_{VFA}/A_{IS}) \times (W_{IS}/RRF_{VFA}) \times 1/w$$

Where w = the weight of milk in milligrams.

7.2.6. Long chain fatty acid determination by gas chromatography (GC)

7.2.6.1. Milk fat extraction

Milk fat for long-chain FA determination was extracted according to the method as described by Feng *et al.* (2004). Milk samples (40 mL) were placed in a water bath at 40°C for 20 min and shaken periodically to disperse the milk fats. Approximately 30 mL of the milk was transferred into a 50 mL conical plastic tube (Nalgene® 3110-9500 Round-Bottom Centrifuge Tube, Capitol Scientific, Inc, USA). Samples were then centrifuged at 17,800 X g for 30 min at 4°C, using a Rotina centrifuge 46 R (Hettich Zentrifugen, Andreas Hettich GmbH & Co. KG, Fohrenstr 12, D-78532 Tuttlingen, Germany). An aliquot (1 g) of the fat cake layer of each sample (the samples were kept in a centrifuge at 4°C and taken out one by one) was transferred to a 2.5 mL micro-tube (Fisher Scientific Ltd, Loughborough, Leicestershire, UK) and left at room temperature (20°C) for

approximately 20 min to allow the fat to melt. After melting, each fat sample was centrifuged at 19,300g for 30 min at room temperature using a micro-centrifuge (Thermo Fisher Scientific, Im Heiligen Feld 17, 58239 Schwerte, Germany). The fat cake layer was separated into 3 layers (a top layer: lipid, a middle layer: protein, fat and other water-insoluble solids and a bottom layer: water). The lipid layer was carefully transferred into a 0.5 mL microcentrifuge tube and placed into a freezer at -20°C to be methylated at a later date.

7.2.6.2. Milk fat methylation

7.2.6.2.1. Methylation reagent

In a small tube, 1.75 mL of methanol was mixed with 0.4 mL of 30% sodium methoxide solution and vortexed which gave approximately 1M NaOMe for use within 24 h.

7.2.6.2.2. Termination reagent

A reagent bottle (50 mL) for the preparation of the termination reagent was placed in an oven at 120°C for 30 min to remove any water then cooled in a desiccator. Oxalic acid (1g) was weighed into the reagent bottle then 30 mL of diethyl ether was added, before the bottle was closed, shaken and stored in a dark place to be used within 2 weeks.

7.2.6.2.3. Methylation process

The milk lipid methylation process followed was as described by Christie (1982) with modifications occurring as described by Chouinard *et al.* (1999). Extracted milk lipid samples were taken out of the freezer and placed in an incubator at <60°C for 20 min until the lipid was melted. Exactly 50 mg of the extracted lipid was weighed into a 10 mL extraction tube which had been pre-rinsed with hexane. Hexane (2 mL) and methyl acetate (40 µL) from Sigma Aldrich UK were added to the extracted lipid and vortexed (FB 15013 Topmix®, Fisher Scientific, UK) for 30 seconds. Then 40 µL of the methylation reagent was added, the tube closed and then vortexed for 2 min. The samples were left to stand for a further 8 min for the reaction to be completed. After the reaction was complete, 60 µL of the termination reagent was added and the tube closed and then vortexed for 30 seconds. Approximately 200 mg of calcium chloride was added, and the tube was then vortexed and left to stand for 1 h. After one hour the samples were centrifuged (Rotina centrifuge 46R, Hettich Lab tech, Tuttlingen, Germany) at 2600 rpm for 30 min at 5°C. The solvent layer containing methyl esters was transferred to a GC vial and then stored at -35 °C in a freezer until injection into the GC.

7.2.6.3. Quantifying long-chain fatty acids

Long-chain fatty acids were identified using a GC (Hewlett-Packard – HP 7820A GC System, Agilent Technologies Inc. Germany) fitted with an automatic sampler, flame ionization detector, (CPSil 88, Agilent Technologies, UK) and equipped with a CP-SIL 88 fused silica capillary column (100 m × 0.25 mm with 0.2 mm film thickness; Varian, Inc., Walnut Creek, CA) as described by Lock *et al.* (2006). The oven temperature started at 70°C, was held for 2 min, followed by an increase of 8°C/min until it reached 110°C. This temperature was held for 4 min, then increased by 5°C/min to reach 170°C. It was held for 10 min, and finally increased at 4°C/min to 225°C and held for 15 min until all of the peaks were analysed. Each sample had a run time of 61.75 min and a post-run time of 1 min at 70°C. Peaks were identified by comparison of the retention time of FA with individual FAME standards (Sigma-Aldrich, Ltd, UK) and a mixed reference standard was used as a routine check for recoveries and correction factors for individual FA.

7.2.7. Milk composition profile

Milk samples were analysed to quantify total solids, total protein, total fat, and lactose using a Milko-Scan Minor analyser (Foss, Denmark) calibrated according to AOAC (2012) for cow's milk. The samples were thawed by placing them in a water bath at approximately 35°C and shaken well to ensure that all of the milk contents were mixed well.

7.2.8. Artificial insemination and pregnancy diagnosis

All of the cows were artificially inseminated 12 hours after detection of oestrus using frozen-thawed semen from one of six bulls. Cows that did not return to oestrus within 30 days of insemination ($n = 29$; 90.6%) were presented for pregnancy diagnosis by a veterinarian using a transrectal ultrasonic scanner device (Easi Scan-3, BCF Technology, UK). Cows were designated pregnant (PD+) or non-pregnant (PD-). Cows that had returned to oestrus 18 to 30 days after AI were also considered to be non-pregnant ($n = 3$; 9.4%). Overall, of the 32 cows, 56.3% ($n = 18$) were diagnosed as being PD+ and 43.7% ($n = 14$) were diagnosed PD- (see section 3.7).

7.2.9. Statistical analysis

The milk concentrations of short-chain FA, long-chain FA, total fat, total protein, lactose, total solids and fat/protein ratio on the day of oestrus were compared with day 14 using a paired t-test (Genstat statistical software package, Genstat 17th edition, 17.1.14713, VSN International Ltd, UK). Fatty acid concentrations in pregnant (PD+) and non-pregnant (PD-) cows were also assessed. Linear regression analysis was used to determine the relationship between GEA activity and the number of steps taken per day (from the IceQubes). Regression analyses were used to determine the relationship between the response variable, the number of steps taken per day (from the IceQubes) and the explanatory variables: acetic acid, caproic acid and valeric acid on the day of oestrus. All of the data sets analysed were normally distributed. Differences are reported as significant at $P < 0.05$ and tendencies are reported when P was between 0.1 and >0.05 .

7.3. Results

7.3.1. Oestrous characteristics

Thirty two spontaneous oestrous events were detected using the GEA pedometers during the study period. The average physical activity during oestrus as recorded by GEA was 768.5 ± 38 AU (Mean \pm SEM; range 412 - 1220 AU). The average duration of oestrus as determined by the GEA pedometers was 12.8 ± 0.6 h (range 7 - 19 h). On the day of oestrus, the average number of steps recorded using the IceQube accelerometers was 2714.5 ± 213 steps (range 1054 - 5381 steps). Based on the number of steps taken by cows, the average duration of oestrus was 12.6 ± 0.6 h (range 7 - 18 h). There was a positive correlation ($P < 0.001$) between the GEA activity (AU/d) measurements and the number of steps recorded by the IceQube accelerometers ($y = 0.162x - 329.84$; $r^2 = 0.821$) during the day of oestrus (Figure 7.1).

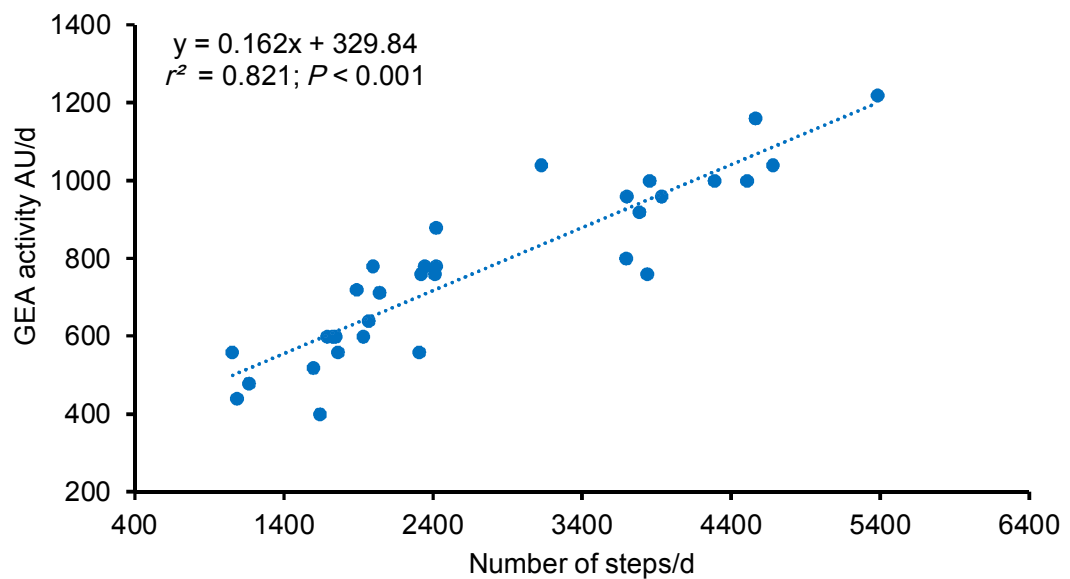


Figure 7.1. The relationship between GEA activity (AU/d) recorded by the GEA pedometers and the number of steps/d recorded by the IceQube accelerometers on the day of oestrus in lactating Holstein Friesian cows ($n = 32$).

7.3.2. Milk volatile (short-chain) fatty acids profile

The concentrations of acetic acid (C2:0; $P < 0.001$), valeric acid (C5:0; $P = 0.016$) and caproic acid (C6:0; $P < 0.001$) in milk were higher on the day of oestrus in comparison to day 14 (Table 7.3). The concentration of butyric acid (C4:0) was not higher ($P = 0.131$) on the day of oestrus compared to day 14 and there was no difference ($P = 0.713$) between

the concentration of isovaleric acid (iso-C5:0) on the day of oestrus and day 14 after oestrus. Propionic acid (C3:0) was not detected in the milk samples, on either the day of oestrus or day 14 (Table 7.3).

There were a positive ($P < 0.001$) quadratic relationships between both the milk concentrations of acetic acid ($y = 0.03x^2 - 9.68x + 2886.8$; $r^2 = 0.40$; Figure 7. 2) and caproic acid ($y = 6.02x^2 - 307.68x + 5574.5$; $r^2 = 0.75$; Figure 7.3) and the number of steps recorded by the IceQubes during the day of oestrus. In addition, a positive linear relationship ($P = 0.004$) was observed between milk concentrations of valeric acid and the number of steps recorded by the IceQubes ($y = 83.57x + 352.91$; $r^2 = 0.25$; Figure 7.4) during the day of oestrus.

Table 7. 3. Milk short-chain fatty acid (mg/100 mL) concentration (Means \pm SEM) on the day of oestrus and day 14 after oestrus of lactating Holstein Friesian dairy cows ($n = 32$).

VFA	Lipid number	Oestrus mg/100mL	Day 14 mg/100mL	P-value
Acetic Acid	C2:0	297.0 \pm 18.5	229.0 \pm 11.9	0.001
Propionic acid	C3:0	N/A	N/A	N/A
Butyric acid	C4:0	179.1 \pm 6.8	157.3 \pm 11.4	0.131
IsoValeric acid	Iso C5:0	389.0 \pm 18.7	380.0 \pm 18.1	0.713
Valeric acid	C5:0	28.3 \pm 1.3	23.7 \pm 1.0	0.016
Caproic acid	C6:0	35.3 \pm 1.7	28.7 \pm 1.1	0.001

VFA = Volatile fatty acids

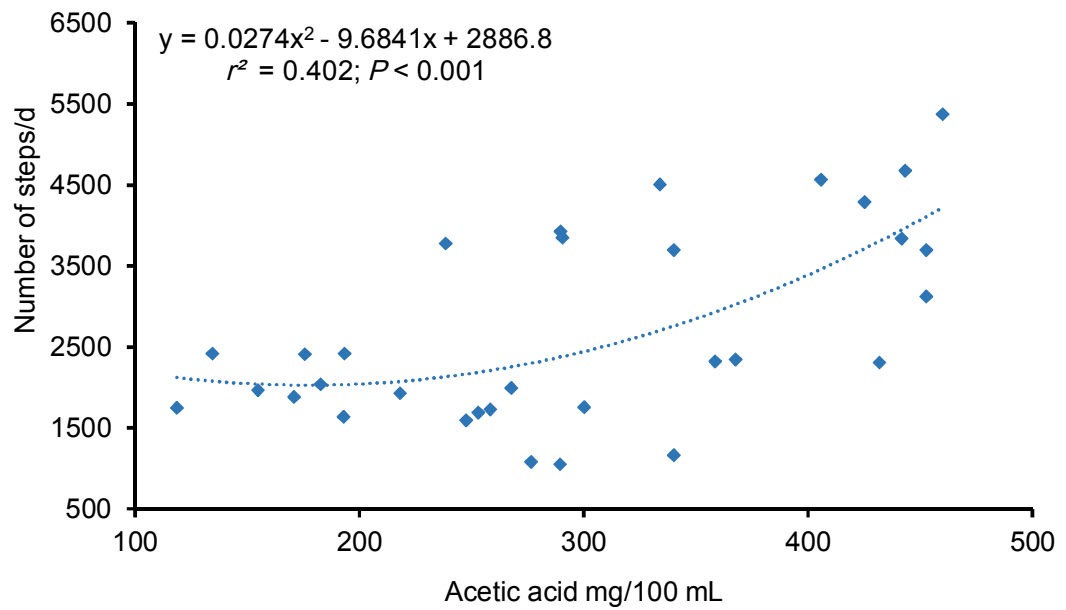


Figure 7. 2. The relationship between acetic acid concentrations (mg/100 mL) in milk and the number of steps recorded by IceQube accelerometers on the day of oestrus in lactating Holstein Friesian cows ($n = 32$).

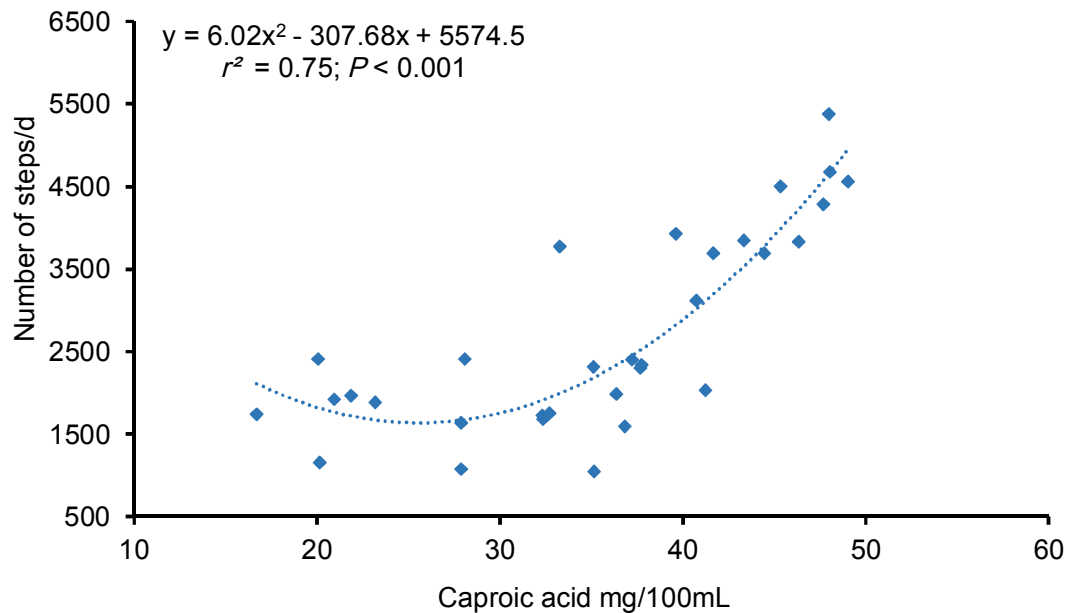


Figure 7. 3. The relationship between caproic acid concentrations (mg/100 mL) in milk and the number of steps recorded by IceQube accelerometers on the day of oestrus in lactating Holstein Friesian cows ($n = 32$).

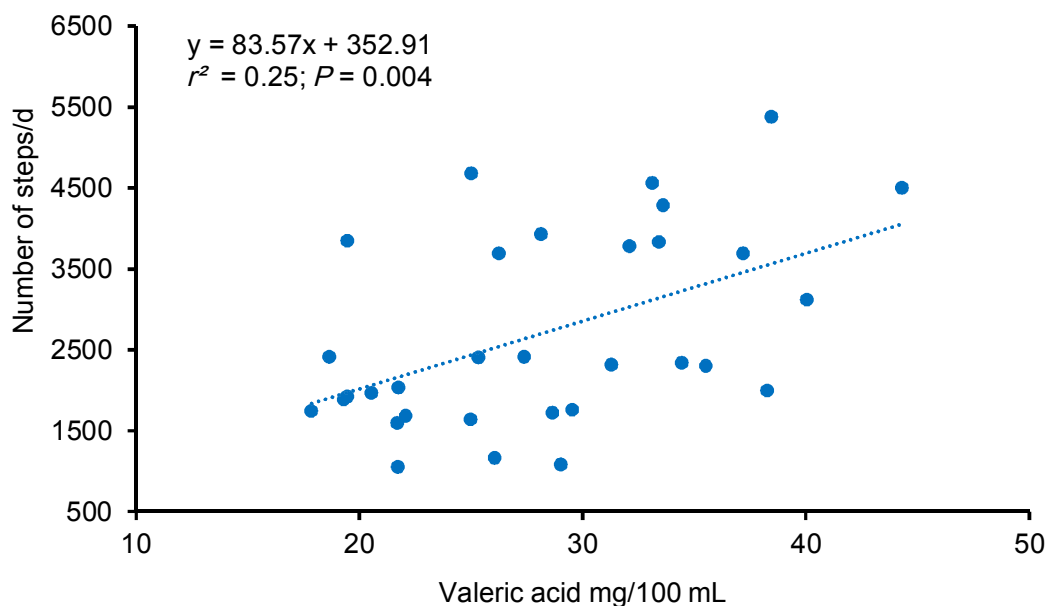


Figure 7. 4. The relationship between valeric acid concentrations (mg/100 mL) in milk and the number of steps recorded by IceQube accelerometers on the day of oestrus in lactating Holstein Friesian cows ($n = 32$).

7.3.3. Milk long-chain fatty acid profile

On the day 14 after oestrus, arachidonic acid (C20:4n6c) concentrations in milk samples were higher ($P = 0.004$) in comparison to the day of oestrus. Furthermore, arachidonic acid concentrations in day the 14 milk samples from PD+ cows were lower ($P = 0.002$; 0.53 ± 0.02 mg/100 mL; mean \pm SEM) compared with PD- cows (0.64 ± 0.02 mg/100 mL). The concentration of undecanoic acid (C11:0) also tended ($P = 0.066$) to be higher on day 14 compared to the day of oestrus. In contrast, the concentration of myristoleic acid (C14:1) was higher ($P = 0.035$) on the day of oestrus, and the concentration of elaidic acid (C18:1n9t; $P = 0.097$) and lignoceric acid (C24:0; $P = 0.063$) also tended to be higher on the day of oestrus compared to day 14. There were no significant differences in the concentrations of the other long-chain FA that were assessed (Table 7.4) and no other differences in fatty acid concentrations of PD+ compared to PD- cows.

7.3.4. Milk composition profile

The concentrations of milk total fat, total protein, lactose and total solids were not significantly different on the day of oestrus compared to day 14 (Table 7.5). There was also no effect of oestrus ($P = 0.990$) on the fat/protein ratio of milk compared to day 14 (Table 7.5).

Table 7. 4. Milk long-chain fatty acid (g/100 g of FA) concentration (Mean \pm SEM) on the day of oestrus and day 14 after oestrus of lactating Holstein Friesian dairy cows ($n = 32$)

Long chain FA	Lipid number	Oestrus	Day 14	P-Value
Caprylic acid	C8:0	1.13 \pm 0.02	1.13 \pm 0.03	0.965
Capric acid	C10:0	2.70 \pm 0.06	2.62 \pm 0.07	0.353
Undecanoic acid	C11:0	0.23 \pm 0.01	0.25 \pm 0.01	0.066
Lauric acid	C12:0	3.44 \pm 0.08	3.38 \pm 0.09	0.470
Tridecanoic acid	C13:0	0.11 \pm 0.01	0.09 \pm 0.00	0.171
Myristic acid	C14:0	10.28 \pm 0.21	10.50 \pm 0.18	0.289
Myristoleic acid	C14:1	0.62 \pm 0.12	0.32 \pm 0.03	0.035
Palmitic acid	C16:0	29.16 \pm 0.50	29.12 \pm 0.50	0.928
Palmitoleic acid	C16:1	1.34 \pm 0.07	1.39 \pm 0.06	0.361
Heptadecanoic acid	C17:0	0.13 \pm 0.01	0.16 \pm 0.03	0.335
Cis-10- Heptadecenoic	C17:1	0.53 \pm 0.01	0.51 \pm 0.01	0.164
Stearic acid	C18:0	10.01 \pm 0.38	9.85 \pm 0.33	0.743
Oleic acid	C18:1n9c	22.08 \pm 0.55	21.33 \pm 0.60	0.230
Linoleic acid	C18:2n6c	2.65 \pm 0.10	2.65 \pm 0.09	0.998
Elaidic acid	C18:1n9t	0.95 \pm 0.15	0.65 \pm 0.08	0.097
Arachidic acid	C20:0	0.13 \pm 0.01	0.14 \pm 0.01	0.308
Gadoliec acid	C20:1n9t	0.39 \pm 0.03	0.42 \pm 0.01	0.250
Arachidonic acid	C20:4n6c	0.41 \pm 0.05	0.60 \pm 0.03	0.004
Henicosanoic acid	C21:0	0.17 \pm 0.06	0.10 \pm 0.01	0.102
Behenic acid	C22:0	0.15 \pm 0.01	0.16 \pm 0.01	0.655
Tricosanoic acid	C23:0	0.06 \pm 0.01	0.07 \pm 0.01	0.509
Lignoceric acid	C24:0	0.09 \pm 0.01	0.07 \pm 0.01	0.063

Table 7. 5. Milk composition (g/kg) and fat/protein ratio (Means \pm SEM) on the day of oestrus and day 14 after oestrus of lactating Holstein Friesian dairy cows ($n = 32$)

Milk composition	Oestrus	Day 14	<i>P</i>-value
Total fat	35.2 \pm 1.4	36.8 \pm 1.4	0.431
Total protein	32.1 \pm 0.6	33.7 \pm 1.2	0.215
Lactose	45.5 \pm 0.7	46.1 \pm 0.6	0.484
Total solid	147 \pm 2.1	150.5 \pm 2.3	0.355
Fat/Protein	1.1 \pm 0.0	1.1 \pm 0.0	0.990

7.4. Discussion

7.4.1. Oestrous characteristics and their relationship with milk fatty acid profile

An increase in physical activity is an important external sign of oestrus in dairy cattle (Firk *et al.*, 2003). In the present study, a concurrent increase in physical activity was recorded with use of the GEA pedometer and the IceQube accelerometers. The increase in number of steps recorded using the IceQubes was positively correlated with the concentration of acetic acid, caproic acid, valeric acid and myristoleic acid in milk on the day of oestrus. Using pedometers, Roelofs *et al.* (2005) detected a similar increase in the number of steps (2080) on the day of visually observed oestrus. In the present study, the physical activity recorded using the GEA pedometers was positively correlated ($y = 0.162x + 329.84$; $r^2 = 0.82$; $P < 0.001$) with the number of steps recorded by the IceQubes on the day of oestrus. Environmental conditions including the type of housing and management conditions may affect the extent of walking activity (López-Gatius *et al.*, 2005a; Yániz *et al.*, 2006).

Previously, the duration of standing oestrus in dairy cows was considered to be approximately 18 h (Valenza *et al.*, 2012). Oestrous duration in the present study, as recorded by the GEA pedometers and the IceQube accelerometers, was 5 to 6 h shorter (12.8 ± 4.6 and 12.6 ± 2.6 h, respectively). Roelofs *et al.* (2005) reported similarly oestrous duration (12.3 h) to the present study, while in a study by Silper *et al.* (2015) oestrous duration of 14.3 ± 4.1 h was detected using a neck mounted accelerometer. However, the duration of oestrus activity of this study was shorter than that reported by Valenza *et al.* (2012) of 16.1 ± 4.7 h detected using a physical activity monitoring system.

7.4.2. Volatile (short chain) fatty acids profile

The results of the current study showed that the average concentration of milk C2:0 was 263 mg/100mL. This was within the range of 79 to 364 mg/100mL found by Yang and Choong (2001) in fresh cow's milk. The average concentration of milk C5:0 was 26.0 mg/100mL in the present study is slightly above the range of 20.6 to 20.9 mg/100mL reported by Liu *et al.* (2016). Milk C4:0 was 168.2 mg/100mL was also higher than the previously published range (124.32 to 161.4 mg/100mL) in dairy cows milk reported by Or-Rashid *et al.* (2009), as were the concentrations of milk Iso-C5:0 (mean 384.5 mg/100mL. This was also higher than the range (Iso-C5:0; 109 to 237 mg/100mL) in spoiled milk found by Yang and Choong (2001). The result of the present study showed that the average concentration of milk C6:0 was 32 mg/100mL, this was higher than the range 13.1 mg/100mL reported by Liu *et al.* (2016). The higher concentrations of some of the short-chain fatty acids measured in milk in the present study may due to the present

study fatty acid profile being measured in fresh whole milk. Yang and Choong (2001) and Liu *et al.* (2016) used milk fat for the determination of milk fatty acids profile.

The findings of the present study indicated that milk concentrations of certain FA vary according to the stage of the oestrous cycle. It is believed that the chemical signals from different body fluids including urine (Kumar and Archunan, 2006; Archunan, 2012), blood (Klemm *et al.*, 1994), milk (Bendall, 2001), vaginal mucus (Sankar and Archunan, 2004) and saliva (Sankar *et al.*, 2007) during oestrus in cattle have an important roles as attractant pheromones so that the bull can differentiate between oestrous and non-oestrous cows. In the present study, significantly higher concentrations of acetic acid (C2:0), valeric acid (C5:0) and caproic acid (C6:0) were recorded in milk on the day of oestrus compared to day 14. As far as I am aware, this is the first study where differences were reported in milk FA concentrations during different stages of spontaneous oestrous cycles. However, several studies have shown similar differences in faecal concentrations of FA during oestrus. Mozūraitis *et al.* (2017) reported that there were higher concentrations of acetic acid (36 ± 8.0 ng/0.5g faeces) and pentanoic (valeric) acid (125 ± 57.6 ng/0.5 g faeces) concentrations in oestrous cows compared to those of non-oestrous cows (19 ± 5 ng/0.5g faeces and 22.96 ± 9.9 ng/0.5g faeces, respectively). The results of the present study are also consistent with those of Sankar and Archunan (2008) who found that acetic acid was present only in cow faeces during oestrus compared to pro-oestrus and post-oestrus. Furthermore, Gnanamuthu and Rameshkumar (2014) found that valeric acid (C5:0) and caproic acid (C6:0) were present only during oestrus but not in faecal samples of *Bos indicus* cattle collected during pro-oestrus or dioestrus. The role of these FA in relation to oestrus remains to be determined. However, milk, urine and vaginal secretions of volatile FA from oestrous cows may have roles as pheromone because urinary FA have this role in mammals (Kumar and Archunan, 2006). Bendall (2001) also reported that volatile compounds in cow's milk such as γ -12:2 lactone functioned as an active odorant in cows. Klemm *et al.* (1994) found that acetaldehyde has an important function as an attractant in cows during oestrus. These findings are consistent with those from the results of the present investigation and suggest that the higher concentration of short-chain FA during oestrus may function as pheromones and sexual attractants in cattle. Furthermore, Vyas *et al.* (2012) reported that cows produce a specific volatile compound in markedly greater concentrations in faeces during oestrus as a sexual attractant.

The higher concentrations of acetic acid on the day of oestrus may relate to its role as a precursor of E2 (Robinson *et al.*, 2002) and milk concentrations and may simply reflect high blood acetic acid concentrations during oestrus (Frateschi *et al.*, 1980). Results from previous studies indicate the synthesis of E2 by the mammary gland of cattle and the secretion into both milk and mammary venous blood (Janowski *et al.*, 1988; Janowski *et*

al., 2002). However, without measures of milk E2 in the present study, it is not possible to explain these findings further.

Propionic acid has previously been reported to be present at high concentrations in various media. However, in the present study, propionic acid (C3:0) was not present in fresh cow's milk on either the day of oestrus or day 14 in dioestrus. This result is consistent with those reported by Bevilacqua and Califano (1989) that showed that propionic acid was not present in the whole milk of cows but was present in yoghurt and blue cheese. From the results of the present study, there are indications that the concentration of butyric acid were numerically higher on the day of oestrus but not significantly different in comparison to day 14 and there were also no differences in the concentration of isovaleric on the day of oestrus compared to 14 after oestrus. Inconsistent with the present findings, Mozūraitis *et al.* (2017) reported that there were higher concentrations of butanoic (butyric) acid in faecal samples of cows in oestrus compared to those in anoestrus. This may be due to the fact that Mozūraitis *et al.* (2017) measured butanoic acid in the faecal samples of oestrous-synchronised cows.

7.4.3. Long chain fatty acids

Dairy cow milk fat contains relatively greater amounts of long chain FA than non-fat constituents (Or-Rashid *et al.*, 2009). In the present study, only two of the long chain FA measured differed in concentrations between samples collected during oestrus and day 14. These were myristoleic and arachidonic acid, and while myristoleic acid was higher during oestrus, the concentration of arachidonic acid was higher on day 14 of dioestrus. Several other studies have shown differences in FA concentration in both faecal and urine samples of cattle. Myristoleic acid was also found to be in higher concentrations in the milk of oestrous cows in the present study. As far as I am aware, this is the first report of differences in milk myristoleic acid concentrations, and the reason for these differences remains to be elucidated. Similar to the findings of the present study, in a study conducted with Holstein heifers by Lukaszewska and Hansel (1980) found that the concentrations of arachidonic acid was higher in plasma on the day 18 of the oestrous cycle in heifers compared with pregnant cows. The results of the present study are also consistent with those of Gnanamuthu and Rameshkumar (2014) who found that arachidonic acid was present in faecal samples in dioestrus but not present on the day of oestrus in cows. These findings are consistent with the finding that there are increases in plasma arachidonic acid and PGF2 α before luteolysis in non-pregnant cows (Mattos *et al.*, 2000; Mattos *et al.*, 2003).

Another interesting finding of the present study is that arachidonic acid concentrations were lower in samples from PD+ cows compared to PD- cows on day 14 after oestrus.

The mammary gland has been shown to synthesise PGF₂α in goats (Walker and Peaker, 1981), and arachidonic acid is a precursor of PGF₂α (Mattos *et al.*, 2000). Lukaszewska and Hansel (1980) reported that the concentration of PGF₂α was lower in the uterine vein plasma of pregnant cows compared to oestrous cyclic cows during dioestrus. This may be due to luteal tissue converting arachidonic acid to PGF₂α and suggests that the products of the arachidonic acid cascade, produced within or accumulated by the CL, may have an important role in the regulation of the oestrous cycle (Lukaszewska and Hansel, 1980).

In the present study, the concentration of undecanoic acid tended to be lower on the day of oestrus compared to day 14. The lower concentration of undecanoic acid may be due to a NEB because undecanoic acid is generally involved in amino acid metabolism and fat metabolism (Li *et al.*, 2014). The results of the current study indicate that there is a tendency for there to be a greater concentrations of myristoleic, elaidic and lignoceric acids in milk on the day of oestrus compared to day 14 of dioestrus. Similarly, Kumar and Archunan (2006) also found that the urinary concentration of tridecanoic, myristic and pentadecanoic acids were significantly higher on the day of oestrus in comparison to the pre-pubertal and pregnancy periods in cattle. The results of the current study are consistent with those reported in a study conducted on Umblachery cattle by Gnanamuthu and Rameshkumar (2014) who found that myristic and gadoleic acid were only present on the day of oestrus, but were not present in pro-oestrus and dioestrus. The presence of certain FA in cow's milk in higher concentrations during oestrus compared to day 14 indicates there may be a role as a chemical signal to attract bulls.

From the results of the current study, there appear to be no differences in the concentrations of the other FA measured on the day of oestrus in comparison to day 14. However, in a recent study conducted by Toledo-Alvarado *et al.* (2018), the concentrations of milk myristic acid (C14:0) and palmitic acid (C16:0) were significantly lower on the day of oestrus compared to dioestrus. In addition, these authors reported that the concentrations of stearic acid (C18:0) and oleic acid (C18:1 *cis*-9) were higher on the day of oestrus compared to other phases of the oestrous cycle. This may reflect a large number of samples analysed in their study. Similar to the findings of the present study, Gnanamuthu and Rameshkumar (2014) found that there were no significant differences in the concentration of faecal stearic acid in oestrus and dioestrus cows. Also, Gnanamuthu and Rameshkumar (2014) reported the concentration of palmitic, elaidic and behenic acids in cattle faeces did not differ among pro-oestrous and dioestrous. In another study conducted in bovines (*Bos taurus*) by Kumar and Archunan (2006) analysing FA in urine, it was found that lauric, tridecanoic, myristic and stearic were present in pro-oestrous, oestrus and dioestrous samples but were not different in concentration. Although Megalac is a source of C16:0 and C18:1n9c FA (Scollan *et al.*, 2001), and DM intake has been

previously shown to be reduced during oestrus (Zebari *et al.*, 2018; chapter 4) C16:0 and C18:1n9c FA concentrations were similar during oestrus and day 14 in the present study.

7.4.4. Milk composition profile

From the results of the current study, total milk fat, protein, lactose and solids, as well as the fat/protein ratio, were not significantly different on the day of oestrus in comparison to day 14 of dioestrus. However, Gnanamuthu and Rameshkumar (2014) found that the total concentration of protein, carbohydrate and lipid in faecal samples on the day of oestrus were significantly higher in comparison to the pro-oestrous and the post-oestrous phase of the oestrous cycle in cows. Although in the results of the present study there were no differences in the composition of milk between oestrus and day 14 after oestrus, it has previously been reported that lipid (Poddar-Sarkar and Brahmachary, 1999; Kumar and Archunan, 2006) and protein (Zhou and Rui, 2010) in mammalian urine and faeces have an important role as a carrier of olfactory chemical signals in sexual attraction. Protein and lipids, therefore, may also have an important role as carriers for the ligands and for transportation of these chemical signals in mammals, while the role of milk carbohydrates in sexual attraction is unknown (Gnanamuthu and Rameshkumar, 2014).

7.5. Conclusion

This is the first study to show an increase in the concentrations of some milk FA during behavioural oestrus in dairy cows undergoing spontaneous oestrous cycles. Further research is needed to establish the potential for using milk FA profiles as part of an on-farm oestrous detection arsenal. In addition, the findings of the present study suggest that it may be possible to use the concentration of certain long-chain FA in milk to determine cows likely to return to oestrus following insemination.

CHAPTER 8

General discussion and conclusions

8.1. General discussion

8.1.1. The proportion of oestruses detected

The results of the study in Chapter 4, found that the percentage of oestruses detected by continuous camera recording was 65.5%. In addition, the percentage of oestruses detected by IceQubes, GEA Rescounter II, visual observation and tail paint were, 52.4%, 41.1%, 35.0%, and 15.0%, respectively (Chapter 4) of all oestruses which were detected during the study using milk P4 profiles. These results concur with the findings of other researchers (Isobe *et al.*, 2004; Roelofs *et al.*, 2005; Palmer *et al.*, 2010 and Dolecheck *et al.*, 2015). Milk P4 profiles were used to show oestrus cyclicity in Chapter 4, 5 and 6. Milk P4 measurements (Chapter 4, 5 and 6) suggested that approximately 40.1% of oestruses were silent and missed by the other methods used in the present studies. The results described in Chapter 4 also found that automated oestrus monitoring using IceQubes and GEA Rescounter II pedometer were better than visual observation or tail paint in continuously cubicle housed cows in a cubicle free-stall yard. These results are consistent with the findings of other researchers (Roelofs *et al.*, 2005; Dolecheck *et al.*, 2015).

The percentage of *behavioural* oestrus during 1st, 2nd and $\geq 3^{\text{rd}}$ oestrus PP were 55.2%, 72.7% and 80% respectively (Chapter 4). Based on IceQube accelerometer data, the studies presented in this thesis have shown that the percentage of behavioural oestrus was 52.4% in cubicle housed cows (Chapter 4) and, for cows that were put out to graze during the day (10:00 to 14:15) in the summer and continuously housed during winter was 55.6% (Chapter 5). The percentage of behavioural oestrus in cows housed with the main herd in a free stall yard (Chapter 6) was 71.8%. The percentage of behavioural oestrus among cows used in Experiment four which is described in Chapter 7 was higher, may be due to the cows being in their 2nd and 3rd oestrus PP. These results agree with those reported in a study conducted by Firk *et al.* (2002) and Isobe *et al.* (2004).

8.1.2. Oestrous duration

The studies presented in this thesis have shown that the duration of oestrus varied among different individual cows ranging between 6 to 24 h (Chapter 4, 5 and 6). These results concur with the findings of other researchers (Roelofs *et al.*, 2005; Sveberg *et al.*, 2011; Valenza *et al.*, 2012). In addition, the results presented in Chapter 4 shows that the average duration of oestrus in dairy cows varied depending on the method of oestrous detection. The duration of oestrus determined by activity monitor (IceQubes accelerometer and GEA Rescounter II pedometer) was found to be approximately 3 h longer than that detected by visual observation. In addition, the study discussed in Chapter 7 showed that the average oestrous duration was longer in oestruses of cows

that became pregnant following AI (15.5 h) compared to those that did not (10.1 h). The cows that became pregnant following AI also took more steps on the day of oestrus compared to cows that were PD- (Chapter 6). This may be because cows that showed longer and stronger oestrous activity were more accurately detected, following AI at the right time.

8.1.3. Oestrous activity

Restlessness is one of the most important secondary indicators of oestrus in cattle (Firk *et al.*, 2002; Roelofs *et al.*, 2005). A study conducted by Dolecheck *et al.* (2015) found that walking activity increases approximately 179% on the day oestrus compared to non-oestrus day. This thesis found that dairy cows took more ($P < 0.001$) steps on the day of *behavioural oestrus* compared to 3DB and 3DA among the cows used. However, on the proposed day of silent oestrus, there were no differences ($P = 0.225$) in the number of steps during the period of low milk P4 profile in comparison to 3DB and 3DA oestrus (Chapters 4 and 5). Concerning the number of steps taken on the day of *behavioural oestrus*, more ($P < 0.001$) steps were recorded on the day of oestrus in the study described in Chapter 5 (3867 ± 243 steps), about 1.5 times higher compared to the results of the studies described in Chapters 4, 6 and 7 (2095 ± 217 , 2831 ± 224 and 2714 ± 213 steps, respectively). However, the same proportion of increase in the number of steps taken on the day of behavioural oestrus compared to day 14 after oestrus were recorded in the studies described in Chapters 5, 6 and 7. However, there was less of an increase in the number of steps between oestrus and day 14 after oestrus in the cows used in Chapter 4 compared to those used in the studies described in Chapters 6 and 7. This may be due to the space per cow which was about 6 times more for the cows used in the studies described in Chapters 6 and 7 compared to those that were used in the study described in Chapter 4. In contrast a greater increase in the number of steps was recorded in Chapter 5. This may be due to the cows that were used in experiment two (described in Chapter 5) being put out to graze during the day (10:00 to 14:15) in the summer months, while the cows used in the study described in Chapter 4 were housed continuously in cubicle housing (Figure 8.1). These results are supported by Palmer *et al.* (2010) who found that housed cows express less activity during oestrus compared to cows that are at pasture.

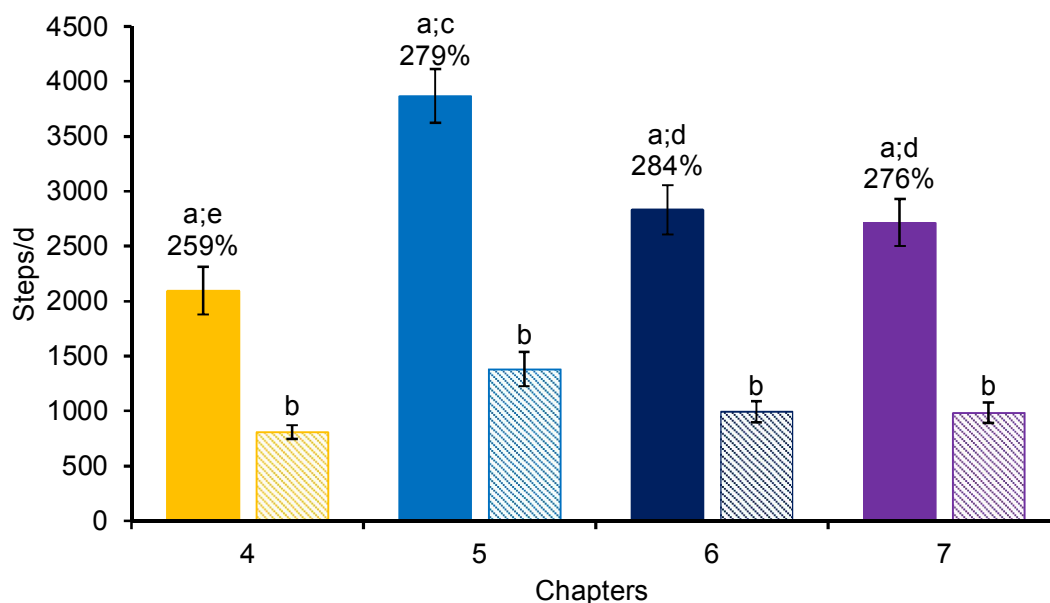


Figure 8. 1. Mean (\pm sem) number of steps/d, on the day of behavioural oestrus (D0; solid bar) compared to day 14 (D14; striped bar) after oestrus in Chapter 4 ($n = 40$; ■), Chapter 5 ($n = 138$; ■), Chapter 6 ($n = 28$; ■) and Chapter 7 ($n = 32$; ■) in lactating Holstein Friesian dairy cows. The percentage difference in step count (D0 vs D14) is shown above the D0 bar. Letters (a vs b) indicate significant difference ($P < 0.05$) means number of steps between the D0 and D14. Letters (c vs d; c vs e) indicate significant differences ($P < 0.05$) in the number of steps taken on the D0 in each of the 4 studies.

During the day of behavioural oestrus, cows spent less time lying (7.1 ± 0.3 h/d; Chapter 4 and 6.8 ± 0.3 h/d; Chapter 5) and had fewer lying bouts (9.1 ± 0.5 bouts; Chapter 4 and 7.0 ± 0.3 bouts; Chapter 5) in comparison to the day of *silent oestrus* (lying time: 9.3 ± 0.5 h/d; Chapter 4 and 9.5 ± 0.2 h/d; Chapter 5; lying bouts: 13.0 ± 1.1 bouts; Chapter 6 and 10.3 ± 0.4 bouts; Chapter 5). These results concur with those found in a recent study conducted by Dolecheck *et al.* (2015) using oestrous-synchronised Holstein cows. The present thesis found that cows which had access to pasture (Chapter 5) spent less time lying with fewer lying bouts compared to cows that were continuously housed in cubicle housing in the study described in Chapter 4, and these results are consistent with those found by Palmer *et al.* (2010).

8.1.4. Feeding behaviour and milk yield (kg/d)

In addition to the changes in activity, reduced DMI and time spent feeding (Reith *et al.*, 2014) can be used as an indicator of oestrus (Reith and Hoy, 2012). Although present dairy farmers do not recording feeding behaviour, while in the future farmers may use DM intake sensor technologies, such as Smart Bow (Smart-bow GmbH Weidern, Jutogasse,

Austria) to monitor cow rumination during oestrus, as an indicator of oestrus. As the dairy cow in oestrus spends more time walking in comparison to resting and feeding time (Hurnik *et al.*, 1975), this disrupts her normal daily time budget (Valenza *et al.*, 2012; Dolecheck *et al.*, 2015). The study described in Chapter 4 found that DMI was lower on the day of *behavioural oestrus* (19.8 ± 0.41 kg/d) in comparison to 3DB (22.4 ± 0.5 kg/d) and 3DA (22.6 ± 0.5 kg/d). The occurrence of *behavioural oestrus* reduced the mean duration of feeding and the number of visits to the RIC feed compared to 3DB and 3DA. These data suggest that increased activity at oestrus diverts cows from their normal time budget with more steps replacing both feeding and resting time (Walker *et al.*, 2008b). This is exacerbated in more active cows which had a greater reduction in DMI demonstrated by the negative correlation between the number of steps taken and DMI ($y = -0.0014x + 22.46$; $r^2 = 0.46$) during the day of *behavioural oestrus* (Figure 8.2).

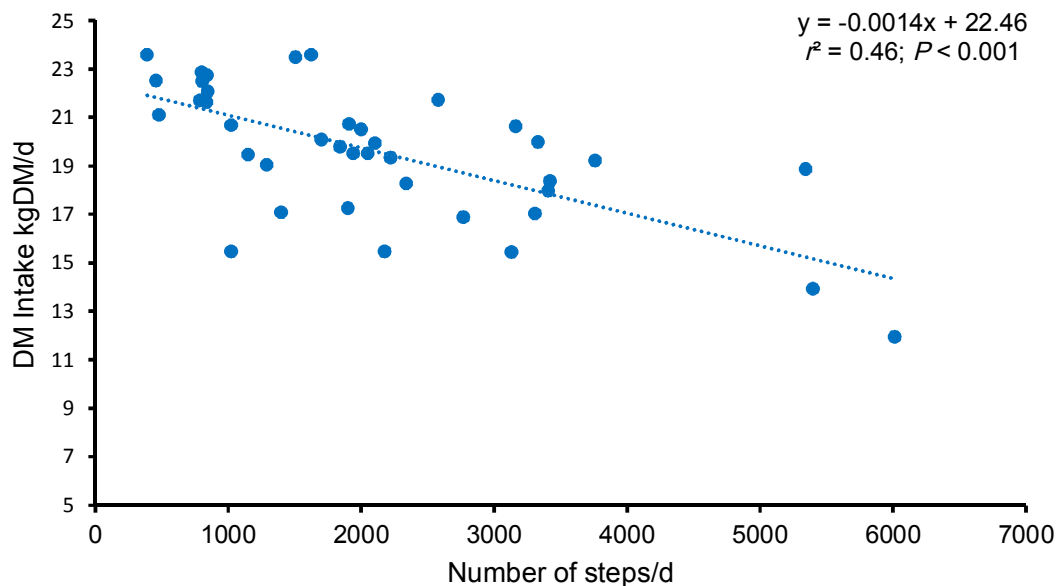


Figure 8. 2. The relationship between DMI (kgDM/d) and the number of steps/d recorded by IceQube accelerometers on the day of behavioural oestrus in lactating Holstein Friesian cows ($n = 40$).

The results presented in Chapter 6 show that milk production was reduced ($P < 0.001$) by approximately 11% on the day of *behavioural oestrus* in comparison to non-oestrus days, while no significant reduction in milk yield was found in cows with *silent oestrus*. These results are supported by studies conducted by Hurnik *et al.* (1975) and Gwazdauskas, *et al.*, (1983). A reduction in milk yield in lactating dairy cows on the day of oestrus may be due to lower feed intake, from the results presented in Chapter 4. Further to this, the results presented in Chapter 5 found a negative ($P < 0.001$) correlation between the number of steps taken and milk yield on the day of behavioural oestrus. The results presented in Chapter 6 confirmed this and also found a negative ($P < 0.001$) relationship

between milk yield and the concentrations of E2 in milk, suggesting E2 stimulates and increase in activity, resulting in lower milk yields. Alternatively, E2 has been shown to suppress feed intake because an increase in circulating E2 leads to substantially reduce appetite (Ingvarsen and Andersen, 2000).

8.1.5. Factors affecting oestrous expression

As described above, these studies highlighted the great variation in oestrus duration and activity of individual cows and of the same cow with time PP. Traditionally, on dairy farms, cows were visually observed for oestrous detection three times daily for periods of 20 to 30 min (Van Eerdenburg *et al.*, 2002). The primary and most definitive sign of oestrus is standing to be mounted. However, the results presented in Chapter 4 found that only 50% of dairy cows showed this behaviour and also only 52.4% of cows can be detected in behavioural oestrus using IceQube accelerometer. These results concurs with those found by previous studies (At-Taras and Spahr, 2001; Roelofs *et al.*, 2005; Palmer *et al.*, 2010).

Many factors contribute to oestrous expression in dairy cattle, which results in poor oestrous detection (Orihuela, 2000). The effects of cow factors such as a number of cows in oestrus simultaneously, BCS, LS, parity and milk yield, and environmental factors like season and day length which can affect oestrous activity were explored in Chapter 5a and 5b.

One of the notable findings of the present studies was the significant effect of the number of cows in oestrus simultaneously. Significantly more ($P < 0.001$) steps were recorded when ≥ 3 cows were in oestrus compared to when 2 and 1 cows were in oestrus simultaneously (Figure 8.3). These results are supported by the findings of the experiment described in Chapter 4. As described in Chapter 5a (section 5a.4.3), an increase in activity with an increased number of cows in oestrus may be due to an increase in walking around sexual partners during oestrous periods as a result of sniffing the anogenital region of herd mates, chin-resting, flehmen, attempts to mount and mounting (Van Vliet and Van Eerdenburg, 1996), which are reported in observational studies of cows in oestrus (Van Eerdenburg *et al.*, 2002).

There was also a significant effect of BCS on the number of steps taken on the day of behavioural oestrus. Cows that had BCS 2.75 took more ($P < 0.001$) steps on the day of behavioural oestrus compared to cows that had BCS ≤ 2.5 and BCS ≥ 3 (Chapter 5). These results are supported by the findings of the experiments described in Chapter 4, Chapter 6 and Chapter 7 (Figure 8.4). As described in chapter 5a (section 4.4), the lower activity of cows that had a BCS ≤ 2.5 or BCS ≥ 3 may be due to the NEB of the cows that had BCS ≤ 2.5 and the heavy body weight of cows that had BCS ≥ 3 (Madureira *et al.*, 2015).

However, there were no significant differences between cows that had BCS ≤ 2.5 and cows that had BCS ≥ 3 with regards to the number of steps taken on the day of behavioural oestrus.

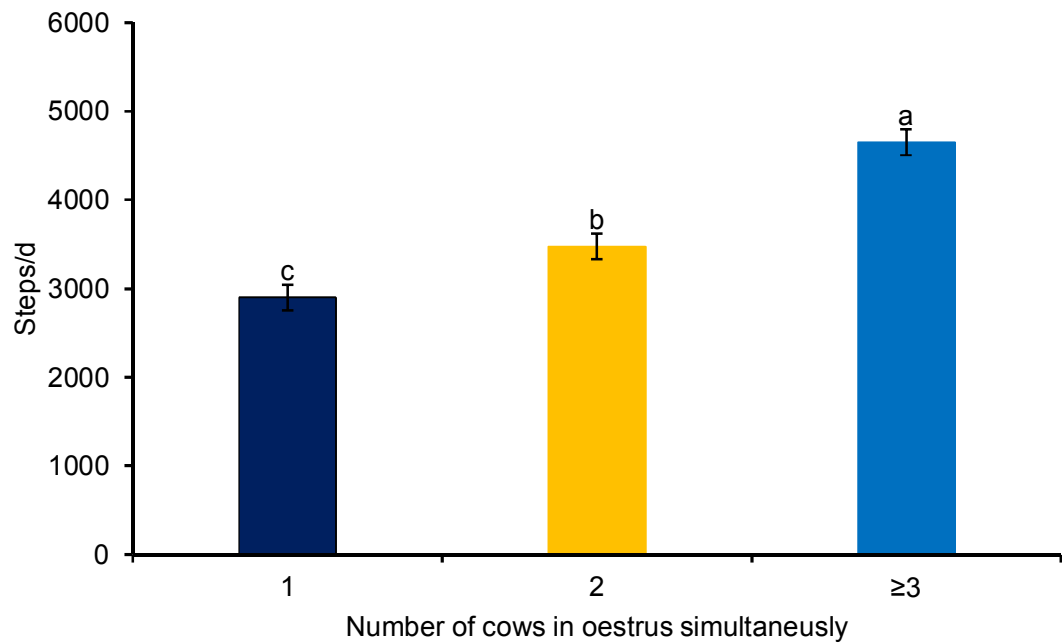


Figure 8. 3. Mean (\pm sem) number of steps/d, on the day of behavioural oestrus between the number of cows in oestrus at the same time (SG 1; $n = 36$, SG2; $n = 52$ and SG ≥ 3 ; $n = 50$) in lactating Holstein Friesian cows ($n = 40$). Letters (a vs b) indicate significant differences ($P < 0.05$) in the mean number of steps with the number of cows in oestrus at the same time (SG 1, SG2 and SG ≥ 3).

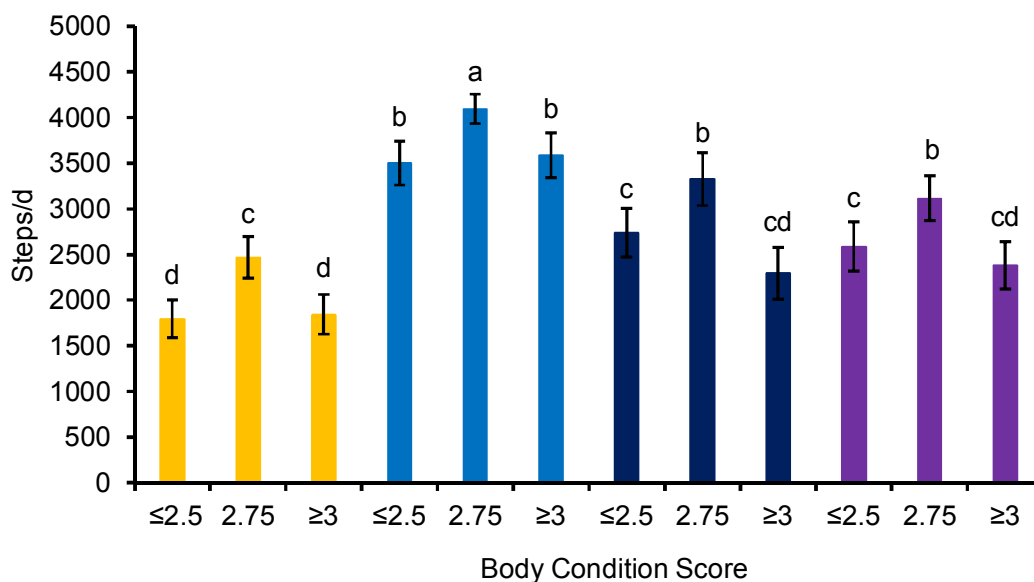


Figure 8. 4. Mean (\pm sem) number of steps/d during the day of behavioural oestrus of lactating Holstein Friesian dairy cows with different BCS, Chapter 4 (BCS ≤ 2.5 ; $n = 9$, BCS = 2.75; $n = 17$ and BCS ≥ 3 ; $n = 14$; ■), Chapter 5 (BCS ≤ 2.5 ; $n = 22$, BCS = 2.75; $n = 81$ and BCS ≥ 3 ; $n = 35$; ■), Chapter 6 (BCS ≤ 2.5 ; $n = 10$, BCS = 2.75; $n = 10$ and BCS ≥ 3 ; $n = 11$; ■) and Chapter 7 (BCS ≤ 2.5 ; $n = 9$, BCS = 2.75; $n = 12$ and BCS ≥ 3 ; $n = 11$; ■). Letters (a, b, c and d) indicate significant differences ($P < 0.05$) in the number of steps taken on the day of behavioural of cows with different BCS of the 4 studies.

The present studies also found there was an effect of LS on the number of steps taken on the day of behavioural oestrus. Cows which were LS 1 took more ($P < 0.001$) steps (6406 ± 178 steps) compared to cows which were LS 2 (3780 ± 133 steps) and LS 3 (3363 ± 202 steps). This also agrees with Chapters 4, 6 and 7 (Figure 8.5). As discussed in Chapter 5a (section 4.5), these results also concurred with the findings reported by Walker *et al.* (2010) who found that non-lame cows recorded a higher total points score of oestrus behaviour signs than lame cows. On the day of non-oestrus (normal day), cows which were LS1 also took more ($P < 0.001$) steps (1862 ± 91 steps) compared to cows that were LS2 or LS3. Cows which were LS2 also took more ($P < 0.001$) steps (1635 ± 82 steps) compared to cows that were LS3 (1343 ± 110 steps). This was as expected as lameness is a chronically painful and stressful condition this has previously been associated with poor reproductive performance (Collick *et al.*, 1989). This may be due to the fact that stress hormones has a negative impact on the reproductive hormones from the hypothalamus-pituitary-ovarian axis (Moberg, 1985; Dobson *et al.*, 2003). In addition, an earlier study revealed that in chronically stressed, lame cows, low P4 exposure before oestrus was associated to the low intensity of sexual behaviours during oestrus in dairy cattle (Dobson *et al.*, 2008).

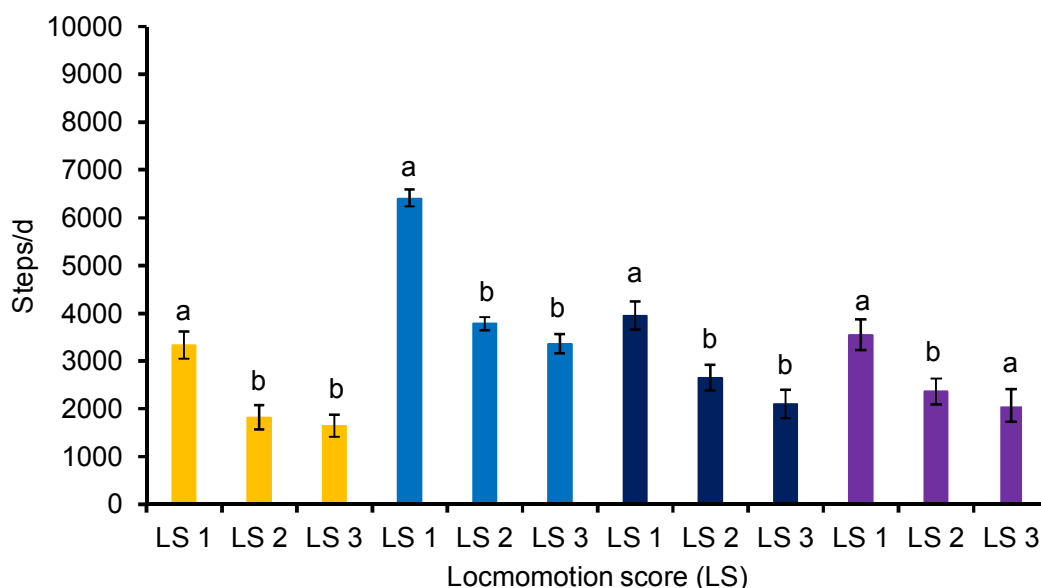


Figure 8. 5. Mean (\pm sem) number of steps/d during the day of behavioural oestrus of lactating Holstein Friesian dairy cows with different LS, Experiment one (LS 1; $n = 9$, LS 2; $n = 19$ and LS 3; $n = 12$; ■), Experiment two (LS 1; $n = 39$, LS 2; $n = 69$ and LS 3; $n = 30$; ■), Experiment three (LS 1; $n = 7$, LS 2; $n = 15$ and LS 3; $n = 7$; ■) and Experiment four (LS 1; $n = 11$, LS 2; $n = 15$ and LS 3; $n = 6$; ■). Letters (a vs b) indicate significant differences ($P < 0.05$) in the number of steps taken on the day of behavioural oestrus with different LS in each of the 4 studies.

8.1.6. Milk E2 and fatty acid profiles

The results discussed in Chapters 4 and 5 showed a great variation in the number of steps taken on the day of oestrus between cows and that the duration of oestrus varied among different individual cows ranging between 6 and 24 h. Therefore, it seems to be important to investigate the physiological reason for this variation. The experiment described in Chapter 6 was designed to investigate the relationships between milk E2 concentrations and activity on the day of behavioural and silent oestrus. The results of Chapter 6 revealed that the concentrations of E2 (pg/mL) in milk were increased by about 300% on the day of behavioural oestrus compared to other days. Regarding *silent* oestruses, in Chapter 6, milk E2 concentrations increased by 75.8% compared to other days. These results are supported by the findings of previous studies conducted by Aungier *et al.* (2015) and Madureira *et al.* (2015) who also found plasma E2 concentrations to be higher in cows that show greater activity on the day of oestrus. While ovarian follicle development was not measured in this study, others (Beam and Butler, 1999; Butler, 2003) have shown that the concentrations of circulating E2 increase with the increasing size of the developing ovarian follicle. Therefore, lower concentrations of E2 on

the day of silent oestrus (Chapter 6) may be due to follicle size because follicle diameter has a positive relationship with the peak concentration of E2 (Perry *et al.*, 2014). Either way, the results in Chapter 6 suggest that lower concentrations of E2 leads to decreased expression of oestrous activity in keeping with the role of E2 in stimulating oestrous behaviour (Mondal *et al.*, 2006). Step-wise multiple linear regression analysis indicated that on the day of behavioural oestrus, the number of steps taken were negatively ($P < 0.001$) associated with milk yield (kg/d) while the number of steps taken on the day of behavioural oestrus were positively ($P < 0.001$) associated with milk E2 concentrations (Chapter 6; see Table 8.1).

Table 8. 1. The relationship between the number of steps on the day of behavioural oestrus and milk yield (kg/d) and milk oestradiol (pg/mL).

Response variables	Explanatory variables			r^2	SE	P-value
	Constant	Milk yield (kg/d)	Oestradiol (pg/mL)			
Steps	5591 (se=790.0)	-87.1 (se=24.7)		0.301	1009	<0.001
Steps	2009 (se=715.0)	-12.4 (se=18.8)	5591 (se=790.0)	0.722	626	<0.001

SE = Standard error of observation

Some published studies have shown greater concentrations of FA in urine (Kumar and Archunan, 2006), faeces (Sankar and Archunan, 2008; Gnanamuthu and Rameshkumar, 2014; Mozūraitis *et al.*, 2017), vaginal mucus (Rajanarayanan and Archunan, 2011) and saliva (Sankar *et al.*, 2007) in oestrous cattle. The appearance of FA at greater concentrations during oestrus may be related to the greater concentrations of circulating steroid hormones and/or may be involved in attracting the opposite sex i.e. acting as pheromones (Kumar and Archunan, 2006). Also, higher levels of acetic acid may reflect as a precursor of E2 (Robinson *et al.*, 2002). The results presented in Chapter 7 show that on the day of oestrus, the concentration of acetic acid was higher in milk compared to 14 days after oestrus. The results described in Chapter 7 also revealed that there was a positive ($P < 0.001$) relationship between the concentration of acetic acid in milk and the number of steps taken on the day of oestrus ($y = 0.03x^2 - 9.68x + 2886.8$; $r^2 = 0.40$). However, at the time of the study presented in Chapter 7, FA had not been quantified in the milk of oestrous cows. Since this study was undertaken Toledo-Alvarado *et al.* (2018) have published a study that reports milk FA levels in agreement with the results presented in Chapter 7. However, they found a high level of stearic acid and oleic acid on the day of oestrus, but they found a lower level of myristic acid and palmitic acid on the day of oestrus compared to dioestrus.

8.2. General conclusions

In summary, the studies presented in this thesis have concluded that:

- 1- On the day of behavioural oestrus, high yielding dairy cows in cubicle housing spend more time walking, less time lying down and have a reduced number of lying bouts, but none of these parameters was affected during silent oestrus.
- 2- On the day of behavioural oestrus, DM intake, feeding duration and number of visits to feed were reduced. However, on the day of silent oestrus, only feeding duration was reduced. Technologies that facilitate the on-farm measurement of feeding duration could potentially be used to help farmers detect silent oestrus in their cattle.
- 3- On the day of oestrus, when more cows were in oestrus at the same time, they took more steps and spent less time resting with a lower number of lying bouts.
- 4- Cows which were BCS 2.75 at behavioral oestrus spent more time active at the expense of the lying time compared to cows which were BCS ≤ 2.5 and BCS ≥ 3 . On the day of oestrus more steps but lower lying time and fewer lying bouts were recorded in cows that were parity ≤ 2 . On the day of oestrus, the number of steps taken increased while lying time and the number of lying bouts increased with increasing LS.
- 5- On the day of behavioural oestrus, the number of steps was higher in cows with higher milk E2 concentrations. While on the day of silent oestrus, although milk E2 was increased, there was no concurrent change in activity or milk yield.
- 6- There were increases in the milk concentrations of some fatty acids during the immediate peri-oestrus period. However, arachadonic acid was higher on day 14.

8.3. Recommendations for practical application

Poor oestrous detection remains one of the major problems in the dairy industry. To improve oestrous expression in dairy cows for increased oestrous detection rates in the dairy herd, it is important to keep cows in BCS at 2.75 and LS1 by providing better daily TMR *ad libitum* and using a good management system e.g. good health system and effectively manage cows locomotion problems by preventative foot care measures and developing the best forms of treatment that fit in well with routines on the farm. In addition, an increase in the number of cows in oestrus simultaneously also helps in the detection of oestrus for cows coming into oestrus or recently have been in oestrus. Using activity monitoring for oestrous detection is also recommended to be used in dairy cattle farms. However, the results of the first experiment (Chapter 4) found that using tail paint or scratched card are less reliable for oestrous detection in cubicle-housed dairy cows.

Future directions

The results of the present thesis revealed that there was a great variation in oestrous expression between cows and a high percentage of silent oestrus in lactating dairy cows. The oestrous detection sensor technologies are moving fast due to the need for oestrous detection in dairy cows and to improve the timing of insemination. Therefore, the following further research will be beneficial to increase oestrous detection rates in lactating dairy cows:

1. Investigate oestrous expression in pasture *versus* housed dairy cows, in dairy cows being managed through an automated milking system and in nulliparous heifers. The study can utilise around 30 primiparous cows at pasture and housed to investigate a comparison of the oestrous behaviour of Holstein Friesian cows at pasture and when housed, from calving to established pregnancy in approximately 4 oestrous cycles spontaneously by using IceQubes, visual observation and tail paint or scratch card.
 - 1.1. Milk hormone profile strategic testing, cow localisation and sensor technology that can measure feeding duration for the detection of silent oestrus in high yielding dairy cows in the Robotic Milking System.
 - 1.2. Identify cows that are silent by using milk hormone profiles such as P4, E2 and cortisol and Smart Bow for localisation of cows in the Robotic Milking System.
 - 1.3. Analysing milk samples for E2 using an enzyme-linked immunosorbent assay kit (ELISA; ALPECO, Salam, NH 03079, USA) and cortisol concentration using an enzyme-immunoassay (Enzo Life Sciences, Inc, Farmingdale, USA).
 - 1.4. Using automated IceQube activity monitors (IceRobotics Ltd., Edinburgh, UK) to measure the following parameters: Number of steps, Lying time, Lying bouts, Motion index.
 - 1.5. Using Smart Bow (Smart-Bow GmbH Weidern, Jutogasse, Austria) to monitor cow location and rumination during oestrus.
 - 1.6. Determine arachidonic acids in milk to predict cows returning to oestrus.
2. Another study that would be beneficial to carry out on Holstein Friesian nulliparous heifers to determine the behavioural changes such as lying time, lying bouts, number of steps and standing time during oestrus by using IceQubes, visual observation and serum hormonal profiles (E2 and P4 concentrations) together with tail paint in approximately 4 oestrous cycles from puberty to established pregnancy to be able to maximize the rate of oestrous detection and to capture more indicators of oestrus.

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Appendix 1. Typical milk P4 profiles shows *behavioural* (generated heat by IceQube; ) and *silent* (non-generated heat by IceQube; ) oestrus in lactating Holstein Friesian dairy cows.

