Stress and subfertility in dairy cows

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Michael Jerome Morris

May 2008

Declaration

Unless otherwise acknowledged, this thesis is all my own work, carried out in the Department of Veterinary Clinical Science, University of Liverpool Leahurst, Neston South Wirral, UK under the supervision of Professor Hilary Dobson. No part of this thesis, in any form, has been submitted to any other university or for any other degree.

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Abstract

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In commercial dairy cattle farming, stressful disease conditions, such as high somatic cell count (SCC), poor body condition score (BCS) or lameness, are associated with subfertility. The objective of this thesis is to identify key areas in the reproductive cycle associated with each condition which contribute to this subfertility.

Cows 30-80 days post partum from two commercial dairy farms were scored for SCC, BCS and lameness over a 5 week period prior to the implementation of an oestrous synchronization programme. Blood and milk sampling, trans-rectal ultrasonography and oestrus behaviour monitoring were conducted.

Follicular development from emergence to deviation was unaffected as all cows produced a dominant follicle, which continued to grow beyond 10mm. In the following period, mean follicular growth, maximum follicular diameter and time to ovulation were not influenced by any of the 3 conditions ($p > 0.05$). In the animals that ovulated (irrespective of clinical status), follicles grew faster and achieved a greater diameter than in the animals that failed to ovulate ($p < 0.05$). Fewer lame animals ovulated compared to healthy animals ($p < 0.05$) and fewer high SCC animals with concurrent lameness ovulated than cows with only high SCC ($p < 0.05$)

In cows with high SCC, progesterone concentrations prior to PG injection, over the peri-ovulatory period, on Days 5 and 7 or during the mid luteal phase after ovulation were unaffected, as was mean oestradiol concentration prior to ovulation. In the cows that ovulated, the dominant follicle grew at the same rate and ovulated at a similar size and at the same time regardless of high SCC, lameness or both. High SCC cows tended to ($p < 0.07$) and lame cows did express an oestrus of lower intensity and had a lower maximum intensity score than healthy animals ($p < 0.05$). High SCC cows started displaying oestrus signs and stood to be mounted (STBM) later than their healthy counterparts ($p < 0.05$) while lame animals displayed oestrus and STBM earlier than non lame cows ($p < 0.05$).

In lame cows, progesterone concentration prior to the follicular phase (Days -5 to 0 PG) was lower than in normal animals ($p < 0.05$). In the following mid luteal phase, ovulating animals had a higher progesterone concentration than those that failed to ovulate ($p < 0.05$). BCS had no effect on progesterone concentration throughout the cycle ($p > 0.05$). In animals bled every 15mins, all those that ovulated had a prior LH surge and all non ovulating animals had no LH surge. Maximum LH surge concentration was similar in lame and healthy cows ($p > 0.05$). LH pulse frequency and oestradiol concentration was greater in ovulating animals than in non ovulating ones ($p < 0.05$).

Supplementation with exogenous progesterone during synchronization did not influence follicular growth and ovulation nor did it influence progesterone or oestradiol concentrations at specific times in the oestrous cycle. Monitoring oestrus behaviour identified time relationships between STBM and the active behaviours: Sniff, Chin Rest and Rear Mount ($p < 0.05$). There were also positive correlations between the frequencies of STBM and the passive behaviours: Sniffed and Chin Rested ($p < 0.05$).

In conclusion, this thesis identifies the specific areas in the reproductive cycle by which the common management disease conditions of high SCC, poor BCS and lameness lead to poor oestrus expression and subfertility in dairy cows in the UK.

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Finally, I would like to thank God that this PhD is done!

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Abbreviations

A4 - androstenedione

DF- dominant follicle

FSH- follicle stimulating hormone

GnRH – Gonadotrophin releasing hormone

IFN-Tau - Interferon Tau

IGF - insulin-like growth factor

IGFBP- insulin-like growth factor binding proteins

LH – luteinizing hormone

LPS- Lipopolysaccharide

PG - Prostaglandin F2α

Literature Review

Stress and Subfertility: Stress can be defined as a change in an animal's environment that prevents expression of full genetic potential (Dobson et al., 2000a). Many dairy cows disease conditions adversely affect subsequent fertility: cystic ovarian disease (Oltenacu et al., 1984); milk fever (Zamet et al., 1979); retained placenta (Borsberry et al., 1989; Kaneko et al., 1997) and uterine infection (Kinsel et al., 1998; Sheldon et al., 2000). High somatic cell count (SCC), low body condition score (BCS) and lameness have been selected for investigation due to their high incidence in the dairy industry. High SCC is easily measurable and consistent (Jayarao et al., 2004) and BCS scoring relies on a robust, repeatable scale (Edmonson et al., 1989; Chamberlain et al., 1996); fig 1. Lameness is a relevant clinical stress 'model' because the onset of pain can be precisely pin-pointed by changes in behaviour and also because there is a well accepted scoring system for severity (Murray et al., 1996; Sprecher et al., 1997a); see fig 2).

The stress of clinical mastitis has an adverse effect on dairy cow fertility (Schrick et al., 2001). The resumption of ovarian cyclicity after calving is delayed by an extra 7 days in mastitic cows (Huszenicza et al., 2005). Also, calving-to-pregnancy interval is increased in mastitic cows to 18 days in another study (Borsberry et al., 1989) and spontaneously occurring mastitis increased the number of days open and reduced pregnancy rates (Santos et al., 2004). There is need to investigate the effect of high SCC on follicle development despite the findings of one study which showed that experimentally-induced mastitis does not affect maximum follicle diameter (Hockett et al., 2000). Once ovarian cyclicity has resumed, the ability to express oestrus is also important. Previous work by our group shows a reduced oestrus intensity due to lameness (Walker et al., 2008) and it can be theorized that there may a similar effect due to mastitis/high SCC.

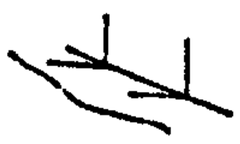
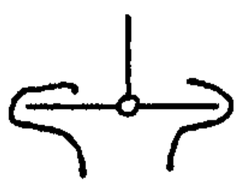
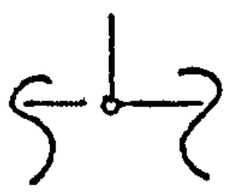
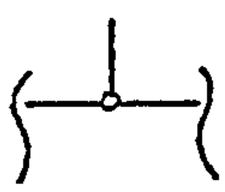
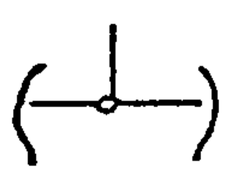
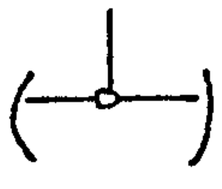
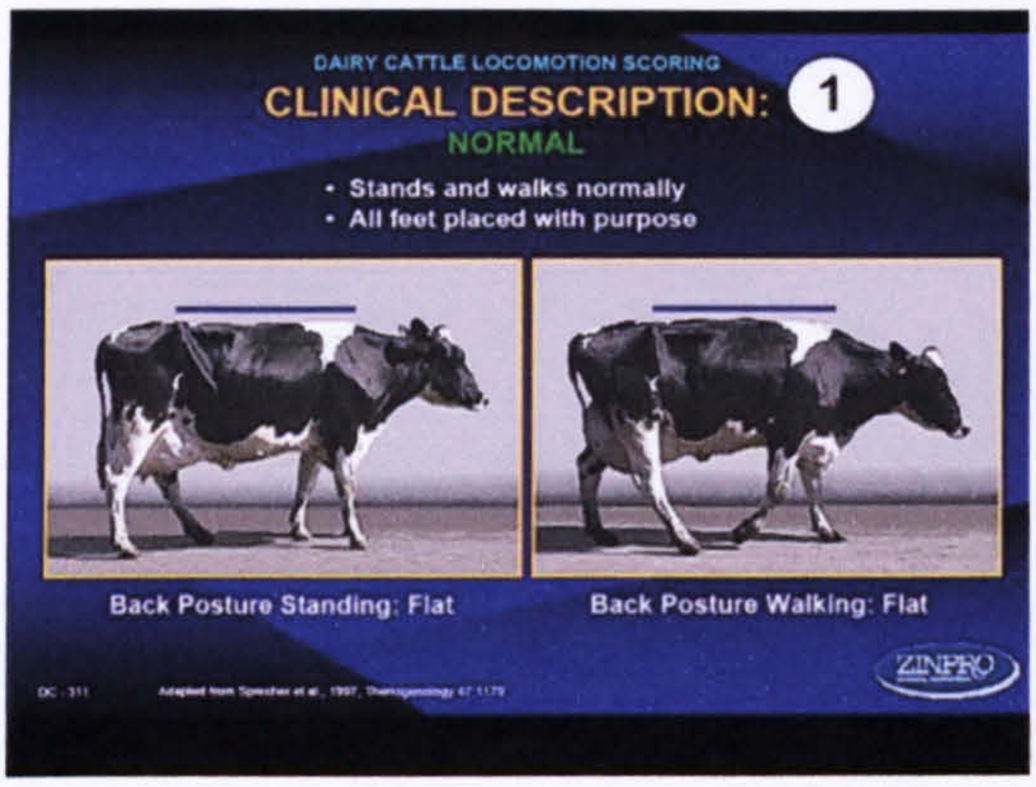
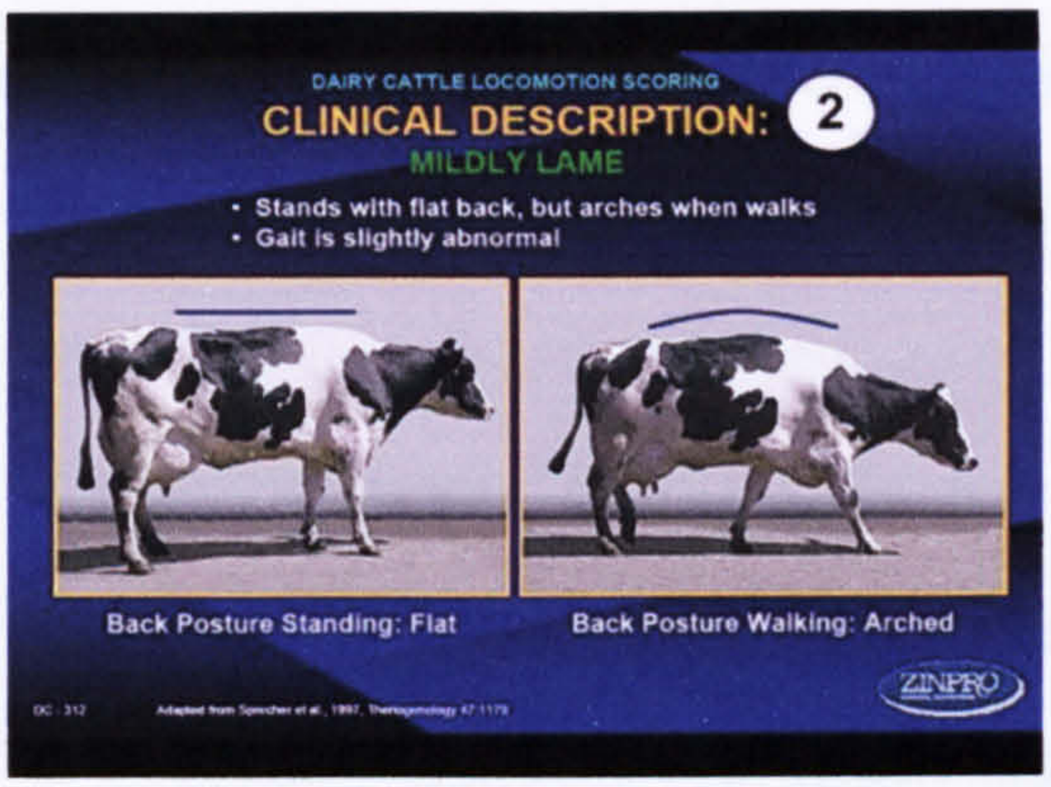
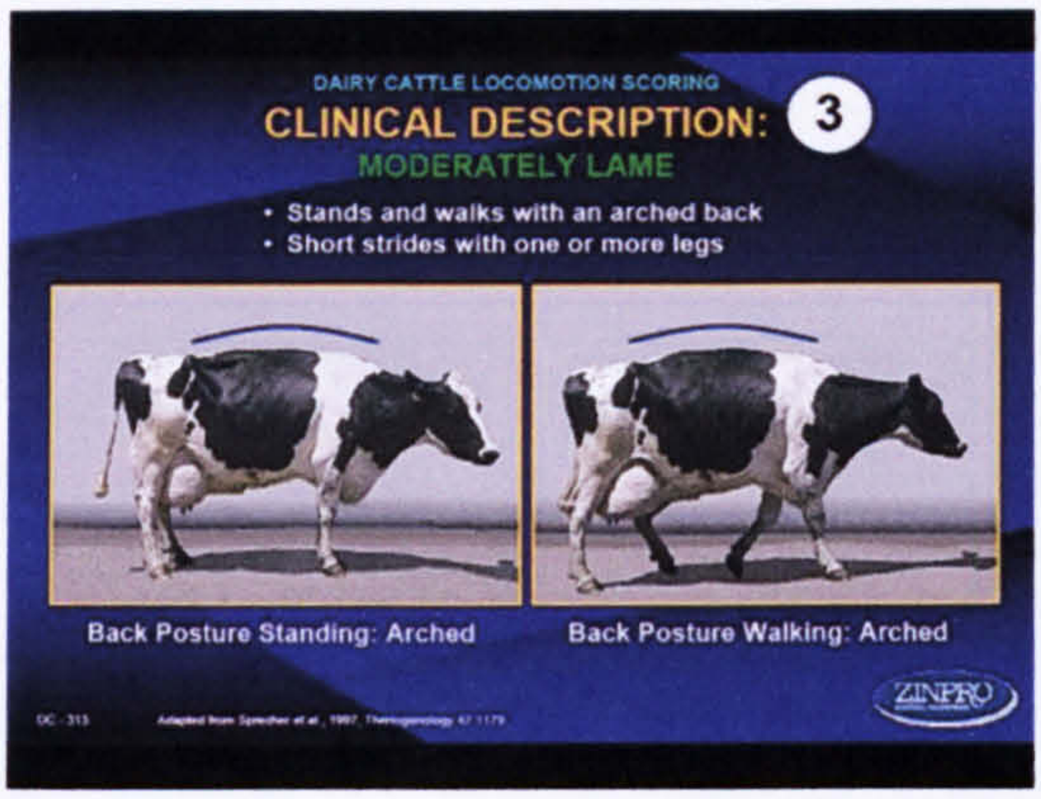
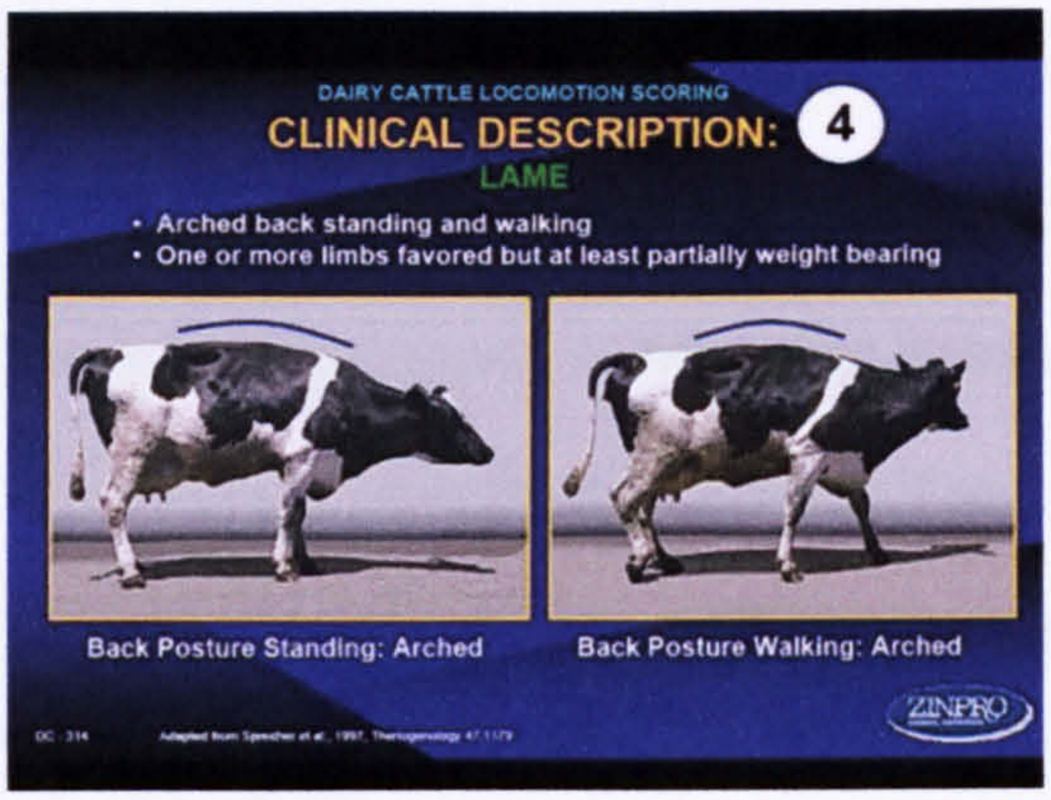
	1	2	3	4	5
General	Emaciated Frame protruding	Moderate condition Frame obvious	Good condition Frame and covering well balanced	Fat Covering more obvious than frame	Obese Severe over-conditioning
Lumbar area Vertebral spinous processes	Sharp ends Little flesh	Easily discernible	Smooth ridge Individual processes not visible	Not discernible Covering almost flat	Buried in fat
					
Transverse processes	Half length visible	Third of length visible	Quarter of length visible	Not discernible Smooth, rounded edge	Buried in fat
					
Transition between transverse processes and para-lumbar fossa	Prominent shelf, gaunt	Overhanging shelf	Slight shelf	None	Bulging
					
Palpation	Transverse processes and vertebral bodies feel sharp	Transverse processes feel sharp	Need to apply pressure to feel transverse processes Smooth	No bones palpable	No bones palpable
Tail head Sacral vertebrae	Individual vertebrae distinct	Individual vertebra not visible	Smooth covering	Smooth covering	Smooth covering
Hook bones (Tuber coxae) P in bones (Tuber ischii) Between hook and pin bones	Extremely sharp No tissue cover Severe depression	Prominent Very sunken	Smooth Depression Little fat deposition	Rounded by fat Slight depression	Surrounded by fat Flat
					
Between hook bones	Severely depressed No flesh	Very depressed	Moderate depression	Flat	Rounded
					
Tailhead	Deep V-shaped cavity under tail	U-shaped cavity under tail	Shallow cavity under tail	Slight depression	Folds of fatty tissue under tailhead.
					
Palpation	No fatty tissues Skin drawn tight over pelvis	Some fatty tissue	Can feel pelvis with slight pressure; fatty tissue over whole area	Bones difficult to feel	Bones difficult to feel

Fig 1 Body condition scoring chart demonstrating scores 1-5 (Edmonson et al., 1989)









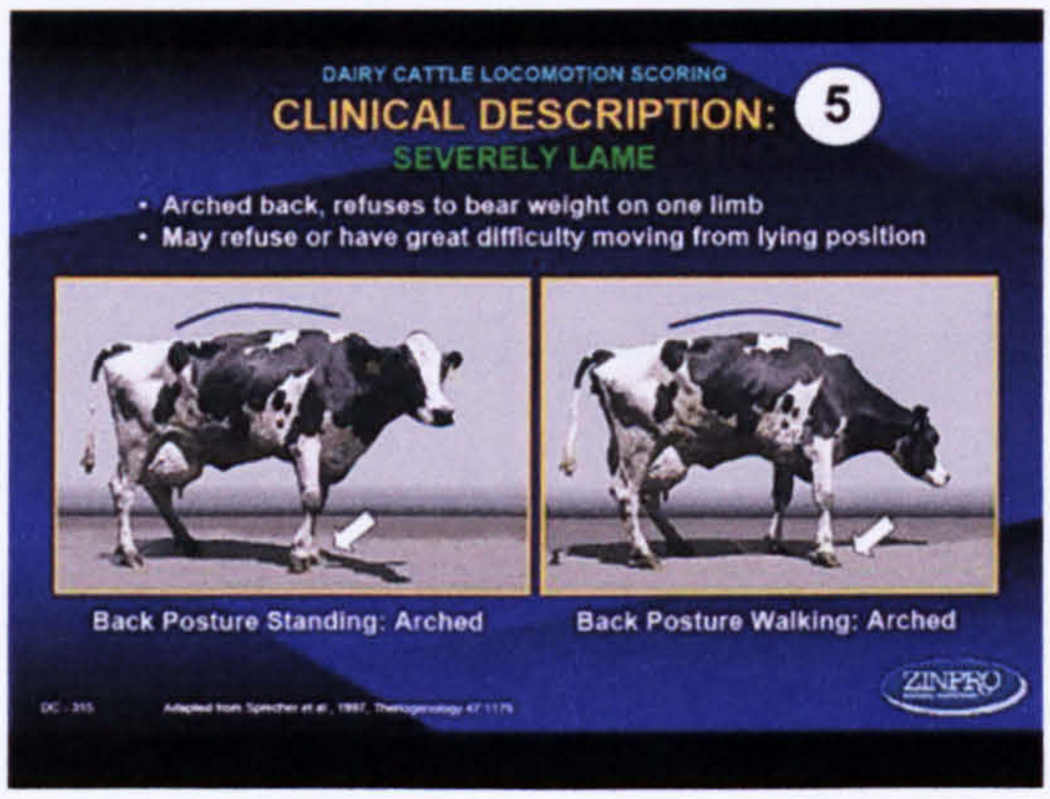


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Fig. 1 Pictures displaying clinical lameness descriptions based on a previously described 5-point scale (Sprecher et al., 1997b). In the following thesis, any cow with an average score of < 1.5 was considered to be nonlame and animals ≥ 1.5 were considered to be lame. Pictures used with the kind permission of Zinpro Animal Nutrition©

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In a commercial farming environment, subclinical mastitis is an economically important condition (Huijps et al., 2008) which may be investigated separately from the clinical situation.

Differing levels of nutrition affect dairy cow fertility (Beam et al., 1999). We know that low BCS in the early postpartum period results in >10 extra days to establish a pregnancy (Lopez-Gatius et al., 2003, Garnsworthy 2006). Similarly, cows in nutritionally-induced anoestrus have smaller dominant follicles (Rhodes et al., 1996). Consequently, there is the possible theory that these smaller follicles are either less likely to ovulate or if indeed ovulation does occur, the resultant ovum released has reduced viability. The investigation of the mechanisms involved with low BCS induced subfertility requires use of an average score over a 5 week period in order to accurately calculate the status of this chronic condition.

Lameness is one of the three major economic diseases affecting dairy cattle in the UK, along with mastitis and subfertility (Whitaker et al., 1983; Kossaibati et al., 1997). The incidence of lameness varies from 17% to 25% (Whitaker et al., 1983) (Esslemont et al., 1996) and the economic impact of lameness is heightened by its secondary role in detrimentally affecting fertility.

Several studies have shown that there is an association between lameness and sub-fertility in dairy cows. Lameness occurring between 36 to 70 days post-partum increased the calving to first service interval by 4 - 17 days (Lucey et al., 1986; Collick et al., 1989) although there was no difference in calving to first service if lameness occurred between 0 and 30 days post partum (Melendez et al., 2003). The calving to conception interval also increased by 14 days in one study (Collick et al., 1989).

Various hypotheses exist to link lameness and sub fertility. A major one is the reduced display of oestrous behaviour simply due to pain. In one series of observations, cows displayed less duration of oestrus, mounting activity and standing activity on hard concrete flooring than on softer dirt yards (Britt et al., 1986). Severely lame cows display less intense oestrous behaviour compared to moderately lame and non-lame cows (Walker et al., 2008). Furthermore, lame cows lie down for longer periods (Weaver, 1985; Singh et al., 1993). Another suggestion is the decrease in appetite associated with lameness leads to reduced energy intake, which has a detrimental effect on reproductive function (Beam et al., 1999). However, the hypothesis of the present study is that there is also an effect on follicular size and steroidogenic potential when animals suffer stressful episodes and this forms the basis of the first section of this review.

Comparatively, heat stress distorts follicular populations due to disruption of normal FSH secretory profiles (Roth et al., 2000). Cows subject to heat stress also have smaller dominant follicles of the first two waves of the oestrous cycle (Badinga et al., 1993). Recent work in transport stressed sheep reveals a delay in the timing of LH release, which could lead to delayed ovulation resulting in reduced fertility (Dobson et al., 2000a) (Smith et al., 2002). The actual concentration maximum of the LH surge is affected but this may have less of an effect on fertility than the actual timing of the surge (Phogat et al., 1997). Studies in cattle have demonstrated that there is a positive correlation between the timing of the LH peak and the time of ovulation (Roelofs et al., 2004b). Delayed ovulation may lead to reduced conception due to the imprecise timing of the resumption of meiosis. This may result in the ovulation of aged follicles which have a reduced capacity for fertilization (Mattheij et al., 1994; Mihm et al., 1994). There may also be a

management component with the timing of AI becoming out of synch with the stage of ovum development.

There may be another proposed link between lameness and sub fertility. The stress of calving affects horn quality (Tarlton et al., 2002) which predisposes to traumatic causes of lameness. This is then associated with increased calving to conception intervals and more services per conception (Collick et al., 1989). However, as primordial follicles can begin to grow two months prior to their actual wave cycle (Webb et al., 2003), acute stressors may have a substantially delayed effect.

Follicle Wave Development

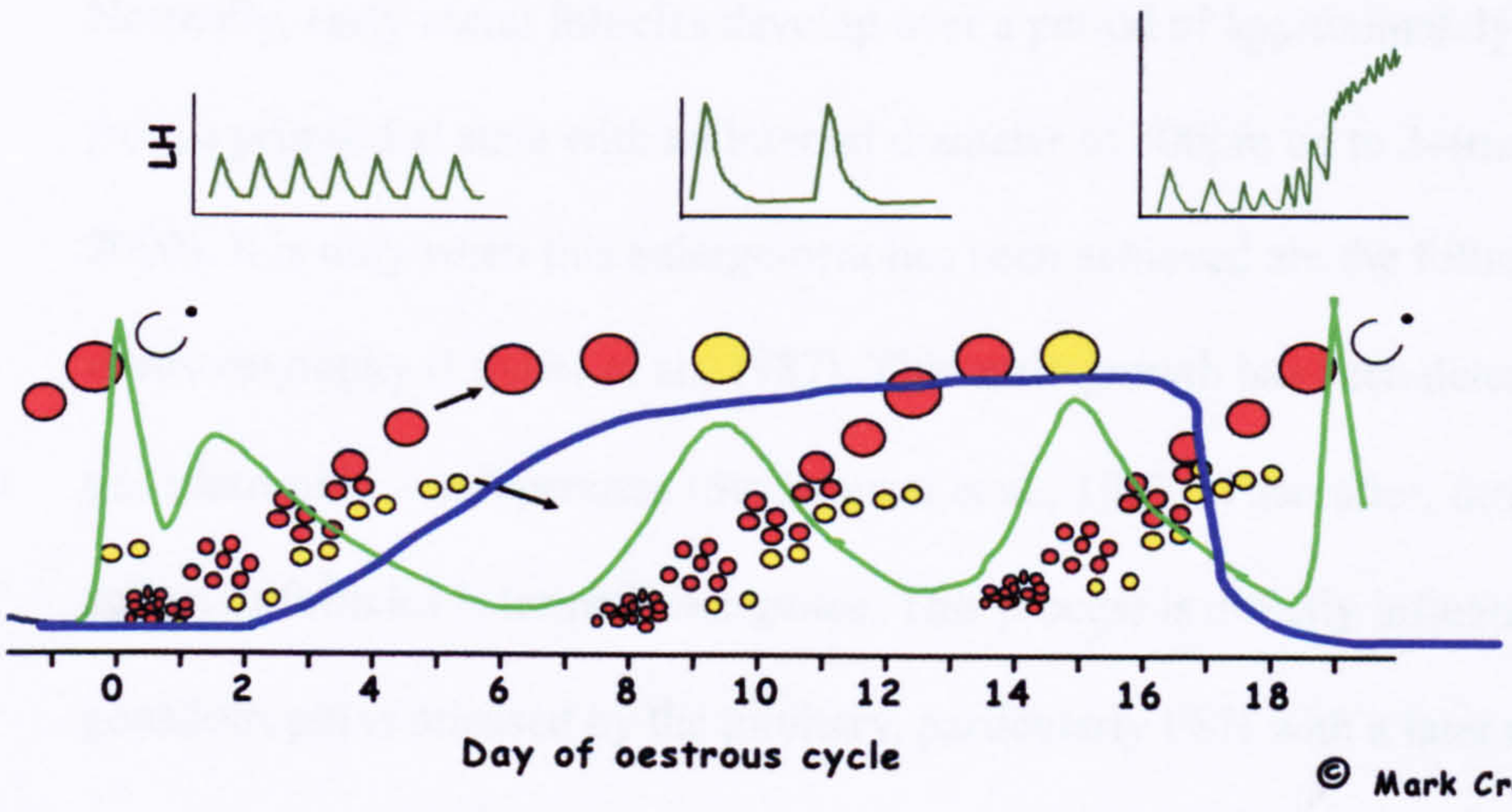

Fig 3 Follicle wave growth in dairy cows showing --- FSH, --- progesterone and -- - LH concentration patterns (Crowe, 2002)

Synopsis: Ovarian follicular development in cattle occurs in a wave pattern usually in two or three sequential waves per oestrous cycle involving up to 20-25 follicles in each wave (Pierson et al., 1988; Sirois et al., 1988); fig 3. These waves are each preceded by an increase in FSH (Adams et al., 1992). Individual follicles stop growing with declining FSH concentration until only the one follicle which is destined to be the selected dominant follicle (DF), grows beyond 8.5mm while the others undergo atresia (Ginther et al., 1997). The DF becomes FSH-independent and LH-dependent, resulting in greater steroidogenic efficiency than the subordinate follicles. Follicular growth is also influenced by intra-follicular factors such as inhibins and the insulin-like growth factor (IGF) family. In the presence of progesterone, the DF will also undergo atresia. However, when luteolysis occurs, progesterone decreases, removing LH pulse inhibition leading to the gonadotrophin surge and ovulation (Cooke et al., 1997).

Normally, early antral follicles develop over a period of approximately three months from a primordial state with an internal diameter of 300μm up to 3-4mm (Campbell et al., 2000). It is only when this enlargement has been achieved are the follicles visible via ultrasonography (Lussier et al., 1987). This early growth has been determined to be gonadotrophin – independent (Scaramuzzi et al., 1993). Thereafter, further growth, of a cohort of follicles is termed *emergence*. This process is directly influenced by the gonadotrophins released by the pituitary, particularly FSH with a later role played by LH (Adams et al., 1992; Gong et al., 1996). Following the first LH and FSH increase, there is a second FSH surge, coincidental with the time of ovulation (Dobson, 1978). This results in the aforementioned emergence of up to 20-25 small antral follicles of which increase in size from approx 4 mm to their largest possible size of approximately 8.5mm at

divergence (Ginther et al., 1997). During this wave growth, there is a progressive loss of follicles which stop growing and undergo atresia and/or apoptosis (Austin et al., 2001). This process persists under the influence of declining FSH concentrations until only one follicle remains, i.e. the DF. Exogenous administration of FSH stimulates the smaller follicles to grow despite the suppressive effect of the DF, resulting in superovulation (Bergfelt et al., 1994). Each domestic species has a predetermined ovulatory number which is equivalent to the number of dominant follicles that undergo deviation in growth rate from the rest of the accompanying cohort (Goodman et al., 1983). Deviation has been described as the process(es) whereby the DF(s) continues to grow and differentiate while the remaining members of the cohort undergo atresia or slow growth preceding eventual atresia (Ginther et al., 2001). The DF produces oestradiol more efficiently than the subordinate neighbours within the cohort (Austin et al., 2001). This is evidenced by the increased gene expression for aromatase, 3β –hydroxy-steroid dehydrogenase and LH-receptors (Bao et al., 1997a; b). This propensity for oestradiol production coupled with enhanced growth rate occurs as FSH concentrations decrease to their lowest concentrations (Austin et al., 2001)

Steroidogenesis: Under the influence of FSH and FSH- receptor interactions, androstenedione (A4) is produced within the theca externa layer of all antral follicles. All stages of antral follicle development exhibit genome expression of the enzymes necessary for A4 production (Xu et al., 1995; Marsters et al., 2003). Post antrum development, A4 diffuses out of the thecal layer and is aromatized to oestradiol within the granulosa cells of individual follicles, particularly the DF in the presence of increased LH binding to its receptor (Austin et al., 2001).Thus, the ovulatory DF becomes FSH- independent and

LH-dependent. Oestradiol production increases along with increasing LH pulsatility until the beginning of the LH surge (Crowe et al., 2001). This contrasts with the first DF growing after the LH surge which reduces high output of oestradiol between days 5- 8 (Badinga et al., 1992). Oestradiol production ceases completely with the loss of dominance of the DF as evidenced by the emergence of a second follicular wave (Ireland et al., 1983; Badinga et al., 1992; Sunderland et al., 1994).

For the first DF in an oestrous cycle, growth in the later stages exists under the progesterone environment supplied by the corpus luteum. The DF increases from 8.5mm at the end of selection to approx 20mm prior to ovulation or atresia (Ginther et al., 1999). The LH pulse generator is then suppressed and the LH- dependent first DF also becomes atretic within the first 3-4 days post selection (Sunderland et al., 1994). This is characterized by a reduction in the numbers of granulosa cells and FSH and LH receptors (Ireland et al., 1983). Another increase in FSH occurs, with a new follicular wave emerging with the selection of a second DF (Sunderland et al., 1994).This second DF also operates within a progesterone- rich environment, but if luteolysis occurs while the DF is in the dominant phase then ovulation will occur (Cooke et al., 1997). Conversely, if the corpus luteum is still physiologically active at this time, atresia will again result and a third DF be selected out of a third emerging cohort. When luteolysis does finally occur, an unsuppressed LH pulse pattern leads to final differentiation of the DF, the LH surge and ultimately, ovulation (Cooke et al., 1997).

Reduced steroidogenic capacity may result from reduction in follicular size although one study suggests that ultrasound echotexture characteristics (which correspond to stage of development of the follicle) may play a more significant role (Vassena et al., 2003).

Selection of the Dominant Follicle: It appears that the successful follicle(s) out of the cohort possess greater steroidogenic capacity (Austin et al., 2001). Oestradiol, in association with inhibin leads to a reduction in FSH release (Ginther, 2000). In this way, the DF exerts a limiting effect on subordinate follicles in the cohort. In an artificially maintained high FSH environment, e.g. superovulation treatment, atresia of the subordinate follicles is suppressed (Bergfelt et al., 1994). Significantly, however, systemic oestradiol concentrations remain low despite the presence of several large follicles within the same cohort (Mihm et al., 1997). The survival of the DF can be attributed not only to its greater ability to produce oestradiol, but also its relative FSH independence although basal levels of FSH are still required for survival (Turzillo et al., 1993; Bergfelt et al., 2000; Ginther et al., 2000).

Progesterone priming dysfunction: It is known that stress may affect progesterone priming which in turn has a detrimental effect on LH surge (Baratta et al., 1994). Fertility is affected by progesterone concentrations in the period 4-7 days before insemination (Meisterling et al., 1987). High pre-follicular phase concentrations of progesterone have a negative feedback effect on LH secretion which adversely affects dominant follicle growth (Ireland et al., 1982; Mihm et al., 2006). Conversely, sufficient progesterone concentrations prior to ovulation are necessary to facilitate hypothalamic responsiveness to oestradiol (Gumen et al., 2005b). In ewes it has been shown that progesterone priming is essential for a conclusive GnRH surge via oestradiol positive feedback (Caraty et al., 1999). In one study the increased progesterone concentration must be in excess of 7 days to allow oestradiol stimulation of the hypothalamus-pituitary axis to effect the LH surge (Ozturk et al., 1998). It has been shown that lameness is associated with delayed

resumption of ovarian cyclicity as characterized by low progesterone concentrations within the first 60 days post partum (Garbarino et al., 2004). This delayed resumption in turn results in lower 100 days AI submission rate, conception rate and pregnancy rate and longer intervals to first AI and to conception (Shrestha et al., 2004).

The role of LH pulses/surge and the implications of pulse/surge dysfunction: LH pulsatility operates at different frequencies depending on the stage of the oestrous cycle. In the mid-luteal phase LH pulses occur every 3-4h, whereas in both the early luteal phase and the follicular phase, pulses occur approximately every hour (Rahe et al., 1980). Hourly pulses of GnRH positively affect LH pulse frequency and amplitude which then stimulate the increased secretion of oestradiol from large follicles (Vizcarra et al., 1997). In the follicular phase, LH pulses facilitate the growth of follicles beyond 9mm (Gong et al., 1996) and exogenous LH pulses stimulated steroidogenesis even beyond the concentration attributed to the naturally occurring DF (Crowe et al., 2001). Time to first post partum ovulation in anoestrous cows is also reduced following pulsatile LH administration (Hampton et al., 2003). Steroid hormone concentrations can actually be controlled by varying LH pulse frequencies by the administration of exogenous progesterone (Kojima et al., 2003). LH surge has been described as a sudden increase in LH concentrations in excess of 10ng/ml and immediately after behavioural oestrus (Imwalle et al., 2002a).The naturally occurring surge lasts approximately 10h in the cow (Rahe et al., 1980). Ovulation occurs approximately 25h after LH peak (Roelofs et al., 2004b). Following the pre-ovulatory gonadotrophin surge there is a decrease in the LH receptor concentration in both thecal and granulosa cells in ewes (Webb et al., 1982).

Pulses of GnRH in the pituitary circulation increased the LH concentration in plasma as compared to continuous release, thereby promoting follicular growth.

Crucially, in support of the study hypothesis, it is also known that the administration of norepinephrine results in a reduced LH response to GnRH, therefore identifying norepinephrine as an LH inhibitor at the pituitary level (Hardin et al., 1983). LH pulses increased the production of oestradiol in the GnRH immunized heifer and in another study, despite the presence of artificially maintained high FSH concentration (Crowe et al., 2000; Crowe et al., 2001). This highlights the greater role of LH compared to FSH in that final pre-ovulatory increase in oestradiol production. Also, in the latter stages of the follicular phase with increased growth of the DF and increased oestradiol production, LH pulse amplitude was increased (Evans et al., 1997). Recent work now suggests that in sheep, there is a lack of change in LH pulsatility coincident with emergence and growth of the DF. This may support the theory that there may be a change in the response of the steroidogenic cells in the ovary and not in LH secretion (Duggavathi et al., 2005). Stressors, such as transport, delay the LH surge and its magnitude in sheep (Dobson et al., 1999b). In heifers, the concentration of oestradiol-17β is decreased one day following Lipopolysaccharide administration (Karsch et al., 2002). LH pulse frequency was also decreased while mean concentration and pulse amplitude were increased within 6 hours of administration. Consequently, the pre-ovulatory LH surge is delayed and subsequently ovulation (Suzuki et al., 2001). Does stress reduce follicular oestradiol production, either at the level of the hypothalamus, the pituitary or at ovarian level with the associated detrimental effect on fertility parameters?

Oestrus Synchronization Techniques: There are many oestrus synchronisation techniques which act at different points in the oestrous cycle. For any technique to be successful, one or more of the following must be controlled: luteal lifespan; emergence of a normal cohort of follicles and closely related to the latter; suppression of FSH secretion. Also essential is the presence of a functionally normal dominant follicle at the end of the treatment regime. The varied protocols fall into groupings: prostaglandin treatments; prostaglandin and progesterone treatments; prostaglandin, oestradiol and human chorionic gonadotrophic combinations; GnRH- Prostaglandin treatments and a variant which also utilises estrogens.

Prostaglandin F2α (PGF) causes the regression of mature corpora lutea thereby bringing about oestrus. However, luteal tissue is unresponsive within the initial five days of the oestrous cycle and consequently double treatment regimens at 7, 11 or 14 day intervals were developed to ensure the presence of a susceptible mature CL at the time of the second PGF treatment. The 14 day protocol, however, produces the best synchrony of the three (Folman et al., 1990). The time interval from PGF to ovulation is dependent on the size of the most recently emergent DF. If the DF was at the static phase (8mm) at the time of PGF injection, then the interval was 3.7 days, whereas if a new DF had emerged at luteolysis, then the interval increased to 4.5 days (Kastelic et al., 1991a; b). The Ovsynch method of synchronization is a GnRH and PGF programme with a specific schedule (100μg GnRH given on day 0 followed by 25mg PGF followed 48h later by another dose of 100μg GnRH (Pursley et al., 1995). GnRH however, causes the cessation of oestradiol secretion from the pre-ovulatory follicle (Kobayashi, 1995) cited by (Stevenson et al., 1999) thus preventing its use in certain study situations. There was also

a slower rate of progesterone increase following AI suggesting an effect on subsequent luteal function (Perry et al., 2005).

A protocol (Select- Synch) using GnRH followed by PGF but omitting the second GnRH injection was compared to the original Ovsynch regimen. Time from PG injection to oestrus increased from 55 to 63h in the protocol without the second GnRH (Stevenson et al., 1999).This compares to the original Ovsynch study which produced a 24 - 32h window for ovulation to occur following the second GnRH dose (Pursley et al., 1995). The choice of synchronization technique is important with respect to its effect on the subsequent follicular wave as it is known that the diameter of the resultant corpus luteum is affected by the size of the previous ovulatory follicle (Cavalieri et al., 2004). This was also seen in a synchronization regimen using GnRH (Vasconcelos et al., 2001b).

Ultrasonography of the bovine ovary: Prior to the use of ultrasonography, identification of ovarian structures more detailed than palpation per rectum was achieved by removal of ovaries, either post ovariectomy or post slaughter (Rajakoski, 1960). These methodologies provided only snap-shot data and thus were unable to chart ongoing follicular dynamics. The suitability of ultrasonography for ovarian structure examination was first assessed in the late 1970's (Palmer, 1980). Initially excised ovaries were examined in a water bath. The scanned images were positively associated with gross anatomic views of the same specimens sliced in the same plane as the ultrasound view, and clearly distinguished follicles, luteal tissue and ovarian stroma (Pierson, 1984). In large animal reproductive work, the anatomy of the reproductive tract, particularly proximity to the rectal wall lends itself well to the trans-rectal method of scanning. The

frequency of the ultrasound waves determines the resolution with detailed examination of ovarian structures utilising 5MHz or more commonly 7.5MHz linear transducers. It is possible to determine accurately the associations between ovarian follicular growth and hormonal patterns (Kastelic, 1990; Adams et al., 1992; Ribadu et al., 1994). It is an improvement on palpation per rectum in terms of the greater and more accurate information collected (Sprecher, 1989; Ginther, 1995). Significantly, repeated ultrasonography per rectum does not influence oestrous behaviour or peri-ovulatory hormone profiles, which makes it an important tool in follicular wave research (Roelofs et al., 2004b).

Within the ovary, the follicles are identifiable as non-echogenic, roughly circular areas. Other non-echogenic structures include the various blood vessels and the central lacunae of the developing corpora lutea. The latter is distinguishable from the follicle by the well defined border of amorphous luteal tissue (Pierson, 1984).

Emergence, as previously defined, begins when the smallest diameter follicle is identified by ultrasonography to generate follicle growth profiles (Ginther et al., 2001).This diameter had generally been accepted by most researchers to be 4-5 mm when these definitions were accepted in the early 1990s (Adams et al., 1992; Sunderland et al., 1994) (Griffin et al., 1992). In follicles >3mm, a positive correlation exists between anatomic and ultrasonographic measurements of follicle size (Pierson, 1987). While follicles 1-3mm may be detected and measured more or less reliably, it is not possible to accurately map development throughout the oestrous cycle (Pierson et al., 1987; Sirois et al., 1988). However, one paper does suggest that improvements in resolution in modern scanners now allow the mapping of follicles 1-3mm in diameter (Jaiswal et al., 2004).

The time of follicular growth deviation is defined as the point when the growth rate of the DF is greater than that of the second largest follicle. Deviation may also be identified as the point when the largest subordinate follicle undergoes complete cessation of growth. This does not rule out the resumption of growth in cases where the original DF is destroyed (Ginther et al., 1996). One of the main points of interest surrounding deviation is the identification of the exact moment at which the DF can be classed as such. The earlier emergence of the DF by 6 hours may confer an immediate size or functional advantage over the other follicles in the same cohort (Ginther et al., 1997). Deviation begins when the largest follicle attains a diameter of 8.5mm and dominance is exerted within 8 hours. Morphological deviation may be visualized on ultrasound, the DF being physically larger than subordinates, growing on from 8.5mm up to 20mm (Ginther et al., 1999). Presumably, the decisive 8 hour deviation period is accomplished quickly so that the other follicles in the cohort do not simultaneously reach the same critical landmark, enabling a parallel growth spurt. There is a definitive temporal association between the attainment of the decisive 8.5mm diameter and lowered plasma concentrations of FSH. This suggests that reduction of FSH to basal concentrations below subordinate follicle requirements is an important component of the deviation process (Ginther et al., 1997).

Oestrus behaviour: By definition, standing to be mounted (STBM) is a response whereby a cow remains immobile during mounting by a bull or another cow (Allrich, 1993). Although only a percentage of dairy cows display standing heat (Pennington et al., 1986; Mai et al., 2002; Van Eerdenburg et al., 2002), it is still the most commonly used sign of oestrus and considered as the definitive indicator. The frequency of STBM within a single oestrus period is extremely variable (Coe et al., 1989). Furthermore, increase in

the numbers of cows in oestrus at the same time enhances the behavioural display of oestrus, further increasing mounting frequency (Allrich, 1993; Van Eerdenburg et al., 1996). Consequently, there is much variation in the observation of this single behaviour with particular difficulty arising due to the small percentage (< 1%) STBM comprises of the total duration of oestrus behaviour (Senger, 1990).

Therefore, monitoring of the secondary oestrus behaviours assumes greater importance in the identification of oestrus. These include mounting behaviour, sniffing, chin resting, licking and flehmen response (French et al., 1989). These secondary behaviours need to be viewed with caution as they may occur at any stage throughout the oestrus cycle (Allrich, 1993). However, secondary behaviours in combination with observation for STBM may result in a more accurate determination of oestrus in the cow.

Mounting behaviour is very likely to be within the oestrus period although again, this activity can occur at other times (Allrich, 1993; Van Eerdenburg et al., 1996). Conversely, mounting activity may not always be present within the oestrus period (Van Vliet et al., 1996). The other secondary oestrus behaviours generally begin onset approximately 12h prior to oestrus and gradually increase frequency until STBM (Hurnik et al., 1975). Following the cessation of STBM, secondary signs gradually decrease in frequency of appearance until they stop approximately 12h later.

All this wide variation in the occurrence of both primary and secondary oestrus behaviour signs renders interpretation difficult. It is therefore within the remit of this thesis to identify significant relationships between individual oestrus signs both in terms of time of onset and frequency of occurrence in healthy and stressed animals.

Hormone profiles and their measurement

Progesterone: Progesterone profiles following ovulation vary significantly according to luteal size which in turn is associated with the size of the previously ovulated DF (Vasconcelos et al., 2001a). Larger sized follicles result in corpora lutea with increased steroidogenic potential (Robinson et al., 2005). Measurement of the natural ovulatory process is desirable as it has also been suggested that a lack of recognition of LH by the GnRH induced CL may result in a shorter luteal phase (Rutter et al., 1985). This subsequent luteal phase is important in the development of a viable foetus with sufficient IFN-Tau production necessary for maternal embryo recognition (Mann et al., 2001). Further work is required to elucidate the mechanisms whereby luteal function influences embryo development. A model has been developed which allows the inducement of a delayed rise in progesterone via the manipulation of the pre-ovulatory follicular phase (Robinson et al., 2005). The development of a reliable enzyme linked immunoassay has allowed rapid assessment of milk progesterone without costly equipment and disposal facilities required of radioimmunoassays (Munro et al., 1984b). By repeated ultrasound measures of follicular sizes and associated post-ovulatory progesterone profiles this study will seek to compare cows with or without disease conditions with all animals being subject to the same synchronization regime.

Oestradiol: Recent development of a sensitive enzyme immunoassay (EIA) for the estradiol-17beta (E2 beta) has facilitated easier and safer assessment of oestradiol compared to previous radioimmunoassay methods (Takenouchi et al., 2004). When oestradiol concentrations were measured in cows undergoing different synchronization techniques, profiles were markedly different despite similar follicular sizes (Stegner et al., 2004). This may suggest differing rates of maturation of follicle steroidogenic

capacity in the face of differing endocrine environments. The action of oestradiol as a significant stimulator of the LH surge requires that timing of peaks in secretion are accurately mapped (Karsch et al., 1997). The failure of other studies to determine differences in oestradiol concentration between lame and healthy animals or to correlate oestradiol concentration with oestrus behaviour intensity (Walker et al., 2008) may be as a result of insufficient frequency of sampling or the greater accuracy in plasma versus milk analysis. However, others have suggested that once or twice daily sampling is sufficient for identification of peak oestradiol concentration while showing an association with the onset of oestrus behaviour (Lopez et al., 2002). Nevertheless, laboratory facilities are still a requirement for assays. In line milk detectors have been developed for progesterone and until an easy 'cowside' test becomes available for oestradiol, its role in the detection of oestrus will remain limited.

Luteinizing Hormone (LH): The sampling of LH for pulsatility testing in cattle can be a difficult task with suggested sampling intervals being 8, 10, 12 and 15min (Cook et al., 1991; Savio et al., 1993; Stock et al., 1993; Bergfeld et al., 1995; Evans et al., 1997; Fike et al., 2004; Robinson et al., 2006a; Roelofs et al., 2007). Usually, an indwelling jugular catheter is used with the animals in a relatively confined space with sampling occurring following a period of acclimatisation. The intensity of sampling with requirement for some form of restraint prevents normal oestrus behaviour although LH surge sampling ($\approx$ 2 hourly) may be possible. We are unable to find any work in the English speaking literature that disproves this view that frequent blood sampling ($\leq$ 20min) is incompatible with oestrus behaviour monitoring although one study has managed 1.5 hourly sampling (Roelofs et al., 2004b).

Previously, there had been a radioimmunoassay for measurement of bovine LH (Niswender et al., 1969), but this is accompanied by the requirement for specialized expensive laboratory equipment and removal of radio-labelled waste. The initial development of a reliable LH enzyme linked immunoassay (Spearow et al., 1987) has resulted in an accurate, safer, more convenient and less expensive procedure (Graham, 2002). Analysis of pulse data has also provided another difficulty in attempting to derive an accurate repeatable method for determining LH pulses. However, there are now many computer programs developed for this task with the one utilised in the present study being the PC-PULSAR pulse analysis program (Gitzen & Ramirez, University of Illinois, USA. Version 3.0) based on the pulsar algorithm (Merriam et al., 1982).

Aims and Objectives

One of the primary aims of this thesis was to investigate the effect of high somatic cell count (SCC), low body condition score (BCS) or lameness on ovarian follicular growth and ovulation.

A second major aim of this thesis was to investigate hormonal involvement by examining progesterone, oestradiol and luteinizing hormone (LH) profiles, and comparing concentrations at designated times in healthy cows with those in cows with high SCC, low BCS or lameness.

The third objective of the thesis was the assessment of the impact of high SCC, low BCS and lameness on the display and timing of oestrus behaviour.

Finally, it was necessary to test the hypothesis that low progesterone concentrations in lame cows were responsible for the previously observed low intensity of oestrus and aberrations in timings of oestrus and ovulation.

This information will aid in the advancement of knowledge regarding the mechanisms responsible for the deleterious effects of common disease stressors on dairy cow fertility.

General Materials and Methods

Progesterone enzyme immunoassay

Milk progesterone was analyzed by enzyme immunoassay (EIA) using the method developed by (Munro et al., 1984b) and adapted by (Young et al., 2004)

The EIA was carried out in 96 well NUNC Maxisorb plates (Nunc™-Immuno, Maxisorp Surface; Nunc A/S Roskilde, Denmark) using an antibody (monoclonal antiserum against progesterone metabolite Quidel Clone #425 supplied by CJ Munro, University of California, Davis, CA), horseradish peroxidase conjugated label [prepared according to (Munro et al., 1984a)] and the standard preparation was obtained from Sigma -Aldrich, Poole, UK (Cat.#P0130).

The progesterone antiserum cross-reacted with several progesterone metabolites including: 4-pregnen-3, 20-dione (progesterone) 100%, 4-pregnen-3α-ol-20-one 188%, 4-pregnen-3β-ol-20-one 172 %, 4-pregnen-11α-ol-3,20-dione 147%, 5α-Pregnan-3β-ol-20-one 94 %, 5α-Pregnan-3α-ol-20-one 64%, 5α-Pregnan-3, 20-dione 55%, 5β -Pregnan-3β-ol-20-one 12.5% and ≤10% for all other metabolites tested (Graham et al., 2001).

Antibody stock was stored at -20°C 1:50 dilution and further diluted to 1:10,000 (25µl antibody stock to 5 ml coating buffer; 0.05 M $NaHCO_3$, pH 9.6) prior to coating wells overnight at 4°C. Standards (0.78-200 pg/well) in assay buffer (0.1M $NaPO_4$, 0.149MNaCL, 0.1% bovine serum albumin, pH 7.0) or undiluted samples (50 µl/well) were combined with the horseradish peroxidase conjugate (50 µl/well, at a dilution of 1:33,000 in assay buffer). Plates were incubated for 2h followed by 5 washes. Plates were then incubated with 100 µl/well substrate buffer (0.4mM ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt-98%; Sigma Prod. No. A-1888),

1.6mM H_2O_2 and 0.05M citrate pH 4.0) and read at 405nm wavelength using an automated plate reader (Multiskan plate reader, Thermo Electron Corporation, Basingstoke, Hampshire).

Plasma oestradiol radioimmunoassay

Ether for the extraction process consisted of diethyl ether freshly washed in an equal volume of saturated ferrous sulphate solution, separated and washed a second time in distilled water then separated and distilled immediately prior to use. Subsequently the extraction was performed (Mann et al., 1995). Five hundred (500)µl of sample or control plasma was added to 3ml ether and mixed well for 5min. The aqueous phase was then immediately frozen out in an ethanol freezing bath and the organic solvent decanted. Extracts were evaporated to dryness using a Savant Instrument Speedvac© Concentrator (Holbrook, NY, USA) and stored at –20°C. The efficiency of steroid extraction was evaluated by adding ^{3}H-oestradiol (16,000 dpm) prior to extraction. The mean recovery of ^{3}H -oestradiol was 70%.

All stored samples were analyzed in duplicate by a previously described RIA to determine oestradiol concentration (Walker et al., 2008). Briefly, the modified oestrogen RIA was based on the oestradiol MAIA Kit (Adaltis Italis S.p.A, Italy) using a rabbit anti-oestradiol antibody [50µl/tube, diluted 1:3 in assay buffer (0.1M phosphate-buffer saline with 0.1% w/v gelatine, 0.2% w/v NaN_3 and 0.3% w/v EDTA, pH 9.6)], (^{125}I)-oestradiol tracer (50µl/tube; diluted 12,000 dpm in assay buffer), goat anti-rabbit gammaglobulin coupled to magnetic particles (100µl/tube). Standards used were

oestradiol-17β; Sigma-Aldrich, UK; range 0.0625-16 pg/tube reconstituted in 250µl assay buffer. The antibody cross-reacted with oestradiol-17β 100% and ≤ 2.5% with all other metabolites tested: Estrone 1.77% Estriol 0.47%, Testosterone 0.0033%, Aldosterone 0.007%, Progesterone absent up to 100 µg/ml, Desoxycorticosterone 0.00003%, Androstenedione 0.0001%, Dehydroepiandrosterone absent up to 100 µg/ml, DHT 0.0002%, Cortisolo absent up to 50 µg/ml, ß-Estradiol-17ß-Glucoronide absent up to 1 µg/ml, ß-Estradiol-3-Sulfate-17 Glucoronide absent up to 1 µg/ml, 17ß-Estradiol-3ßD-Glucoronide 0.14%, 17ß-Estradiol-3 Sulfate 0.07%, ß-Estradiol-Dipropionate 0.19% 17ß-Estradiol-3 Glucoronide-17 Sulfate absent up to 1 µg/ml, 17ß -Estradiol-3-Benzoate 2.5%, Clomiphene absent up to 50 µg/ml, 17ß -Estradiol-3-disulphate absent up to 1 µg/ml and DHEA-sulfate 0.00049%.

LH enzyme immunoassay

Plasma LH was analyzed by enzyme immunoassay (EIA) using the method developed by (Graham, 2002). Microtitre plates (Nunc™-Immuno, Maxisorp Surface; Nunc A/S Roskilde, Denmark) were coated (250ng/well) with affinity isolated anti-mouse gamma globulin developed in a goat (Sigma M8645) dissolved in coating buffer (1:1,000; 125 µl antibody stock to 25 ml coating buffer; 0.015M Na_2CO_3, 0.035M $NaHCO_3$, pH 9.6) and incubated at room temperature overnight. The wells were then emptied, not washed but refilled with 300µl LH blocking buffer (0.02M Trizma, 0.30M NaCl, 1.0%BSA, 0.01% NaN3; pH 7.5). Plates were then incubated, preferably overnight but for a minimum of three hours, at room temperature prior to use.

Bovine LH (NIH-bLH-B10, AFP-5551B) used for the preparation of standards and ovine LH (NIH-oLH-518) for biotin labelling were obtained from Prof AF Parlow, Torrance, California, USA. Specific anti-LH antibody (LH 518-B7) raised in a mouse against bovine LH (Matteri et al., 1987) was obtained from C. Munro, UC Davis, California, USA. Biotin labelling of ovine LH was performed using an EZ-Link™ Sulpho- NHS-LC- Biotinylation Kit Prod # 21430, Pierce, Rockford, Illinois, USA. Procedure was as follows: A 2mg/ml oLH stock was made by adding 1 ml of PBS to 2 mg of ovine LH (reagent grade - NIADDK-oLH-26). 86µl of a 10µg/µl biotin solution (2mg of biotin in 200µl of dH_2O) was added to 1ml of the 2mg/ml oLH stock. This biotin/oLH solution was then incubated for 30 min at room temperature. 30ml PBS was then run through a desalting column, followed by a layer of 1ml biotin/oLH solution and another layer of 20ml PBS. Twenty 1ml fractions were collected and run on a plate at 1:50,000 to determine in which fraction the label was eluted. Fractions that contained label were then titrated out to determine working concentration.

Assay procedure: Assay buffer used was 0.02M Trizma, 0.30M NaCl, 0.1%BSA, 0.01% Tween 80; pH 7.5. Microtitre plates, coated as previously described, were washed in a wash buffer (1.5M NaCL, 0.5% Tween 20). 50µl plasma samples (neat for surge detection or 1 in 5 dilutions with assay buffer for pulse), standards (range 0.156 – 10 ng/ml in assay buffer) or plasma controls were dispensed in appropriate wells. 100µl anti–LH antibody (working dilution- 1:800,000 in assay buffer) was added to every well and the plates were incubated at room temperature overnight. 100µl biotinylated LH (working dilution 1:100,000 in assay buffer) was added and the plates incubated at room temperature for a further 4h.

1µl of 500 U/ml Streptavidin β-peroxidase conjugate [lyophilized streptavidin (Boehringer 1 089 153, 500U) in 1 ml sterile water] in 24ml assay buffer was prepared and stirred for 15 min using a magnetic stirrer immediately prior to use. After incubation with labeled LH, the plates were washed and 200µl Streptavidin were added to each well and further incubated at room temperature for 40 min.

TMB substrate solution was prepared immediately before use by combining 500 µl 0.4 % TMB (0.016M 3,3', 5,5'-tetramethylbenzedine in dimethylsulfoxide; DMSO), 100 µl 0.6% H_2O_2 and 24 ml substrate buffer (0.016 M CH_3.COONa; BDH Product 30104). The plates were washed, substrate added (200µl/well) and further incubated at room temperature for 40 min. The reaction was stopped by adding 50µl 0.2M H_2SO_4 to each well. Absorbance was measured using a 450nm filter (Multiskan plate reader, Thermo Electron Corporation, Basingstoke, Hampshire).

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Influence of high somatic cell count, low body condition and lameness on follicular growth and ovulation in dairy cows

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Abstract

The objective of this study was to investigate the effect of somatic cell count (SCC), body condition score (BCS) or lameness score on ovarian follicular growth and ovulation in dairy cows. Seventy four animals 30-80 days post-partum were scored for all three conditions before synchronisation of their ovarian follicular phases by administration of gonadotrophin releasing hormone (GnRH) followed seven days later with prostaglandin F2alpha (PG). Ultrasonography of their ovaries twice daily throughout the follicular phase revealed that fewer animals with high SCC and lameness (4/9) ovulated compared to healthy animals (19/21; $P = 0.006$) or animals with only high SCC (11/11; P = 0.004) or only lameness (21/27; P = 0.06). Additionally 2/6 animals which also showed low BCS, ovulated. Overall, regardless of the presence of other conditions, fewer lame cows ovulated compared to non lame animals (30/42 and 30/32; $P = 0.015$). Mean follicular growth rate and maximum follicular diameter were unaffected by any of the three conditions. However, dominant follicle growth and maximum diameter were greater in the 60 animals that ovulated compared to the 14 that did not; (1.83 ± 0.16 *versus* 0.96 ± 0.26 mm/day P = 0.014) and (19.4 ± 0.4 *versus* 16.4 ± 1.2 mm; $P = 0.003$), respectively. In conclusion, lameness reduced the proportion of cows that ovulated and the synergistic effect of high SCC and lameness further reduced that proportion. However, follicular growth and maximum follicular diameter were unaffected by high SCC, low BCS or lameness.

Introduction

The stress of various production diseases has an adverse effect on dairy cow fertility with clinical mastitis (Schrick et al., 2001), low body condition score (Beam et al., 1999) and lameness (Lucey et al., 1986; Collick et al., 1989; Melendez et al., 2003) being especially deleterious. Subclinical mastitis is also an economically important disease stressor (Huijps et al., 2008). This prompted us to investigate high somatic cell count (SCC) animals rather than clinical cases of mastitis. It has been suggested that stressors disrupt gonadotrophin support required for optimal fertility, with consequent effect on follicular growth and factors controlling timing of ovulation (Dobson et al., 2000a).

Recent studies have indicated that lame cows inherently have lower progesterone concentrations in the luteal phase prior to expressing oestrus (Walker et al., 2008). Thus, to facilitate frequent ultrasound examinations from a sufficient number of cows, ovarian follicular phases were synchronised using a hormonal regime that did not involve administration of exogenous progesterone to avoid disrupting the endogenous progesterone milieu. Consequently, in the present study, an injection of gonadotrophin releasing hormone (GnRH) was used followed seven days later by prostaglandin F2alpha (PG).

The use of frequent real time trans-rectal ultrasonography allows close monitoring of ovarian activity, especially enabling the precise measurement of follicular diameter, mean growth rate and time of ovulation, without itself influencing the results (Roelofs et al., 2004b). Using this technique, heat stress in cattle has been shown to result in slower follicular growth concomitant with atypical steroidogenic profiles and smaller dominant

follicles (Badinga et al., 1993; Wilson et al., 1998). Similarly, cows in nutritionally-induced anoestrus have smaller dominant follicles (Rhodes et al., 1996).

Stressors often occur in combination; therefore the present study investigated the role of the common production stressors (high SCC, low body condition and/or lameness) on follicular growth and ovulation in dairy cows. The cumulative effect of diseases may synergise, pushing the affected animals "over the edge" with respect to follicular growth and the ability to ovulate. Furthermore, to ensure that the data are meaningful to the dairy industry, the study was carried out on commercial farms with as little disruption as possible to normal management practices.

We hypothesise that dairy cows with the most common production stressors (high SCC, low body condition and/or lameness) have smaller follicles growing at a slower rate, ultimately resulting in a lower proportion of ovulations than in healthy counterparts living under the same conditions.

Materials and Methods

The study was conducted from May to November in three successive years on two commercial farms with 200 and 130 cows, respectively. The 90 multiparous lactating Holstein cows enrolled in the study had an average milk yield of 8800 kg per lactation with milking (including study cows) starting at 06.00 h and 16.00 h each day. Animals were enrolled from the whole herd as those without any confounding clinical conditions except high SCC, low body condition and/or lameness. Cows randomly entered the study only once between 30 – 80 days post partum and, at any one time, no more than 12 cows

were monitored. During the study periods, animals on one farm were out at grass with a supplementary Total Mixed Ration (TMR) fed indoors for one hour immediately after each milking. Pastures were of seasonal ryegrass, Italian ryegrass and white clover. Cows on the second farm were kept inside throughout and fed TMR *ad libitum*. Cows had been routinely hoof trimmed at the end of the previous lactation. The study was performed under a UK Home Office licence for work on living animals and with the approval of the University of Liverpool Ethical Review Process.

Individual cow Somatic cell counts (SCCs) from a pooled milk sample from all quarters of the udder were measured every four to six weeks by commercial companies employed by the individual farms (National Milk Records Plc, Chippenham or the Cattle Information Service, Watford). Animals with clinical mastitis (presence of clots or watery milk, with or without inflamed udder) were excluded from the study. Cell counts of the study cows immediately prior to oestrous cycle synchronisation were used to define the prevailing SCC status of the cow. A cell count < 100,000 cells/ml was classified as low SCC and a count ≥100,000 cells/ml was deemed as high (Dohoo et al., 1993).

Body condition scores (BCS) were determined using a 1-5 system (Chamberlain et al., 1996). Animals with a mean BCS < 1.5 were classified as low BCS and those with a mean score ≥1.5 or more were classified as moderate BCS. The cows were also scored for lameness at the same time as BCS, using a standardized 1-5 system (Sprecher et al., 1997c). An intra coefficient of variation of 0% was determined by multiple observations of the 3 study animals on the same day. Both lameness and BCS were performed weekly from three weeks before oestrous cycle synchronisation for a total of five weeks and the

mean scores over the time period were calculated. The lameness score of > 95% of individuals was the same, or one assessment was within 1 score different over the duration of the study. Animals with a mean score < 1.5 were classified as Non Lame and those with a mean score of ≥ 1.5 or more were classified as Lame.

Oestrous cycle synchronisation: Cows received 100 µg buserelin (GnRH; 2.5 ml Receptal®; Intervet, Milton Keynes, UK) at morning milking followed 7 days later by 500 µg cloprostenol (PG; 2ml Estrumate®; Schering-Plough, Uxbridge, UK).

Milk sampling: Milk samples were taken on alternate days for 3 weeks prior to GnRH administration and then daily until the day after PG injection. From Days 2-7 after PG, during the scanning period, milk collections were increased to twice daily, and then daily for 3 weeks to monitor the subsequent progesterone profile. All samples were taken immediately before milking and promptly stored at -20 °C without preservative.

Ultrasonography: The ovaries of all animals were scanned twice daily *per rectum* with a Concept/ MCV Veterinary Ultrasound Scanner using a 7.5 MHz linear array probe (Dynamic Imaging, Livingstone, Scotland) from PG administration until ovulation or until the appearance of a new follicular wave. Follicles were identified as non-echogenic structures with a defined wall between the antrum and normal ovarian tissue. Corpora lutea were identified as grainy echogenic structures with a distinct demarcation from the less echogenic normal ovarian stroma (Pierson et al., 1984) . Diameters were calculated as the average of two perpendicular measurements. Dominant follicles were defined as those that achieved an internal diameter ≥ 10 mm in the absence of other actively growing follicles (Dobson et al., 2000b; Imwalle et al., 2002b). Ovulation was considered

to have occurred when a follicle > 10 mm was absent at the following ultrasonography session 12 h later.

Hormone assays: Progesterone, analysed as 'pregnane metabolites', was measured in 50 µl whole milk samples after vortex mixing using an established EIA assay (Walker et al., 2008). Samples were compared with standards prepared using progesterone from Sigma - Aldrich, Poole, UK (Cat. # P0130). The minimum detectable amount was 0.015ng/ml; and the intra- and inter-assay coefficients of variation were 8.3 % and 14 %, respectively.

Statistical analysis: All data were analysed using Minitab (Version 14, Minitab Inc. Pennsylvania, USA) with data expressed as mean ± SEM or proportions where appropriate. Analyses were corrected for between farm variation. Statistical differences were considered when $P < 0.05$ and reported as a tendency when $0.05 < P < 0.10$. Maximum diameter of the dominant follicle was defined as the diameter just before ovulation (mean = Day 4.5 ± 0.2 after PG), or the diameter on Day 4.5 in those animals that failed to ovulate. Follicular growth was determined as the mean positive change in follicular diameter between time of PG administration and last ultrasound before ovulation. Associations between ovulation and conditions were examined by χ-square analysis. General linear model analysis of variance (GLM ANOVA) was used to analyse the effect of SCC, lameness and body condition scores on follicular growth rate and maximum follicular diameter. Factors affecting time to ovulation were identified using regression with life data, right censored at 7.5 days for animals that did not ovulate.

Results

A progesterone 'baseline' value (0.17ng/ml) has been previously determined as the mean follicular phase concentration +2 SD of 21 non lame dairy cows in an earlier pilot study. Progesterone profiles from all 90 cows revealed 16 animals where progesterone concentrations remained below this baseline value. These animals were deemed not to have responded to oestrous synchronisation and removed from the study. All of these non-responding animals were lame and none ovulated. A total of 74 animals responded and were used for further statistical analysis. Fifteen cows had more than one concurrent condition (mastitis, low body condition and/or lameness); the distributions are shown in Figure 1.

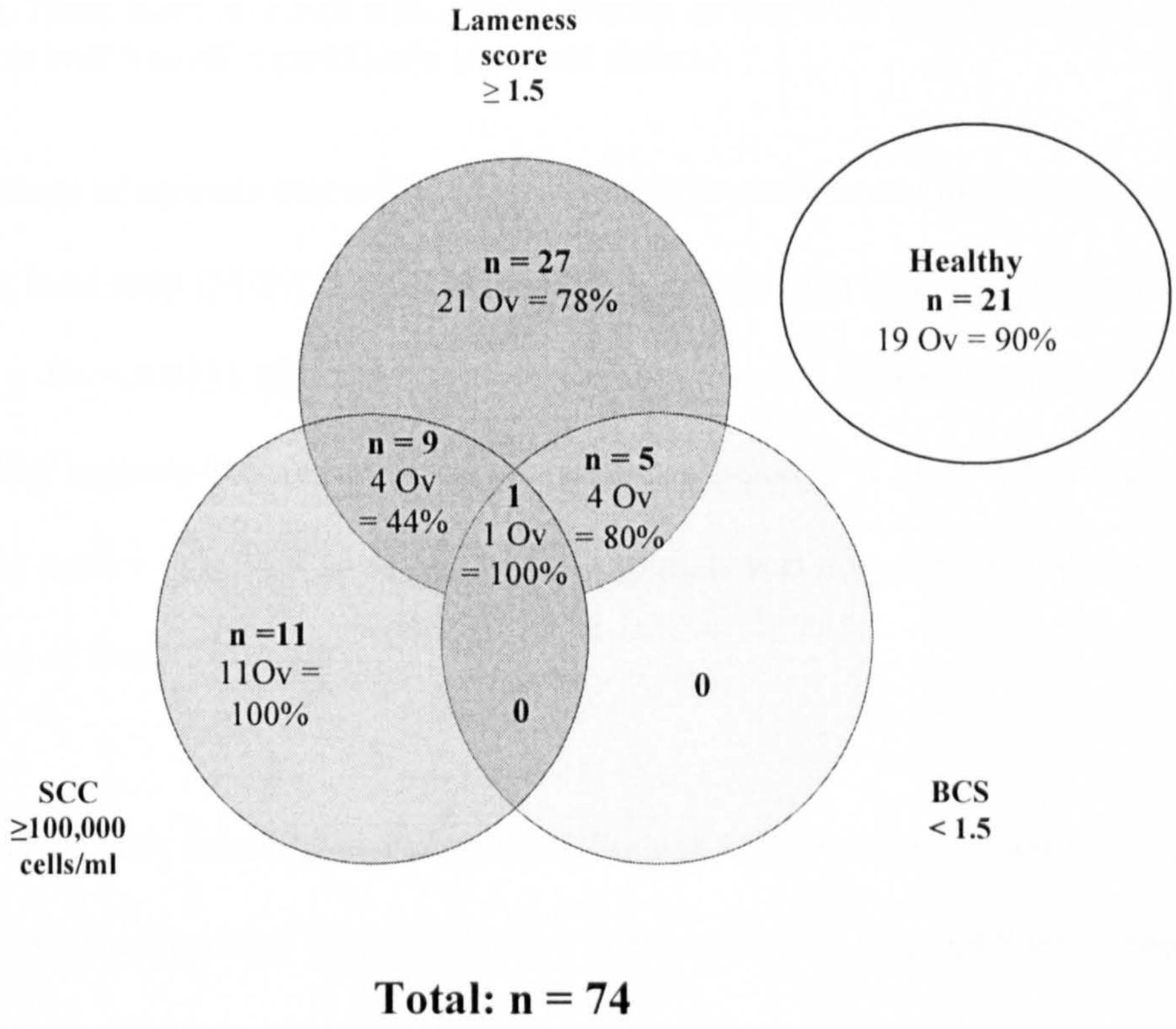

Fig. 1 Numbers of animals (**bold type**) that were healthy or had high SCC, low BCS, lameness or a combination of conditions. Also shown are numbers and percentages within each subgroup that ovulated (Ov).

At the time of PG injection, each cow regardless of group had a follicle of at least 10 mm diameter that grew to a maximum of 14 to 33 mm. The mean follicular growth rate, maximum follicular diameter and time to ovulation were similar in all groups of cows (P > 0.1, GLM ANOVA; Table 1).

Table 1. Mean ± SEM follicular parameters in cows with different conditions.

Follicular parameters	Healthy $n = 21$	High SCCs only $n = 11$	Lame only $n = 27$	Lame & high SCCs $n = 9$	Lame & low BCS $n = 5$
Mean follicular growth rate (mm/day)	1.5 ± 0.2	2.3 ± 0.5	1.8 ± 0.2	1.1 ± 0.5	1.3 ± 0.5
Max. follicular diameter (mm)	19.1 ± 0.6	19.2 ± 0.9	19.4 ± 0.5	16.0 ± 1.8	19.2 ± 2.9
Number of cows ovulating (%)	19 (90%)	11 (100%)	21 (78%)	4 (44%)	4 (80%)
Days to ovulation after PG	4.5 ± 0.2	4.4 ± 0.4	4.0 ± 0.1	4.3 ± 0.2	4.4 ± 0.7

N. B. There were no cows with low BCS only, or low BCS combined with high SCCs. One animal had all 3 conditions (data not shown).

Proportions of animals that ovulated were not different between the healthy group (19/21) and the lame only (21/27; P = 0.242, χ -Sq = 1.371) or high SCC only groups (11/11; P = 0.909, χ -Sq = 0.013). However, fewer lame animals with high SCC ovulated compared to healthy animals (4/9 versus 19/21 $P = 0.006$; χ -square = 7.462). The comparison between lame + low BCS cows and healthy animals was not performed due to the low numbers of low BCS animals.

Fewer cows with lameness and concurrent high SCC (4/9) ovulated compared to those animals with only a high SCC (11/11; P = 0.004, χ -Sq = 8.15); and there tended to be fewer cows with both conditions that ovulated compared to cows that were only lame (21/27; P = 0.06 χ -Sq = 3.54).

There were no differences between the proportion of cows ovulating in the lame + low BCS group (4/5) compared with the lame only cows (21/27; P = 0.91; χ -square = 0.012); or the lame + high SCC cows (4/9; P = 0.20; χ -square = 1.659).

Overall analysis of the individual diseases conditions regardless of the presence of other conditions), revealed that the proportion ovulating were similar between animals with high or low SCC (P = 0.5; χ -square = 0.46; 16/21 and 44/53); and low or moderate BCS (P = 0.88, χ -square = 0.02; 5/6 and 55/68). However, fewer lame compared to non lame animals ovulated (P = 0.015; χ -Sq = 5.89; 30/42 and 30/32).

The dominant follicle grew faster in animals that ovulated compared to those that did not (1.83 ± 0.16, (n = 60) *versus* 0.96 ± 0.26 mm/day (n=14); P = 0.014). The maximum diameter of the dominant follicle was also larger in the ovulating cows (19.42 ± 0.39 *versus* 16.43 ± 1.22 mm; P = 0.003).

Discussion

Overall, in animals with the studied conditions (high SCC, low BCS and/or lameness), only lameness reduced the proportion of animals that ovulated. Furthermore, in the presence of both lameness *and* high SCC, the proportion of ovulated animals was lower than with each condition alone. Dominant follicles grew at the same rate to the same maximum diameter and ovulated at the same time as healthy animals.

Of all the cows subjected to the synchronisation regime, 72 % of lame animals failed to respond, with progesterone concentrations remaining at or below baseline for the duration

of the study. This response rate is comparable with others (70-80%; (Thatcher et al., 1989; Twagiramungu et al., 1992) and could be associated with failure of commencement of luteal activity (CLA). Spontaneous delays in CLA affect 15 % of post partum cows and had been found to be 18 days later in lame animals, increasing intervals from calving to first AI, and to conception (Petersson et al., 2006a; Petersson et al., 2006b).

Per-rectum ultrasonographic measurements of both follicular growth and maximum diameter were similar to other studies in postpartum cows (Ginther et al., 1989; Sunderland et al., 1994; Mann et al., 2007). Also in agreement with the present study, maximum follicle diameters were not different in healthy and in animals with experimentally induced mastitis (Hockett et al., 2005).The lack of difference in follicular growth in the present study suggests that while follicles may grow normally in the face of the three conditions, the ability of the follicle to ovulate is detrimentally affected. The failure of high SCC, low body condition and/or lameness to affect growth rate of dominant follicles and maximum diameter suggests different mechanisms are involved compared to heat stress whereby follicles grow more slowly and achieve smaller maximum diameters (Badinga et al., 1993; Wilson et al., 1998; Roth et al., 2000). Low dietary DM intake also resulted in smaller follicles reported in other studies (Murphy et al., 1991; Diskin et al., 2003). As body condition was not reported in these studies it is not clear how this relates to long-term low body condition score.

The lower proportion of lame cows ovulating may explain the decreased fertility seen in various epidemiological field studies (Lucey et al., 1986; Collick et al., 1989; Fourichon et al., 2000; Melendez et al., 2003). The sub fertility associated with high SCC (Santos et al., 2004) does not appear to be due to the same mechanism, as high SCC did not

influence the proportion of cows ovulating in the present study. However, the two conditions together had a more profound effect on ovulation suggesting that the stressful effects were synergistic. On the two farms in the present study, there was a low incidence of low BCS, reducing the power of analysis. We have been unable to find other data in the literature linking the incidence of ovulation with low BCS although dietary restriction lowers the proportion of animals ovulating (Diskin et al., 2003) and low BCS reduces the conception rate to first insemination (Garnsworthy *et al* 1999). Furthermore, a reduction in BCS increases the days from calving to first ovulation (Beam et al., 1999). However, the present study was carried out over a relatively short period of time, preventing detection of changes in BCS.

In ovulating animals, irrespective of conditions, rates of growth and maximum follicular diameter were greater than in non-ovulating animals and agreed with those seen in previous studies (Beam et al., 1999; Maquivar et al., 2007).

In conclusion, we have identified that three common conditions in dairy cows (high SCC, low BCS and lameness), that reduce fertility in the dairy cow; do not act via mechanisms involving follicular growth rates nor attained maximum diameter. The current study supports the hypothesis that lame cows have a reduced ability to ovulate; although this was not proven for animals with either high SCC or low BCS alone. Nevertheless, we have shown for the first time a synergistic effect of lameness and high SCC that further reduces the likelihood of ovulation. Determining the mechanism by which this occurs requires further study.

Acknowledgements

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Influence of concurrent lameness on reproductive hormones and oestrus behaviour in dairy cows with high somatic cell counts in milk

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Abstract

Dairy cows with high somatic cell counts (SCCs) and other production diseases are less fertile than cows with low SCCs and this study aims to establish the reason(s). The objective of Part One was to determine whether differences in steroid hormone profiles could explain the low incidence of ovulation in cows with combined high SCC and lameness. The objective of Part Two was to establish whether cows with high SCC display different behaviour in terms of oestrus intensity or timing. In Part One, 45 animals 30-80 days post-partum were scored for SCC and lameness and divided into three groups: healthy, high SCC alone or high SCC + lame. The ovarian follicular phases of all cows were synchronised by administering gonadotrophin releasing hormone (GnRH) followed seven days later by prostaglandin F2alpha (PG). Milk samples were collected throughout the entire study period and, twice daily during the follicular phase, blood samples were taken and the ovaries scanned using ultrasonography. Fewer high SCC + lame cows ovulated compared to healthy animals (4/9 *versus* 19/21 $P = 0.006$). Progesterone concentrations were similar in all 3 groups during each of five specific time periods, i.e. throughout the five days before PG injection, the peri-ovulatory period, on Day 5 and on Day 7, and during the mid luteal phase 12-17 days after ovulation ($P > 0.13$). Mean plasma oestradiol concentrations during the 36 h period before ovulation were similar in all groups (healthy, 2.80 ± 0.30; high SCC only, 3.82 ± 0.48; high SCC +

lame 2.94 ± 0.51; $P = 0.175$). In Part Two, a different group of cows with high SCC (scored and synchronised as above) tended to have a less intense oestrus and a lower maximum oestrus score per 30-min period than the low SCC cows (P = 0.063 and 0.066, respectively). Also, intervals from PG to the onset of oestrus or to the first stand-to-be-mounted (STBM) were longer for the high SCC cows than the low SCC animals (P = 0.011 and 0.002, respectively). In conclusion, high SCC ± lameness did not affect progesterone nor oestradiol profiles although the onset of oestrus was delayed and oestrus tended to be less intense.

Introduction

Individual health conditions have a detrimental effect on various fertility parameters in dairy cattle (Lucey et al., 1986; Collick et al., 1989; Melendez et al., 2003). For example, days to first insemination and pregnancy rates were negatively affected by ketosis, dystocia or retained placenta, while mastitis, milk fever or abomasal displacement had little effect (Fourichon et al., 2000). However, in another study, spontaneously occurring mastitis increased the number of days open and reduced pregnancy rates (Santos et al., 2004). Furthermore in our previous studies, the combination of high SCC and lameness reduced the proportion of cows that ovulated compared to either condition alone (Morris et al., 2008a). In a commercial farming environment, subclinical mastitis is an economically important condition (Huijps et al., 2008), particularly due to its insidious nature in comparison to clinical mastitis cases. This prompted us to study animals with high somatic cell count (SCC) rather than clinical cases of mastitis. Part One of the present study seeks to determine whether there are differences in steroid hormone concentrations in cows with high SCC with or without concurrent lameness.

Ovarian follicular phases were synchronised to facilitate frequent ultrasonography examinations and blood collection in a large group of animals. Additionally, this increases the numbers of cows in oestrus during the study period, enhancing the behavioural display of oestrus (Allrich, 1993; Van Eerdenburg et al., 1996). As we have previously observed that endogenous progesterone concentrations are lower in the late luteal phase of lame animals (Walker et al., 2008) exogenous progesterone was not used to synchronise cattle. In the present study, groups of cows were synchronised with an injection of gonadotrophin releasing hormone (GnRH) followed seven days later by prostaglandin F2alpha (PG).

We have recently shown that, although there is no difference in the incidence of oestrus in lame and non lame cows, the intensity of oestrus expression is reduced in lame animals (Walker et al., 2008). Thus, we hypothesise that high SCC may also have a negative effect on the intensity and timing of oestrus. Therefore, in Part Two of the present study, detailed observations of oestrus were additionally compared in high and low SCC animals.

In summary, the present study examines whether high SCC ± lameness affect oestradiol or progesterone concentrations at physiologically important times, i.e., oestradiol throughout the follicular phase and progesterone at particular times that have previously been associated with sub fertility: the late luteal phase (Walker et al., 2008), the peri-ovulatory phase (Bage et al., 2002b), and Days 5 and 7 after ovulation (Mann et al., 2003). In addition, the effects of SCCs on the intensity and timing of oestrus were examined.

Materials and Methods

Part One

The study was conducted from May – October over three years on two commercial farms with herd sizes of 200 and 130 cows, respectively. All enrolled cows were multiparous lactating Holsteins which had an average milk yield of 8800 kg per lactation with milking (including study cows) starting at 06.00 h and 16.00 h each day. During the study periods, animals on one farm were out at grass with a supplementary Total Mixed Ration (TMR) fed indoors for one hour immediately after each milking. Pastures were of seasonal ryegrass, Italian ryegrass and white clover. Cows on the second farm were kept inside throughout and fed TMR *ad libitum*. Cows had been routinely hoof trimmed at the end of the previous lactation. Study animals were selected from within the whole herd based on lack of disease conditions other than high SCC with or without concurrent lameness. Individual cows entered the study once between 30 – 80 days post partum and at any one time, no more than 12 cows were monitored. The study was performed under a UK Home Office licence for work on living animals and with the approval of the University of Liverpool Ethical Review Process.

Individual cow Somatic cell counts (SCCs) from a pooled milk sample from all quarters of the udder were measured every four to six weeks by commercial companies employed by the individual farms (National Milk Records Plc, Chippenham or the Cattle Information Service, Watford). Animals with clinical mastitis (presence of clots or watery milk, with or without inflamed teats) were excluded from the study. The SCC of the study cows immediately prior to oestrous cycle synchronisation were used to define the prevailing status of the cow. A cell count < 100,000 cells/ml was classified as low and

a count ≥ 100,000 cells/ml was deemed a high SCC, reflective of either none or possible udder infection respectively (Dohoo et al., 1993). The cows were also lameness scored using a 1-5 system (Sprecher et al., 1997c). An intra coefficient of variation of 0% was determined by multiple observations of the 3 study animals on the same day. This was performed weekly from three weeks before oestrous cycle synchronisation for a total of five weeks to determine a mean score. The lameness score of > 95% of individuals was the same, or one assessment was within 1 score different over the duration of the study. Thus, animals with a mean score < 1.5 were classified as Non Lame and those with a mean score of ≥ 1.5 or more were classified as Lame. Animals which were not lame and had a low SCC were deemed 'healthy.'

Oestrous cycle synchronisation: Cows received a GnRH analogue (100 μg buserelin; 2.5 ml Receptal®; Intervet, Milton Keynes, UK) at morning milking followed 7 days later by a PG analogue (500 μg cloprostenol; 2ml Estrumate®; Schering-Plough, Uxbridge, UK).

Milk and blood sampling: Milk samples were taken on alternate days for 3 weeks prior to GnRH administration and then daily until the day after PG injection. Between 2-7 days after PG, the frequency of milk collections increased to twice daily, and then daily for 3 weeks to monitor progesterone profiles. All samples were taken immediately before milking and promptly stored at -20 °C without preservative. Blood samples (10ml) were taken from the tail vein by venepuncture using a Vacutainer®, between 2-7 days after PG immediately following each milking. Samples were centrifuged at 1000 g for 15 min and the plasma stored at -20 °C until oestradiol analysis.

Ultrasonography: The ovaries of 45 animals were examined twice daily *per rectum* with a Concept/ MCV Veterinary Ultrasound Scanner (Dynamic Imaging, Livingstone,

Scotland) using a 7.5 MHz linear array probe from PG administration until ovulation or the appearance of a new follicular wave. Follicles (F) were identified as non-echogenic structures with a defined wall between the antrum and normal ovarian tissue. Corpora lutea (CL) were identified as grainy echogenic structures with a distinct demarcation from the less echogenic normal ovarian stroma (Pierson et al., 1987). Diameters were calculated as the average of two perpendicular measurements. Dominant follicles were defined as those that achieved an internal diameter ≥ 10 mm in the absence of other actively growing follicles (Dobson et al., 2000b; Imwalle et al., 2002b). Ovulation was considered to have occurred when a follicle > 10 mm was recorded on an ovary and absent at the ultrasound session 12 h later.

Hormone assays: Progesterone, analysed as 'pregnane metabolites', was measured in 50 µl whole milk samples after vortex mixing using an established EIA assay (Walker et al., 2008). Samples were compared with standards prepared using progesterone from Sigma-Aldrich, Poole, UK (Cat. #P0130). The minimum detectable amount was 0.015ng/ml; and the intra- and inter-assay coefficients of variation were 8.3 % and 14 %, respectively. Oestradiol was extracted in ether (Mann et al., 1995) and measured in plasma (0.5 ml) with an MAIA kit manufactured by Biodata S.p.A, Roma, Italy, using a previously described modification (Walker et al., 2008). Standards were from the MAIA kit using oestradiol in human serum matrix buffered with Tris and containing sodium azide, <0.1% w/w and glycerol (2.5-10%). The minimum detectable amount was 0.2 pg/ml; the intra- and inter-assay coefficients of variation were 14 % and 17 %, respectively.

Statistical analysis: Differences were considered significant when $P < 0.05$ and reported as a tendency when $0.05 < P < 0.10$. All data were analysed using Minitab (Version 14,

Minitab Inc. Pennsylvania, USA). Analyses corrected for between farm variation. The association between animals that ovulated and high SCC and lameness category was examined by χ-square analysis. Behavioural differences between groups were examined with general linear model analysis of variance (GLM ANOVA) using Bonferroni simultaneous *post hoc* comparisons with the factors of lameness and BCS in the model. Analysis of the timings of various oestrus behaviours utilized "Regression with life data" (survival analysis).

Part Two

Visual observations of oestrus behaviour: A simultaneous parallel study was conducted on 45 cows, 19 of which were included in Part One. All cows were subject to the same conditions and methodologies as cows in Part One but were *also* closely monitored for signs of oestrus. The frequency and intensity of eight different behavioural signs of oestrus were observed for 30 min every 3 h for 7 days following PG injection. Behavioural signs of oestrus were attributed points (Van Eerdenburg et al., 1996); based on the number of times each was observed in a 30-min observation period (behaviours and points shown in Table 1). When the sum of points in a consecutive 30-min observation period exceeded 100 points, an animal was considered to be in estrus, and no longer in estrus when <100 points for 2 or more consecutive periods. Due to the 3-hourly observation regime, the beginning or end was respectively defined as the first (- 1.5 h) or last (+ 1.5 h) 30-min observation period the animal exhibited a particular behavioral sign. The total 'points' received over a whole oestrus period were considered to be a measure of 'intensity'.

Table1. Behaviour scoring scheme: Each recorded observation of an oestrus sign was scored according to the weightings adapted from (Van Eerdenburg et al., 1996)

Behaviour	Points
Restlessness	2
Flehmen	3
Vulval sniffing	10
Mounted but did not stand	10
Chin resting	15
Mounting the rear of another cow	35
Mounting the head of another cow	45
Standing to be mounted (STBM)	100

Results

Part One

A 'baseline' progesterone concentration (0.17ng/ml) was calculated as the mean follicular phase concentration +2 SD of 21 non lame dairy cows from a pilot study. An animal was considered not to have responded to the oestrus synchronisation regime if progesterone concentrations remained below baseline (non luteal) from GnRH injection to Day 4 after PG. Progesterone profiles from all 45 cows revealed 4 animals that did not respond to oestrous synchronisation, (all of which were lame, had a high SCC and failed to ovulate). To avoid bias, these 4 non-synchronised animals, were removed from the study, leaving a total of 41 animals for statistical analysis.

Of the 41 cows remaining in the study, there were 21 healthy animals and 20 with high SCC, nine of which were concurrently lame (High SCC + lame); leaving 11 cows that only had high SCC. The above results have been previously summarized (Morris et al., 2008a), but day-by-day follicle data follow for comparison with hormone profiles.

Follicles: In the high SCC + lame animals, fewer cows ovulated compared to the healthy animals (4/9 *versus* 19/21 $P = 0.006$; χ -square = 7.462). However, high SCC alone did not affect the proportion of cows ovulating (11/11 *versus* 19/21; P = 0.909, χ -Sq = 0.013). High SCC, alone or in combination with lameness, had no effect on the growth, maximum follicular diameter or time of ovulation in the animals that ovulated (Fig.1). The non ovulating animals had smaller follicles on Day 4.5 than ovulating animals regardless of disease status ($P < 0.04$).

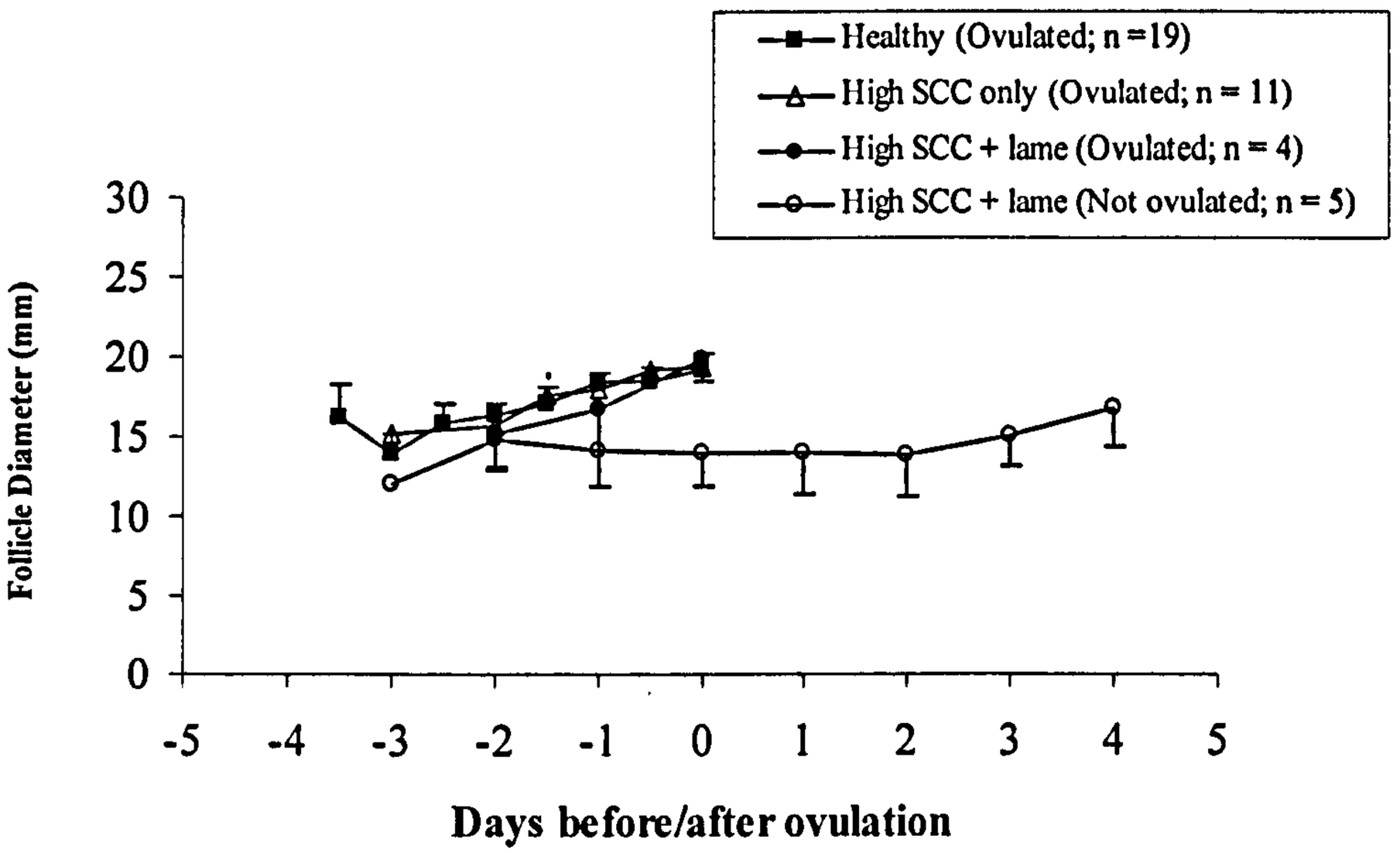


Fig 1. Mean follicular diameter (error bars: SEM) in cows with or without high SCC and lameness, following synchronised follicular phases (GnRH followed 7 days later by PG). Data for non ovulating groups have been aligned to Day 4.5 after PG (mean day of ovulation in ovulating groups).

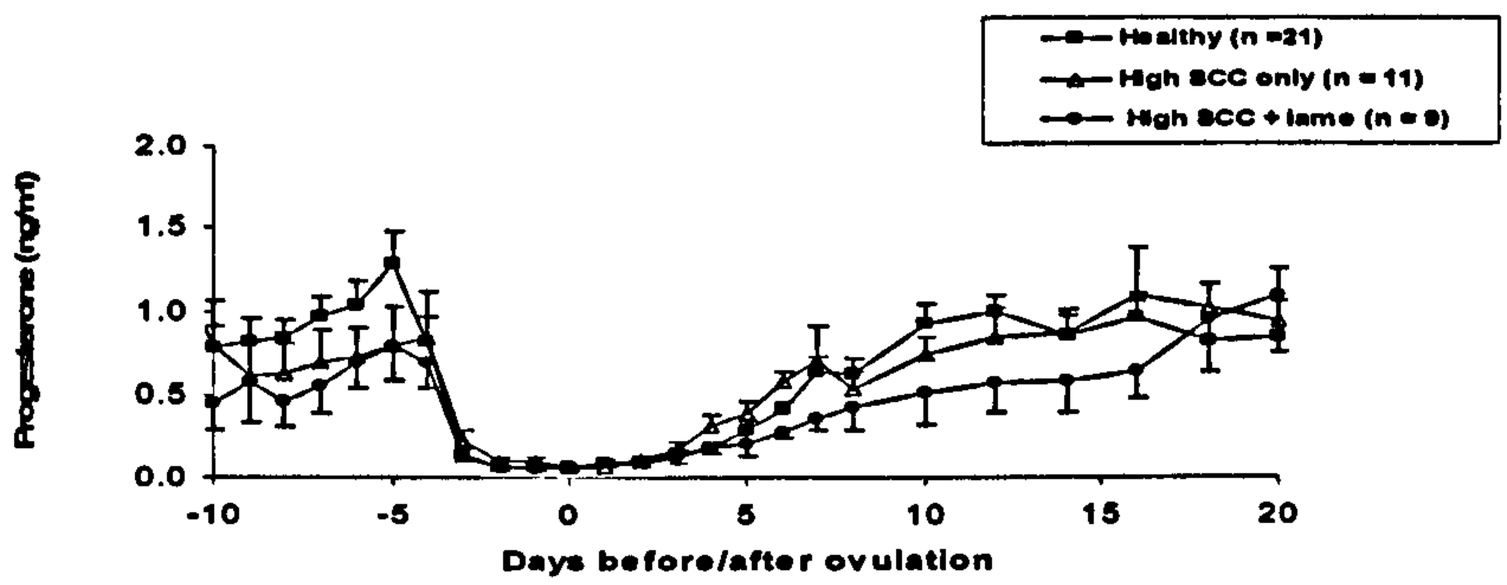
Hormones: Progesterone concentrations were similar in all 3 groups during each of the specific time periods, i.e. days prior to PG injection, the peri-ovulatory period, Day 5, Day 7 and during the mid luteal phase 12-17 days after ovulation ($P > 0.13$, GLM ANOVA; Table 2; Fig. 2a).

Table 2. Milk progesterone concentrations (mean ± SEM; ng/ml) in cows of differing disease status at key times of the study (see text for definitions).

Mean milk progesterone	Healthy $n = 21$	High SCC only $n = 11$	High SCC + lame $n = 9$
5 days before PG	1.01 ± 0.12	0.99 ± 0.22	0.63 ± 0.16
Day -1 to +1 ovulation	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
Day 5 after ovulation	0.33 ± 0.05	0.44 ± 0.06	0.25 ± 0.05
Day 7 after ovulation	0.57 ± 0.07	0.65 ± 0.12	0.38 ± 0.06
Days 12-17 after ovulation	0.83 ± 0.08	0.89 ± 0.11	0.67 ± 0.18

Mean plasma oestradiol concentrations in blood samples collected twice daily during the 36 h period before ovulation were similar in all groups ($P = 0.175$, GLM ANOVA; healthy, n = 21, 2.80 ± 0.30; high SCC only, n = 11, 3.82 ± 0.48; high SCC + lame, n = 9, 2.94 ± 0.51; Fig. 2b). Graphically, mean oestradiol concentrations followed the pattern of mean follicular diameters except for the period 0-12 h before ovulation when oestradiol concentrations in all 3 groups declined, but follicular diameters did not (Fig 1 and Fig 2b).

a.



b.

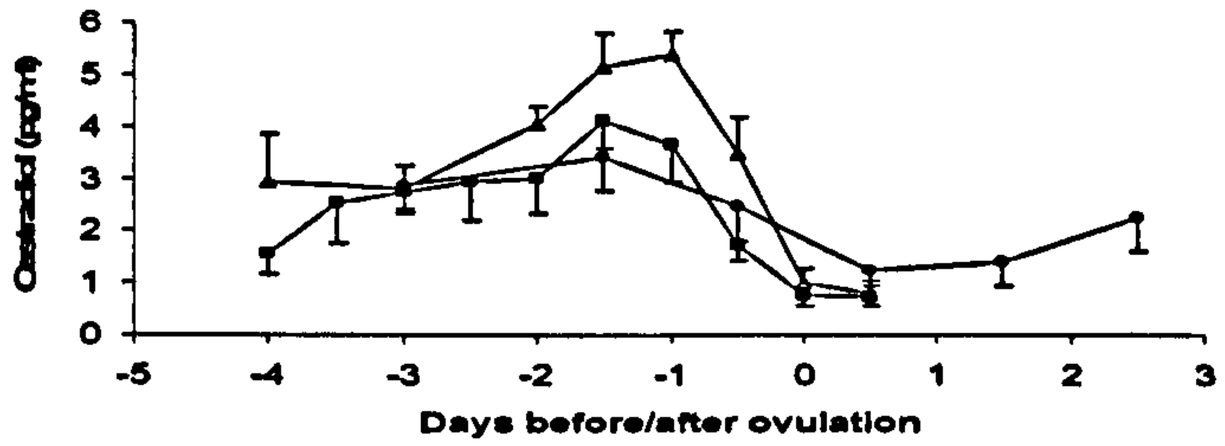


Fig 2. Mean (error bars: SEM) concentrations of a) progesterone in milk (ng/ml) and b) oestradiol in plasma (pg/ml) in cows of differing disease status, prior to and following

hormonal synchronisation (GnRH followed 7 days later by PG). Data for non ovulating groups were aligned to Day 4.5 after PG (mean day of ovulation in ovulating groups).

Part Two

Visual observations of oestrus behaviour: Of the 45 animals that were observed for oestrus, 11 failed to respond to synchronisation, leaving 34 cows in the study for statistical analysis. Of these 34, SCCs were low in 23 cows and high in 11 animals. Out of the total, 28 cows (82%) displayed signs of oestrus; but the proportions of cows displaying oestrus were not different between the high and low SCC groups (8/11 *versus* 20/23; $P = 0.309$; χ -square = 1.037). When lameness was also considered in the group displaying oestrus, there were 8 healthy cows (not lame, low SCC), 5 with only high SCC and 3 with high SCC + lameness.

Including lameness as a random variable within statistical models, cows with high SCC tended to have a less intense oestrus and a lower maximum oestrus score per 30-min period than the low SCC cows (Table 3). Also, intervals from PG to the onset of oestrus and to the first stand-to-be-mounted (STBM) were longer for the high SCC cows than the low SCC animals (Table 3; Fig 3a & b).

All animals that displayed oestrus ovulated and those that did not display oestrus failed to ovulate, irrespective of SCC status. Furthermore, the time to ovulation was not different between SCC groups ($P = 0.11$).

Oestradiol concentrations in twice daily plasma samples during the 36 h period before ovulation were not affected by SCC ($P = 0.25$). Oestradiol concentrations in animals that displayed oestrus was higher than those that did not (3.94 ± 0.30 *versus* 2.38 ± 0.42; p =

0.021). However, there was no correlation between oestradiol concentration and maximum intensity in the 28 ovulating animals (r = -0.03, P = 0.878).

Table 3. Parameters of oestrus behaviour (mean ± SEM) in cows with low or high SCC that were monitored for behaviour every 3 hours.

	Low SCC (n = 20)	High SCC (n = 8)	*P*-value
Interval PG to onset of oestrus (h)	71.6 ± 2.9	84.4 ± 6.1	0.011
Interval PG to first STBM (h)	74.2 ± 3.2	89.6 ± 6.5	0.002
Total oestrus intensity points	1917 ± 247	1437 ± 334	0.063
Maximum points per 3 h period	766± 74	601 ± 135	0.066

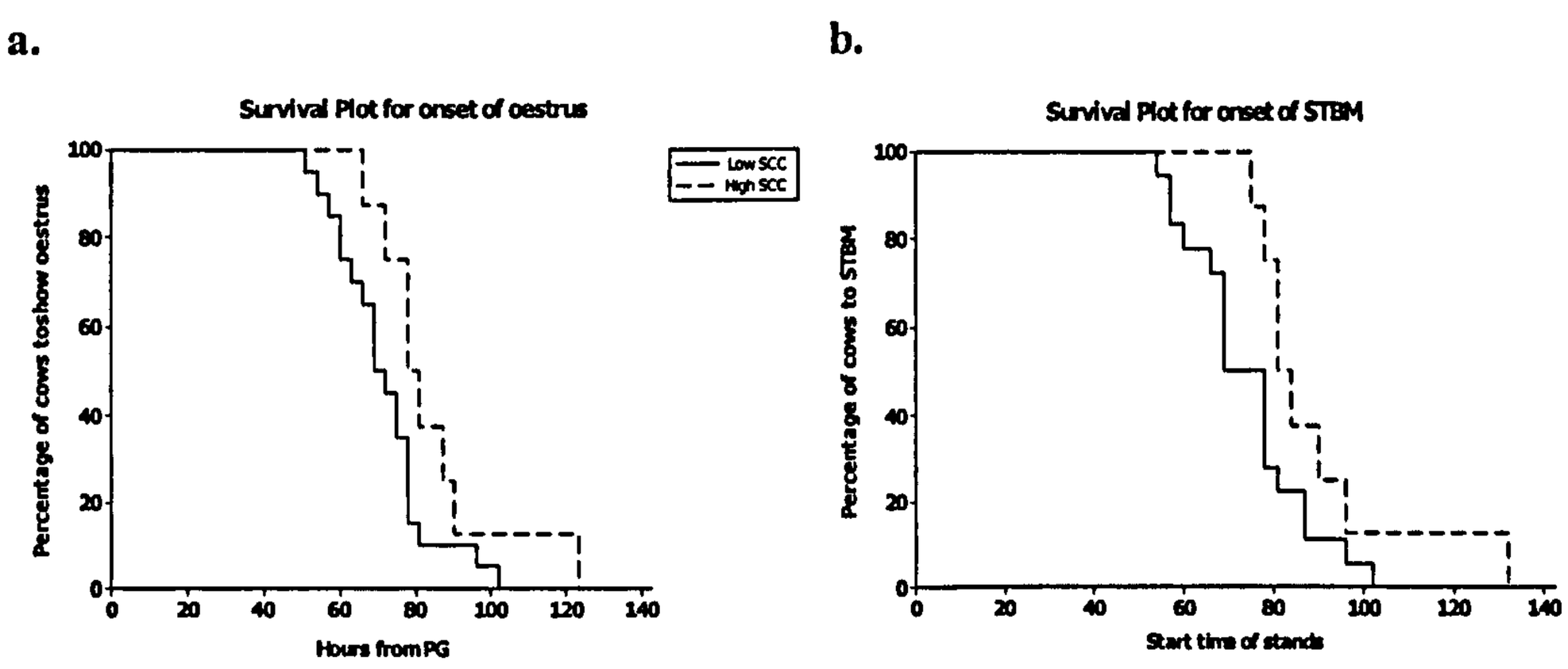


Fig 3. Survival plots generated by regression with life data for start of a) oestrus or b) STBM behaviour in 20 low and 8 high SCC cows.

Discussion

Concurrent high SCC and lameness lowered the number of animals ovulating, although high SCC alone did not decrease the proportion of cows that ovulated and displayed oestrus. These effects were not explained by changes in progesterone and oestradiol concentrations but high SCC did delay the onset of oestrus behaviour and tended to reduce the intensity.

High SCC by itself does not reduce the ability of an animal to ovulate, but the addition of concurrent lameness plays a significant role (Morris *et al.* 2008). The synergistic effect of mastitis and lameness is particularly important as there is a high incidence of both in UK dairy herds (Esslemont et al., 1996). Indeed, there is an association between the prevalence of the two conditions (Sogstad et al., 2006) along with an associated risk of occurrence of both diseases before first insemination in individual animals (Peeler et al., 1994). Thus, the combination of high SCC and lameness is not a rare event and, therefore, the fewer cows that ovulate with the combined conditions as revealed by the present study are important in identifying a main cause of the lowered fertility.

The present study importantly shows that oestrus and ovulation are disrupted in groups of cows with high SCC. Subclinical mastitis is of higher incidence and easily overlooked, while contributing significantly to a reduction in welfare and financial profit (Huijps et al., 2008). Both subclinical and clinical mastitis increase the interval to first insemination, days open and inseminations per conception, especially if subclinical mastitis precedes the clinical condition (Schrick et al., 2001). Clinical mastitis reduces conception rates and increases the number of days open (Santos et al., 2004).

High SCC ± lameness in the present study did not affect maximum follicle diameter, in agreement with similar work using experimentally-induced clinical mastitis (Hockett et al., 2005). Additionally, the present study showed that follicles are smaller in the non ovulated animals compared to those that ovulate, agreeing with (Hockett et al., 2005). Thus, if an animal is going to ovulate, follicular growth rates, maximum size of the dominant follicle and time to ovulation are unaffected, but only in 50% high SCC + lameness animals. The question remains: What are the mechanisms involved that

determine whether a high SCC + lameness cow grow follicles normally and thus ovulate or not?

Clinical mastitis delays the resumption of ovarian activity (Huszenicza et al., 2005). However, in the present study, high SCC did not affect progesterone concentration in animals once they had resumed cyclicity or been stimulated to do so by a synchronisation regime. Progesterone priming prior to the follicular phase plays a key role in establishing oestradiol receptors in the hypothalamus (Gumen et al., 2005b), thus lowered progesterone concentrations may harmfully affect behavioural centres in the brain. Indeed, in other studies on both synchronised and unsynchronised lame cows, progesterone concentrations were lower in the late luteal period (Walker et al., 2008) but results from the present study suggest that this is not the mechanism involved in the different expression of oestrus in high SCC cows. Suprabasal progesterone concentrations have been associated with failure to conceive and a "repeat breeder" syndrome (Bage et al., 2002a) but in the present study progesterone concentrations were low around ovulation (± 1day), in high SCC cows with or without lameness suggesting a different mechanism is responsible for mastitis induced sub fertility. In the luteal phase following ovulation, progesterone concentrations on Days 5 and 7 were similar in all groups of cows, not supporting the hypothesis that high SCC ± lameness is associated with the low early luteal phase progesterone concentrations that lead to early embryonic death or failure of conception (Mann et al., 2001).

There was no difference in plasma oestradiol profiles between normal cows and those with high SCC, with or without concurrent lameness. Also, there was no correlation with oestrus intensity scores. However in retrospect, the sampling frequency in the current study may not have been sufficient with a follicular phase lasting only around 2 days.

Fewer cows with high SCC and concurrent lameness ovulated in both Parts One & Two. In the latter, the high SCC animals also tended to display oestrus with reduced intensity and maximum score. This concurs with effects of lameness on oestrus behaviour in spontaneously cyclic cows (Walker et al., 2008). In the modern farming environment, with emphasis on oestrus detection for artificial insemination (AI), any reduction in behaviour is almost as detrimental as the absence of ovulation. The delay in the onset of oestrus, and especially STBM, highlights another problem. As onset of oestrus was delayed but the time of ovulation was not affected by high SCC ± lameness, there would be a resultant asynchrony of AI with ovulation resulting in the fertilization of aged follicles which may have a reduced capacity for establishing a pregnancy (Mattheij et al., 1994; Mihm et al., 1994).

In conclusion, oestrus was delayed and tended to be of lower intensity in cows with high SCC. There were no detectable differences in progesterone and oestradiol profiles that could explain the lower proportion of animals that ovulated when cows with high SCC were additionally lame. Whatever the precise cause, both these observations will have considerable detrimental effects on fertility.

Acknowledgements

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Influence of lameness and body condition on follicular growth, ovulation, reproductive hormone concentrations and oestrus behaviour in dairy cows

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Abstract

To explain the poor fertility of lame cows, the objective of this study was to examine the effect of lameness ± low body condition on follicle growth and ovulation, steroid hormone profiles, luteinizing hormone (LH) pulsatility and LH surges. Additionally, we examined the role of lameness in modifying the intensity and timing of oestrus. Seventy cows 30-80 days post-partum were scored for lameness and body condition, then their follicular phases were synchronised by administration of gonadotrophin releasing hormone (GnRH) followed seven days later by prostaglandin F2alpha (PG). However, 15 of the lame animals did not initiate a follicular phase in response to synchronisation. Subsequently, cows were scanned using ultrasonography twice daily throughout the follicular phase and more frequent samples of blood were taken in a subset of 20 animals. In the parallel study 45 cows were observed for oestrus behaviour for 30 min every 3 h for 7 days following PG. Overall, 0/15, 26/36 and 17/18 cows ovulated during the study in the non-synchronised lame, synchronised lame and non-lame groups, respectively ($P < 0.05$). In moderate BCS and low BCS groups, 40/58 and 3/11 cows ovulated respectively ($P = 0.009$). During the period 5 days prior to PG injection, mean milk progesterone concentrations in healthy animals was higher than in lame cows ($P < 0.05$). There were no differences in mean milk progesterone concentrations between all groups in the peri-ovulatory period (ovulation ±1days; $P = 0.21$) and on Day 5 after ovulation ($P > 0.05$).

Furthermore, moderate and low BCS cows had similar progesterone concentrations at *all* the designated time periods ($P > 0.3$). All the 12 cows that ovulated during frequent blood sampling had an LH surge occurring 72 ±10 h after PG and 22.2 ± 1.5 h prior to ovulation but these intervals were not influenced by lameness. The LH pulse frequencies were similar in healthy and lame ovulating animals but lower in lame non-ovulating animals ($P < 0.04$). During the 18 h period before the LH surge peak, oestradiol concentrations were higher in all the animals that subsequently ovulated (healthy + lame) than in all non ovulating cows combined (3.7 ± 0.4 pg/ml *versus* 1.4 ± 0.6 pg/ml, P = 0.006.) and pulse frequency was positively correlated with mean oestradiol concentrations (r = 0.64; P = 0.002). The healthy cows had a more intense oestrus and a higher maximum oestrus score in any 30-min period than the lame ovulating cows (P = 0.023 and 0.047, respectively). The intervals from PG to onset of oestrus and to the first stand-to-be-mounted (STBM) were shorter for the lame cows than healthy cows (70.6 ± 2 *versus* 80.5 ± 5.0, P = 0.004 and 72.7 ± 3.4 versus 85.2 ± 5.0, P = 0.002; respectively). In conclusion, for the first time we have identified several differences in hormonal parameters that may explain the poor fertility of lame cows and those with low BCS.

Introduction

Many studies have tried to elucidate the mechanisms involved during stress-induced sub fertility. They have tended to use acute and repeated acute stress (so-called chronic) on rats, mice and ruminants held under experimental conditions (Nanda, 1988; Rivier et al., 1991; Dobson et al., 1999a; Dobson et al., 2001; Suzuki et al., 2001). However, to be meaningful and relevant to commercial farming, it is necessary to examine truly chronic stressors. Cows that have been continuously lame for several weeks require more

inseminations per pregnancy and have a lower pregnancy rate to the first insemination after calving (Lucey et al., 1986; Collick et al., 1989; Hernandez et al., 2001). Thus lameness would appear to be a good naturally occurring chronic stressor to study. The increased likelihood for the incidence of lameness in animals with low BCS suggests that BCS should be also be included in the study analyses as a significant co-variable (Gearhart et al., 1990).

Our previous studies have shown that the incidence of oestrous behaviour is similar in lame cows compared to healthy herd-mates and is accompanied by similar profiles of oestradiol in daily milk samples although oestrus intensity lowered (Morris et al., 2008c; Walker et al., 2008)). Thus, lame cows do secrete oestradiol, many express oestrus and are inseminated but the pregnancy rate to first insemination is still approximately 10 % lower (Collick et al., 1989). It has been suggested that there is a greater incidence of persistent follicles (cysts) in lame cows (Melendez et al., 2003). This implies a lack of ovulation, and hence the low fertility of lame cows might be due to the absence of an adequate ovulatory surge of luteinising hormone (LH). Furthermore, studies in sheep have indicated that acute stressors, such as transport or insulin during the follicular phase, lower LH pulse frequency and delay or block the LH surge (Dobson et al., 2000a) but the effect of chronic stressors on LH in ruminants has not yet been investigated. Other studies have shown varying levels of nutrition also influence ovulation success but individual body condition scores were not recorded (Beam et al., 1999).

Our recent studies also indicate that lame cows have inherently lower progesterone concentrations in the luteal phase prior to expressing oestrus (Walker et al., 2008). Thus, to facilitate frequent collection of blood samples for LH and oestradiol analysis from a

sufficient number of cows, it would be necessary to synchronise ovarian follicular phases using a hormonal regime that does not involve administration of exogenous progesterone so that any effects would not be altered by the synchronisation protocol. Consequently in the present study, we synchronised follicular phases using an injection of gonadotrophin releasing hormone (GnRH) followed seven days later by prostaglandin F2alpha (PG).

To establish why chronically lame or low BCS cows are less fertile than healthy herd-mates, we hypothesise that in both groups (a) the rates of follicular growth and ovulation are lower; (b) the relationship between oestradiol and progesterone profiles is disrupted (and as a consequence oestrus expression is disturbed); (c) the frequency of LH pulses is lower; and (d) the LH surge occurs later or is more likely to be totally absent.

Materials and Methods

Part One

The study was conducted on two commercial farms with 200 and 130 cows from May to November in two successive years. 70 multiparous lactating Holstein cows were enrolled from within the herds which had no confounding clinical conditions except lameness, low BCS and high somatic cell count (SCC) in milk. Cows were enrolled into the study once between 30 – 80 days post partum and at any one time, no more than 12 cows were monitored. Each herd averaged 8800 kg milk per lactation with milking (including study cows) starting at 06.00 h and 16.00 h each day. During the study periods, animals on one farm were out at grass with a supplementary Total Mixed Ration (TMR) fed indoors for one hour immediately after each milking. Pastures were of seasonal ryegrass, Italian ryegrass and white clover. Cows on the second farm were kept inside throughout and fed

TMR *ad libitum*. Cows had been routinely hoof trimmed at the end of the previous lactation. The study was performed under a UK Home Office licence for work on living animals and with the approval of the University of Liverpool Ethical Review Process.

As it has previously been shown that high SCC influences fertility (M Hendry & H Dobson, *personal communication, (Santos et al., 2004; Morris et al., 2008c)*, individual cow pooled milk somatic cell counts were measured every four to six weeks by commercial companies employed by the individual farms (National Milk Records Plc, Chippenham or the Cattle Information Service, Watford). Animals with clinical mastitis (presence of clots or watery milk, with or without inflamed teats) were excluded from the study. The SCC of the study cows immediately prior to oestrous cycle synchronisation was used to define the prevailing status of the cow. A cell count < 100,000 cells/ml was classified as low SCC and a count ≥100,000 cells/ml was deemed to be high (Dohoo et al., 1993).

BCS was determined using a 1-5 scoring system (Chamberlain et al., 1996). Animals with mean BCS < 1.5 were classified as low body condition score (low BCS) and those with a mean score of ≥ 1.5 were classified as moderate body condition score (moderate BCS).

The cows were lameness scored using a standardized 1-5 system (Sprecher et al., 1997c). An intra coefficient of variation of 0% was determined by multiple observations of the 3 study animals on the same day. Both BCS and lameness scoring were performed weekly from three weeks before oestrous cycle synchronisation for a total of five weeks to allow the calculation of a mean score for these chronic conditions. The lameness score of >

95% of individuals was the same, or one assessment was within 1 score different over the duration of the study. Thus, animals with a mean score < 1.5, were classified as Non Lame and those with ≥ 1.5 or more were classified as Lame. Animals that were non lame and ovulated were defined as 'healthy.'

Oestrous cycle synchronisation: Cows received 100 μg buserelin (GnRH; 2.5 ml Receptal®; Intervet, Milton Keynes, UK) at morning milking followed 7 days later by 500 μg cloprostenol (PG; 2ml Estrumate®; Schering-Plough, Uxbridge, UK).

Milk sampling: Milk samples for hormone measurements were taken on alternate days for 3 weeks prior to GnRH administration and then daily until the day after PG injection. Between 2-7 days after PG, the frequency of collections increased to twice daily, and then daily for 3 weeks to monitor the subsequent progesterone profile. All samples were taken immediately before milking and promptly stored at -20°C without preservative.

Ultrasonography: The ovaries of all animals were scanned twice daily *per rectum* with a Concept/ MCV Veterinary Ultrasound Scanner using a 7.5 MHz linear array probe (Dynamic Imaging, Livingstone, Scotland) from PG administration until ovulation or the appearance of a new follicular wave. Follicles were identified as non-echogenic structures with a defined wall between the antrum and normal ovarian tissue. Corpora lutea were identified as grainy echogenic structures with a distinct demarcation from the less echogenic normal ovarian stroma (Pierson, 1984). Diameters were calculated as the average of two perpendicular measurements. Dominant follicles were defined as those that achieved an internal diameter ≥ 10 mm in the absence of other actively growing follicles (Dobson et al., 2000b; Imwalle et al., 2002b). Ovulation was considered to have

occurred when a follicle > 10 mm was absent at the following ultrasound session 12 h later.

Blood sampling: A subset of animals (n = 20) was used to examine LH pulse and surge secretion patterns. Silastic tubing(used as catheters; STHT-C-040; 0.040 inches ID, 0.086 inches OD, 0.023 inches wall; Sani-Tech®, Bio Pure Technology Ltd., Denmead, UK) were placed in the left jugular vein under local anaesthesia. Prophylactic antibiotic (Ceftiofur 1 mg/Kg (Excenel RTU™ ; Pfizer Ltd., Sandwich, UK) was administered subcutaneously at catheter insertion and again at removal. Blood samples were taken at PG injection and 24 h later. To assess pulse parameters, samples were taken every 15 min from 37-46 h after PG. Subsequently, sampling continued every 2 h until ovulation was identified by ultrasonography. If ovulation did not occur, 2-h sampling continued until the appearance of a new follicular wave (approximately 5 days after PG injection). During the 15 min sampling period, cows were restrained by placing a rope across the back of a free access stall/cubicle in which they could sit or stand with access to food and water. Thereafter, cows were restrained in these stalls for ~10min at each 2 h blood sampling and then immediately released.

Hormone assays: Progesterone, was analysed as 'pregnane metabolites' in 50 µl whole milk samples after vortex mixing using an established EIA assay (Walker et al., 2008). Samples were compared with standards prepared using progesterone from Sigma - Aldrich, Poole, UK (Cat. #P0130). The minimum detectable amount was 0.015ng/ml; and the intra- and inter-assay coefficients of variation were 8.3 % and 14 %, respectively.

Oestradiol was extracted in ether (Mann et al., 1995) and measured in plasma (0.5 ml) with an MAIA kit manufactured by Biodata S.p.A, Roma, Italy, using a previously described modification (Walker et al., 2008). Standards were from the MAIA kit using oestradiol in human serum matrix buffered with Tris and containing sodium azide, <0.1% w/w and glycerol (2.5-10%).The minimum detectable amount was 0.2 pg/ml; the intra- and inter-assay coefficients of variation were 14 % and 17 %, respectively.

Luteinizing Hormone was analysed using 50µl plasma neat for pulsatility, whereas samples for LH surge were diluted 1:5 with phosphate buffer, pH 7.0. The assay was an EIA modified from (Graham, 2002). Briefly, the antibody used was LH 518-B7, obtained from C. Munro, UC Davis, California, USA raised in a mouse against bovine LH (Matteri et al., 1987). The antibody working dilution was 1:800,000. Bovine LH standards (NIH-bLH-B10, AFP-5551B) and ovine LH standards (NIH-oLH-518) for labelling were obtained from Prof AF Parlow, Torrance, California, USA. Enzyme labelling was performed using an EZ-Link™ Sulpho- NHS- LC- Biotinylation Kit Prod # 21430 from Pierce, Rockford, Illinois, USA. The biotynylated ovine LH label was used at a working dilution of 1:100,000. The minimum detectable amount was 0.15ng/ml, and the intra- and inter-assay coefficients of variation were 4 % and 15 %, respectively. An LH surge was defined as an increase in LH concentrations > 8 ng/ml for two consecutive samples taken 2 h apart (Dobson et al., 2000b).

Part Two

Visual observation of oestrus behaviour: A simultaneous parallel study was conducted on 45 cows, 19 of which were included in Part One using the same two farms, conditions and methodology as described above in Part One. *Additionally*, the 45 cows in Part Two

were closely monitored for signs of oestrus. Briefly, the frequency and intensity of eight different behavioural signs of oestrus were observed for 30 min every 3 h for 7 days following PG injection. The signs of oestrus were analysed using a weighted scoring method (Van Eerdenburg et al., 1996); cows received 'points' based on the number of times a behavioural sign of oestrus was observed in a 30-min observation period (behaviours and points shown in Table 1). When the sum of points in a consecutive 30-min observation period exceeded 100 points, an animal was considered to be in estrus, and no longer in estrus when <100 points for 2 or more consecutive periods. Due to the 3-hourly observation regime, the beginning or end was respectively defined as the first (-1.5 h) or last (+ 1.5 h) 30-min observation period the animal exhibited a particular behavioral sign. The total 'points' received over a whole oestrus period were considered to be a measure of 'intensity'.

Table1. Behaviour scoring: The individual oestrus signs were scored based on the frequency of recording and the weightings adapted from (Van Eerdenburg et al., 1996).

Behaviour	Points
Restlessness	2
Flehmen	3
Vulval sniffing	10
Mounted but did not stand	10
Chin resting	15
Mounting the rear of another cow	35
Mounting the head of another cow	45
Standing to be mounted	100

LH Pulse Analysis: Pulses of LH secretion above baseline were identified using the PC-PULSAR pulse analysis program (Gitzen & Ramirez, University of Illinois, USA. Version 3.0) based on the pulsar algorithm (Merriam et al., 1982). G-values used were G1 = 3.8, G2 = 2.6, G3 = 1.9, G4 = 1.5 and G5 = 1.2. Baxter parameters were derived

from individual assays and were specific for each animal (Baxter, 1980). Individual contentious pulses were re-examined and confirmed by plotting graphs with errors derived from G1 x Baxter Standard Deviations (SD) and G1 x real SD of the duplicates. Pulse frequency was then calculated by dividing the number of complete pulses by the duration of the sampling period.

Statistical analysis: Statistical differences were considered when $P < 0.05$ and reported as a tendency when $0.05 < P < 0.10$. All data were analysed using Minitab (Version 14; Minitab Inc. Pennsylvania, USA). Analyses corrected for between farm variation. Maximum diameter of the dominant follicle was defined as the diameter just before ovulation (mean = Day 4.5 ± 0.2 after PG), or the diameter on Day 4.5 in those animals that failed to ovulate. Follicular growth was determined as the mean positive change in follicular diameter between time of PG administration and last ultrasound before ovulation. Lameness score, BCS and SCC were included in the all statistical models; however, SCCs were not further considered in the present study. Correlations were examined using Pearson's correlation or Pearson's ranked correlation for non-parametric data. The association between animals that ovulated and lameness category was examined by χ-square analysis. Behavioural differences between groups were examined with Students *t* test. Further statistical methods included general linear model analysis of variance (GLM ANOVA) using Bonferroni simultaneous comparisons with the fixed factors of lameness and ovulation. For repeated measures GLM ANOVA, lameness/ovulation factors were nested within individual animals. LH pulse frequencies were compared using Mann-Whitney tests for non-parametric data. Analysis of the timings of various oestrus behaviours utilized regression with life data (survival analysis).

Results

Part One

A 'baseline' progesterone value (0.17ng/ml) has been previously determined as the mean follicular phase concentration +2 SD of 21 non lame dairy cows (Morris et al., 2008c).

Progesterone profiles revealed 15 lame animals that did not respond to oestrous synchronisation (progesterone values remained below baseline until Day 4 after PG, and none ovulated). These were categorized as lame non responders. The remaining lame or non lame animals were divided into those that did or did not ovulate, forming the following groups: non lame ovulated ('healthy', n = 17); non lame non ovulated (n = 1; thus this animal was removed from all further analyses); lame ovulated (n = 26); and lame non ovulated (n = 11). Hence, a total of 69 animals were studied further, including the 15 lame non responder cows.

Overall, 26/51 and 17/18 cows ovulated in the lame and non-lame groups, respectively (P = 0.005; χ -square = 7.913). If the 15 lame non responder cows were removed from the analysis, fewer lame cows ovulated (P = 0.042; χ -square = 4.15).

There were totals of 11 low BCS cows and 58 moderate BCS cows but fewer low BCS animals responded to the synchronisation (4/11 *versus* 50/58; P = 0.000, χ -Sq = 13.502). All animals with low BCS were concurrently lame (0/17 non lame *versus* 11/52 lame; P = 0.039, χ -Sq = 4.278) and 7/11 low BCS + lame did not respond to synchronisation. In the low and moderate BCS groups, 3/11 and 40/58 cows ovulated, respectively (P = 0.009; χ -square = 6.85. Due to the small number of low BCS animals, further χ –square analysis was not performed.

Follicles: At the time of PG injection, each cow, regardless of group, had a large follicle of at least 10 mm diameter that grew to a maximum of 14 to 33 mm. However, the mean follicular growth rate from PG to ovulation tended to be higher in non lame than lame cows, whether they ovulated or not (Table 2, Fig. 1; $P = 0.065$; $n = 69$). The maximum diameters of the dominant follicle were similar in healthy and lame ovulated cows (Table 2, Fig. 1; $P = 1$). The maximum diameter of the dominant follicle was also similar in those that did or did not ovulate (Table 2, $P = 0.75$). For those cows which did ovulate, the time to ovulation was longer in non lame cows compared to lame cows ($P = 0.038$; Table 2).

In contrast, moderate BCS cows were quicker to ovulate than low BCS animals ($P = 0.014$; moderate BCS 4.2 ± 0.1 days, n = 40; low BCS 4.7 ± 0.9 days, n = 3).

Table 2. Follicular and ovulation parameters in groups of healthy and lame cows

	Healthy (n = 17)	**Lame ovulated (n = 26)**	**Lame non ovulated (n = 11)**	**Lame non responder (n = 15)**
Mean follicular growth rate (mm/day)	[a]2.1 ± 0.2	[b]1.9 ± 0.2	[c]1.1 ± 0.3	[d]1.3 ± 0.2
Maximum follicular diameter (mm)	19.2 ± 0.8	19.5 ± 0.5	18.9 ± 1.1	20.5 ± 1.4
Time from PG to ovulation (days)	[e] 4.4 ± 0.2	[f] 4.1 ± 0.1	Not applicable	Not applicable

Within rows, values [a]*versus* [b+c+d] $P < 0.07$; [e]*versus* [f] $P < 0.04$.

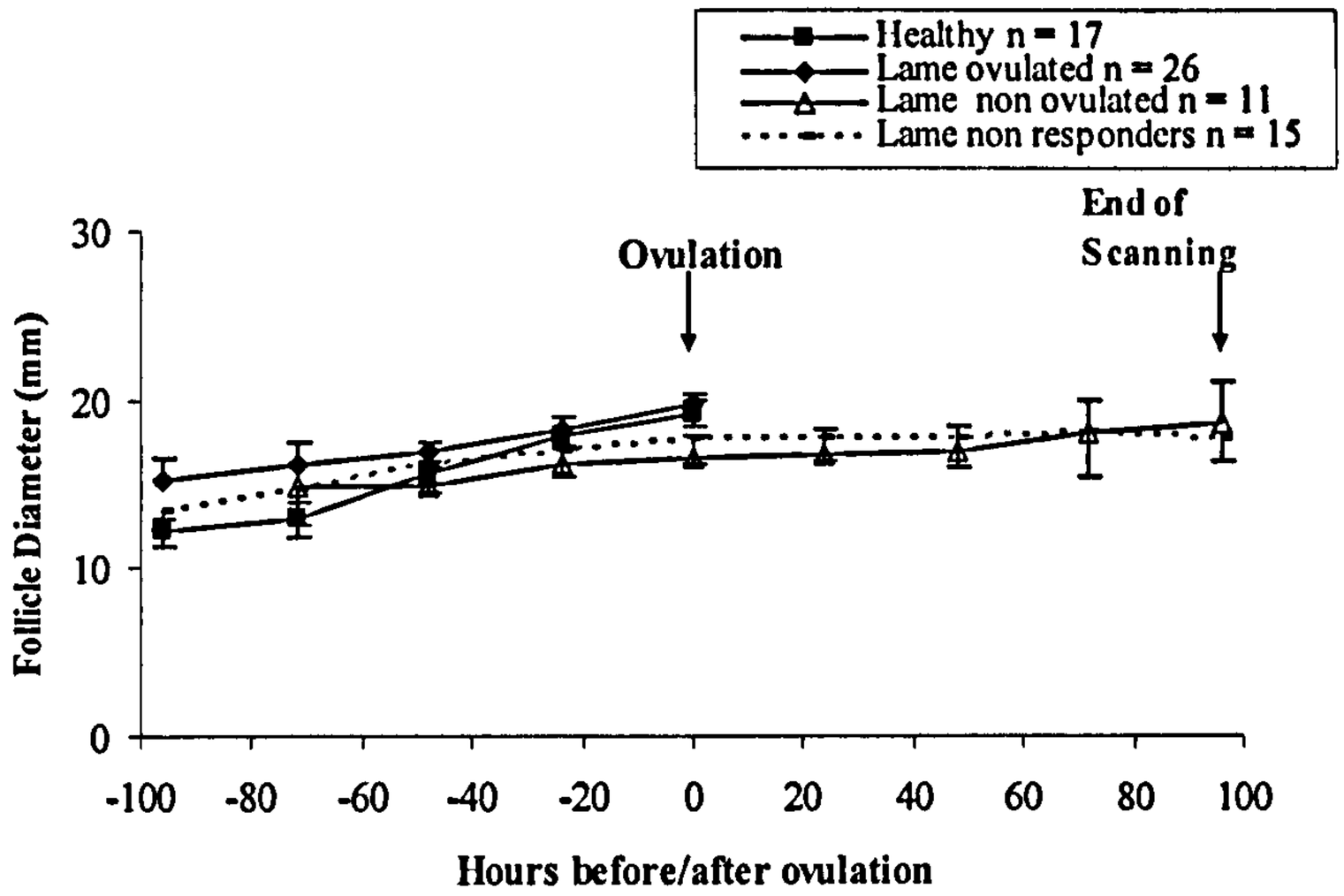


Figure 1. Mean follicular diameter (error bars: SEM) in cows of differing lameness status, around synchronised ovulation (GnRH followed 7 days later by PG). Data were aligned to day of ovulation (or Day 4.5 if ovulation was not observed).

Hormonal analysis in all animals: For the period 5 days prior to PG injection, mean milk progesterone concentrations in healthy animals were higher than in all three groups of lame cows (Table 3; Fig. 2a). Progesterone concentrations were intermediate in both lame ovulated and lame non ovulated cows ($P = 1$); and lame non responder animals had the lowest concentrations (Table 3; Fig. 2a; $P < 0.009$). There were no differences in mean milk progesterone concentrations between all groups in the peri-ovulatory period (Day -1 pre-ovulation to Day +1 post ovulation, $P = 0.21$; Table 3; Fig. 2a). Progesterone concentrations on Day 5 were similar in all groups, although lame ovulated tended to be higher than lame non responder ($P = 0.082$). The concentrations on Day 7 were higher in ovulating animals (healthy and lame ovulated) than in lame non responder cows. The lame ovulated animals also had a higher concentration than lame non ovulated animals ($P < 0.05$; Table 3; Fig. 2a). During the mid luteal phase 12-17 days after ovulation, mean progesterone concentrations were higher in lame ovulated cows than all non ovulated

animals (lame non ovulated and lame non responder; $P < 0.0003$, Table 3; Fig. 2a) and non lame ovulated cows tended to have a higher concentration than lame non ovulated cows ($P = 0.06$, Table 3; Fig. 2a). In 3/11 animals in the latter group, there was a subsequent increase in progesterone which contributed markedly to mean values.

Moderate BCS and low BCS cows had similar progesterone concentrations at all designated time periods ($P > 0.3$, n = 54).

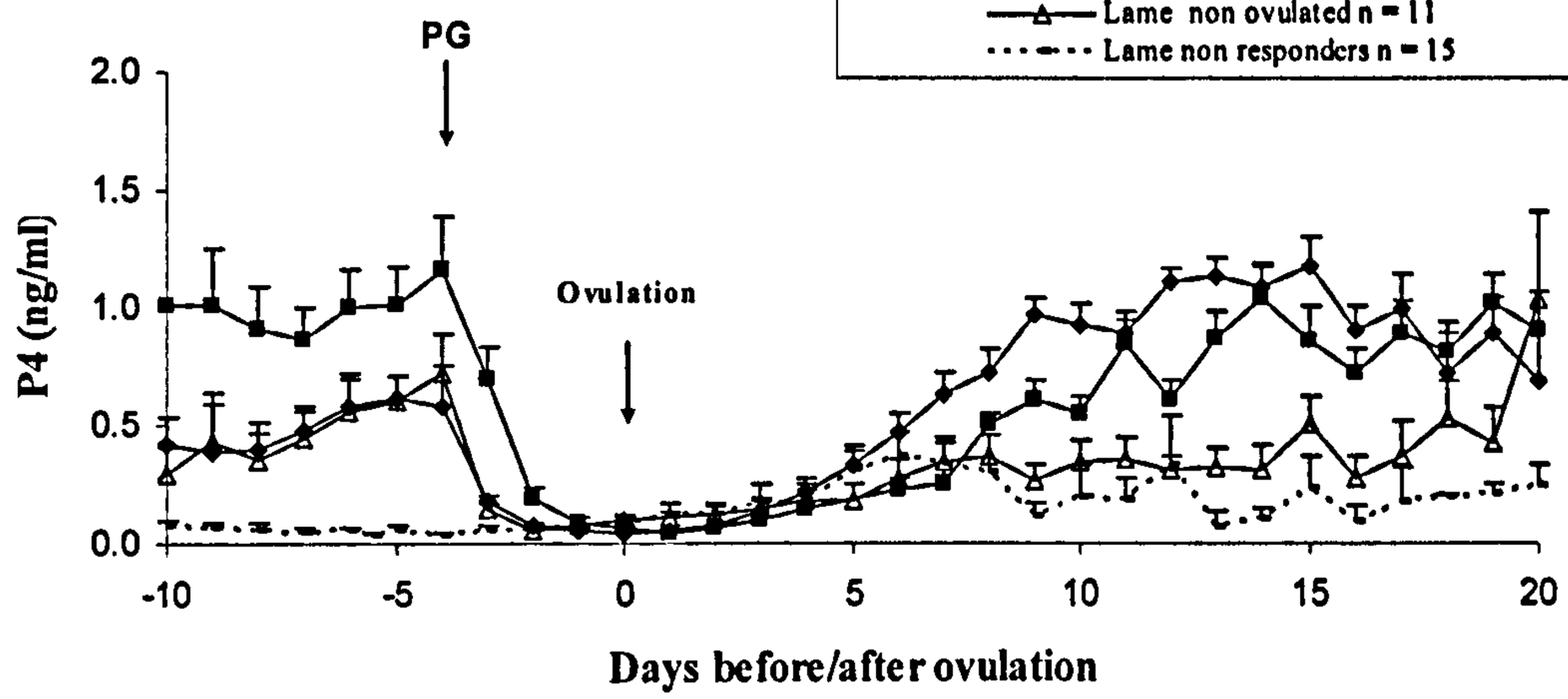
Mean plasma oestradiol concentrations in blood samples collected twice daily during the period 36 h before ovulation were greater in both ovulating groups (healthy and lame ovulated; Fig.2b; $P < 0.03$) than the lame non responder group. Values in the lame non ovulated group were intermediate. Oestradiol concentrations in the low and moderate BCS groups were similar (data not shown).

Table 3. Milk progesterone concentrations (mean ± SEM; ng/ml) in groups of healthy and lame cows.

Mean milk progesterone	Healthy (n = 17)	Lame ovulated (n = 26)	Lame non ovulated (n = 11)	Lame non responder (n = 15)
5 days before PG	[a]0.98 ± 0.14	[b]0.53 ± 0.07	[b]0.52 ± 0.12	[c]0.05 ± 0.01
Day -1 to +1 ovulation	0.06 ± 0.04	0.05 ± 0.01	0.10 ± 0.02	0.30 ± 0.10
Day 5 after ovulation	0.32 ± 0.05	[d]0.37 ± 0.03	0.23 ± 0.06	[e]0.19 ± 0.06
Day 7 after ovulation	[a] 0.59 ± 0.06	[a]0.60 ± 0.04	[b]0.33 ± 0.08	[b]0.29 ± 0.10
Days 12-17 after ovulation	[a] 0.77 ± 0.08	[a]1.03 ± 0.10	[b]0.33 ± 0.08	[b]0.28 ± 0.12

Within rows, values with different superscripts ([a b c]) are different ($P < 0.04$); [d] *versus* [e] $P < 0.09$.
Lame non responder = no response to synchronisation (see text for definition)

a.



b.

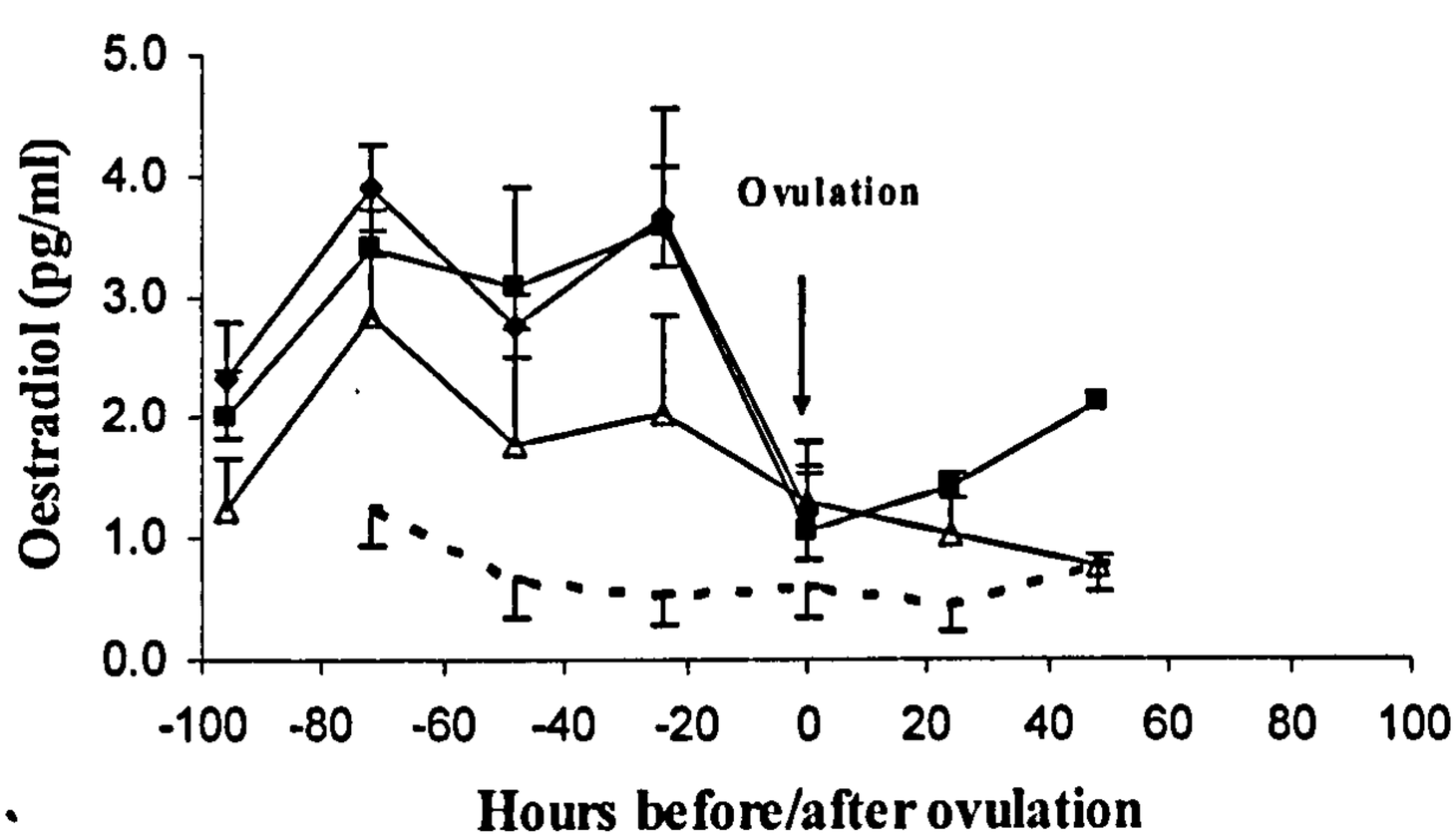


Figure 2. Mean (error bars: SEM) concentrations of a) progesterone in milk (ng/ml) b) oestradiol in plasma (pg/ml) prior to and following synchronised ovulation (GnRH followed 7 days later by PG) in cows of differing lameness status. Data for non ovulating groups were aligned to Day 4.5 after PG (mean day of ovulation in ovulating groups).

Hormonal analysis in frequently bled animals: This subset of animals consisted of 4 healthy and 16 lame cows, and 17 out of all 20 responded to oestrous cycle synchronisation (thus, lame non responder = 3). Twelve of the 17 responding animals

ovulated, comprising 4 healthy and 8 lame ovulated cows; leaving 5 lame non ovulated cows (Table 4). All animals had a BCS ≥ 2, therefore, there were no analyses relating to BCS.

All the 12 cows that ovulated had an LH surge occurring 72 ±10 h after PG and 22.2 ± 1.5 h prior to ovulation. These intervals were not influenced by lameness (healthy *versus* lame ovulated, $P = 0.99$ and 0.24, respectively). Maximum LH (surge) concentrations ranged from 7.3 to 28.8 ng/ml in the ovulating animals but again there was no association with lameness ($P = 0.91$). The 5 lame non ovulated and 3 lame non responder cows had no discernible LH surge (range 4.3 - 6.2 ng/ml) and did not ovulate (Fig. 3a).

During the period 22 to 40 h before ovulation (i.e., during the 18 h period before the LH peak), mean oestradiol concentrations in healthy animals tended to be higher than in the lame non responder group, and values in lame ovulated cows were higher than in the lame non responder group (Table 4; $P = 0.07$ and $P = 0.03$, respectively; Fig. 3b).

Over the same time period, oestradiol concentrations were higher in all the animals that subsequently ovulated (healthy + lame ovulated) than in all non ovulating cows combined (lame non ovulated + lame non responder; 3.7 ± 0.4 pg/ml *versus* 1.4 ± 0.6 pg/ml, $P =$ 0.006.)

Table 4. Mean ± SEM concentrations of oestradiol (E2; pg/ml), maximum LH surge values (ng/ml) and incidence of LH surge and ovulation in groups of healthy and lame cows subjected to frequent blood sampling.

	Healthy ($n = 4$)	**Lame ovulated** ($n = 8$)	**Lame non ovulated** ($n = 5$)	**Lame non responder** (n = 3)
Mean plasma E2 values (pg/ml) during 22- 40 h period before estimated time of ovulation	[a] 3.6 ± 1.0	[c] 3.7 ± 0.4	2.0 ± 0.8	[bd] 0.4 ± 0.2
LH surge observed	4	8	0	0
Max (surge) plasma LH values (ng/ml)	[c] 16.5 ± 4.3	[c] 14.6 ± 2.2	[d] 5.2 ± 0.3	[d] 5.2 ± 0.7
Ovulated	4	8	0	0

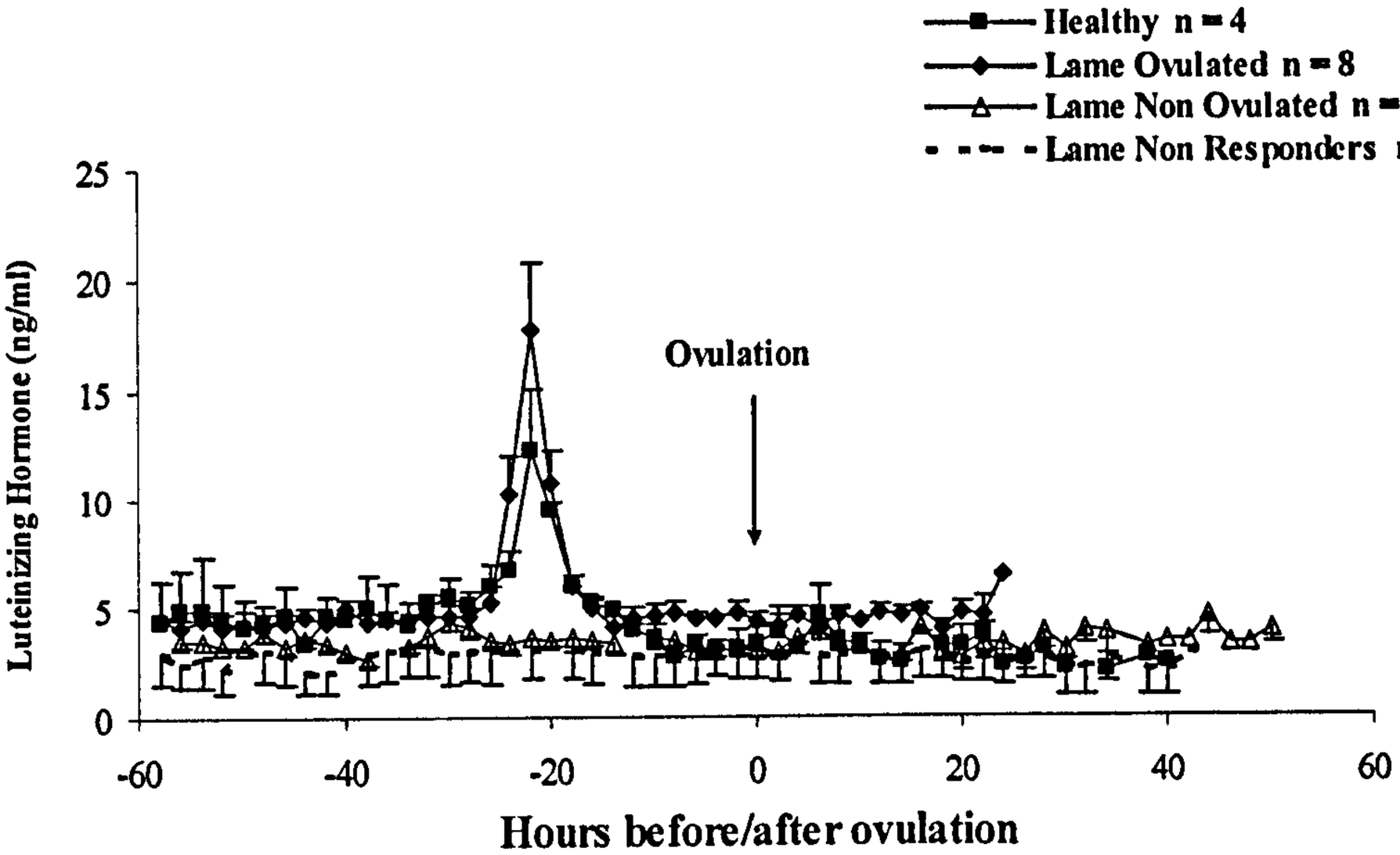
Within rows, values [a] *versus* [b] $P < 0.07$; [c] *versus* [d] $P < 0.03$.

Examples of LH pulse profiles are shown in Fig. 4a-d. The mean LH pulse frequency was similar in healthy and lame ovulated animals ($P = 0.93$) but both were greater than in lame non ovulated animals ($P < 0.04$, Mann-Whitney; Fig. 4e). Lame ovulated animals also tended to have a greater LH pulse frequency than lame non responder cows ($P < 0.06$, Mann-Whitney; Fig. 4e). LH pulse frequency was higher in all the animals that subsequently ovulated (healthy + lame ovulated) than in all non ovulating cows combined (lame non ovulated + lame non responder; Kruskal-Wallis, $P = 0.001$).

LH pulse amplitude (ng/ml) was not different between the different categories of ovulation response by lameness ($P = 0.65$; healthy (4) 0.89 ± 0.17, lame non ovulated (5) 0.68 ± 0.17, lame ovulated (8) 1.3 ± 0.23, lame non responder (3) 0.53 ± 0.16, n = 20).

LH pulse frequency was positively correlated with mean oestradiol concentrations both at the time of pulse sampling (r = 0.59, $P = 0.006$; Pearson ranked correlation), and over the period 18 h before the LH peak (r = 0.64; $P = 0.002$).

a.



b.

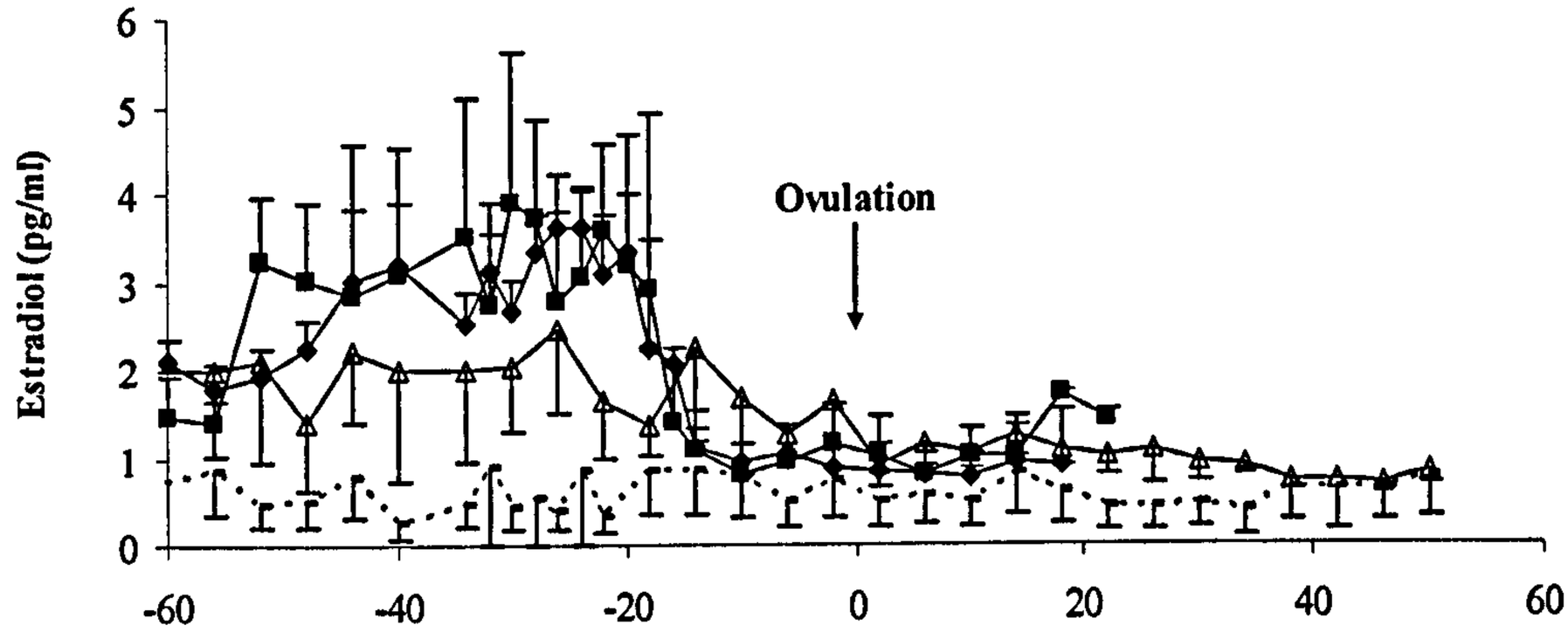


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Figure 3. Mean (error bars: SEM) plasma concentrations of **a)** LH (ng/ml) and **b)** estradiol (pg/ml) in cows of differing lameness status during synchronized follicular phases (GnRH followed 7 days later by PG). Data for non-ovulating groups were aligned to Day 4.4 after PG (mean day of ovulation in ovulating groups).

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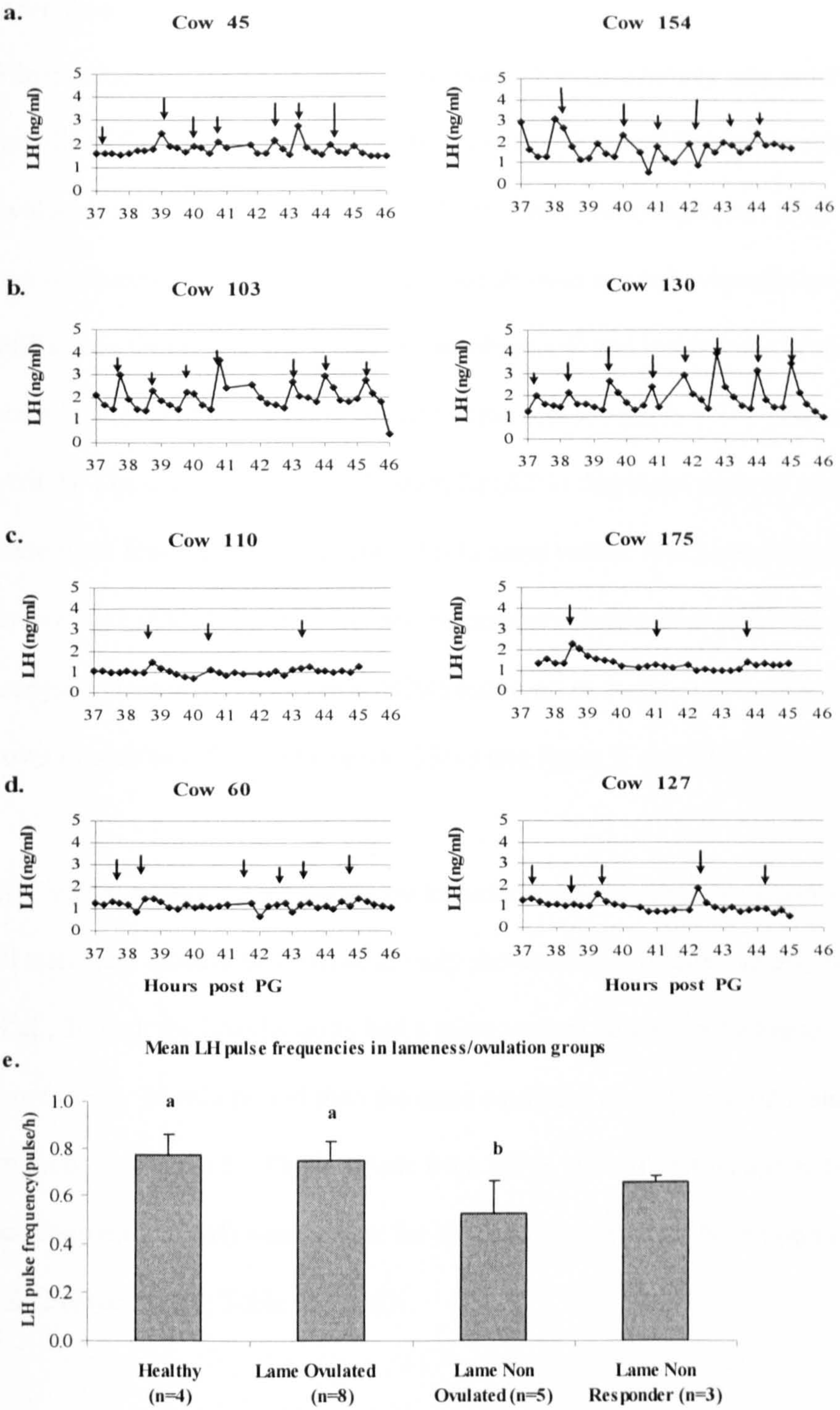

Figure 4. Examples of LH pulses in a) two Healthy cows; b) two Lame Ovulated cows; c) two Lame Non Ovulated cows; d) two Lame Non Responders. e) Histogram of mean LH pulse frequency (pulses/h + SEM) for all animals in each group (values [a] *versus* [b] $P < 0.05$). The arrows represent pulses identified by PC-PULSAR (see text).

Part Two

Visual observations of oestrous behaviour: Oestrus intensity was scored for some (but not all) of the cows in Part One: these included 13 out of 17 healthy animals; 15/26 lame ovulated; 6/11 lame non ovulated; and 11/15 lame non responder. Only 3/26 of the lame non ovulated or lame non responder cows showed any behavioural sign (only 1 or 2 sniffs each throughout the whole period observed) and hence none of these animals were classified to have been in oestrus (minimum score requires > 100 points in a 30-min period). Thus, out of the total 45 cows, 28 (62%) displayed signs of oestrus, of which there were fewer of the lame cows (15/32 lame *versus* 13/13 non lame; $P = 0.001$; χ-square = 11.099). If the 11 lame non responder animals were removed from the statistical analysis, there were 28/34 cows (82%) observed in oestrus but there were still fewer lame cows in oestrus (15/21 lame *versus* 13/13 non lame; $P = 0.034$; χ-square = 4.51).

SCC category was controlled for by included it as a random explanatory variable within all statistical models as we have already shown effects of this variable on oestrus (Morris et al., 2008c); the healthy cows had a more intense oestrus and a higher maximum oestrus score in any 30-min period than the lame ovulating cows ($P = 0.023$ and 0.047 respectively; Table 5). The intervals from PG to start of oestrus and to the first stand-to-be-mounted (STBM) were longer for the healthy cows than lame cows ($P = 0.004$ and 0.002 respectively; Table 5; Fig 5).

All the frequently bled animals that displayed oestrus ovulated and vice versa, irrespective of lameness status. Furthermore, the time to ovulation was not different between lame and non lame animals ($P = 0.104$).

Table 5. Parameters of oestrous behaviour (mean ± SEM) in healthy and lame cows that were monitored for behaviour every 3 h.

	Healthy (n = 13)	Lame ovulated (n = 15)
Total oestrus intensity points	[a] 2254 ± 311	[b] 1368 ± 219
Maximum points per 3 h period	[a] 855 ± 100	[b] 600 ± 77
Interval PG to start of oestrus (h)	[a] 80.5 ± 5.0	[d] 70.6 ± 2.8
Interval PG to first STBM (h)	[a] 85.2 ± 5.0	[d] 72.7 ± 3.4

Within rows, values [a]*versus* [b] $P < 0.05$

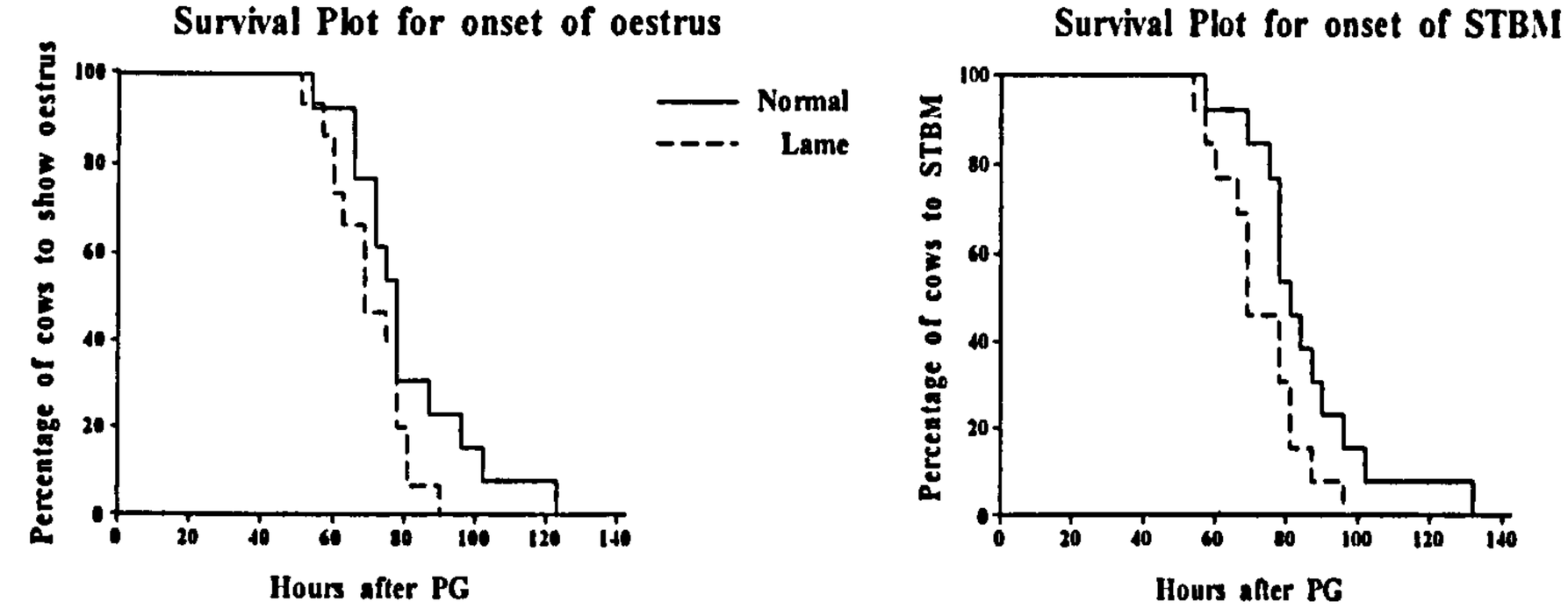

Figure 5. Survival plot generated by Regression with Life tests for a) onset of estrus and b) first STBM in 13 Healthy and 15 Lame cows

Discussion

More lame cows failed to ovulate within the study period than non lame cows. Furthermore, four distinct groups of cows were revealed when categorised by reproductive function and incidence of lameness: namely, 1) healthy (non lame) animals that responded to oestrus synchronisation, all of which ovulated within the study period; 2) lame cows that responded to synchronisation and ovulated – 50% of all lame cows in the study; 3) lame cows that responded to synchronisation but did not ovulate – approximately 20% of all the lame cows; and 4) those lame cows that were totally

unresponsive to the synchronisation regime - approximately 30% of all the lame cows. There appears to be a graded effect of lameness on dairy cows in the postpartum period, ranging from ovarian activity being unaffected by lameness to total shut-down of ovarian response, even to exogenous hormonal stimulation. This was not dependent on the severity of the initial 'lameness score of the system used (data not shown).

Analysis of frequent blood samples in the synchronised follicular phase indicated that LH pulse frequencies and oestradiol concentrations were higher in animals that displayed oestrus and ovulated than those that did not express oestrous behaviour or ovulate. Thus, we propose that the chronic stress of lameness in postpartum cows is associated with a graded dysfunctional ovarian hormone production (ranging from minor to severe as identified above) originating from effects mainly at hypothalamic level because LH pulse frequency is dictated by frequency of GnRH pulses generated from this area of the brain in ruminants (Moenter et al., 1992). The lower LH pulse frequency in lame cows reduces the ability of selected follicles to produce sufficient oestradiol to induce intense oestrous behaviour, an LH surge and ovulation. Indeed, we have previously shown in sheep that acute stressors such as transport or sudden hypoglycaemia reduce LH pulse frequency that ultimately lowers oestradiol concentrations (Dobson et al., 1999b). Similarly in cattle, transport or acute administration of ACTH reduce LH pulse frequency and oestradiol resulting in delayed ovulation (Nanda et al., 1989; Dobson et al., 2000b).

Others have suggested that lame cows have a delayed return to regular ovarian/ovulation cyclicity in the early postpartum period (Garbarino et al., 2004). The present study advances those observations, by showing that 50% of lame cows did not ovulate or express oestrus even after external hormonal stimulation in the postpartum period from

30-80 days after calving. Poor response rates to endogenous or exogenous hormones undoubtedly contribute to the much lower fertility in lame cows after insemination at this economically crucial time (Lucey et al., 1986; Collick et al., 1989). Low BCS also affected the return to cyclicity, with a smaller proportion of the low BCS animals responding to synchronisation, and then a smaller proportion of the responding animals, proceeding to ovulate. This suggests a graded ovarian dysfunction similar to that described above for lameness, although this requires confirmation in a greater number of animals. Nevertheless, similar differences in ovulation success have been seen in cows subjected to varying levels of nutrition (Beam et al., 1999) which may suggest the presence of a common pathway.

All animals in the present study had a follicle greater than 10mm that subsequently grew at similar rate in all groups suggesting that mechanisms controlling follicular emergence, selection and physical growth were unaffected by lameness or low BCS, regardless of prior progesterone status. These mechanisms appear, therefore, to be different from those during heat stress in which growth and size were affected (Wilson et al., 1998; Roth et al., 2000). Furthermore, the maximum diameters achieved were similar in both lame and non lame cows – although, in the non ovulating lame cows, the structure persisted for longer. The large follicles that did not ovulate remained physically present but they were not functional, i.e. hormone concentrations in peripheral plasma were basal and another wave of follicles eventually developed. These observations are consistent with the positive association between lameness and incidence of ovarian cyst diagnosis (Melendez et al., 2003). Interestingly, in Part One of the study, lame cows ovulated earlier than healthy animals. This is in contrast to both Part Two of the study and our previous work showing no difference between lameness groups (Morris et al., 2008a). This may be a

consequence of lower frequency of ultrasound scanning compared to oestrus observations and suggests further work is required to narrow the window for identification of ovulation without the scanning process itself influencing results (Roelofs et al., 2004b).

In the early postpartum period of healthy cows, FSH provokes initial follicle growth; adequate LH pulses stimulate oestradiol production by late follicular phase dominant follicles and this is followed sequentially by oestrous behaviour, an LH surge, ovulation and formation of a good corpus luteum (defined as one producing maximum progesterone concentration for 12-15 days). It requires a fully functional dominant follicle to form a good corpus luteum with a 'mature' progesterone profile (Robinson et al., 2006b). Often, the first postpartum CL is 'immature' and produces lower maximum concentrations of progesterone for a shorter period (7-9 days; (Meisterling et al., 1987; Kawashima et al., 2006). From results of the present study, we suggest that lameness (mediated by an associated low LH pulse frequency) either delays the formation of a good follicle, and hence delays optimum corpus luteum growth and function (Garbarino et al., 2004); or, despite the emergence of a good follicle, subsequent poor function eventually leads to low progesterone production. Evidence for the latter has also been provided in a previous study that revealed low progesterone profiles in unsynchronised lame cows 20-80 days after calving (Walker et al., 2008). The presence of an immature corpus luteum results in reduced progesterone priming at a critical time for the attainment of hypothalamic responsiveness to oestradiol (Gumen et al., 2005b). Whatever the cause, progesterone concentrations in all groups of lame cows in the present study were lower than in healthy animals. Furthermore, follicles that had been luteinised by the GnRH treatment were unable to produce a fully functional corpus luteum before prostaglandin treatment in some lame cows.

Prior exposure to progesterone is undoubtedly important to establish normal oestrous behaviour and ovulation but the lame ovulating and non ovulating groups had similar prior progesterone values, albeit lower than the healthy cows. In the ewe, prior progesterone exposure leads to an increase in the density of oestradiol receptors in the ventro-medial hypothalamus, thus promoting a further increase in LH pulse frequency and oestradiol induction of oestrus behaviour and an LH surge (Caraty et al., 1999).

However, during the few days around ovulation progesterone concentrations were similar in lame and non lame animals, therefore, suprabasal concentrations do not seem to contribute to lameness-induced lowered fertility as suggested in other infertility studies, albeit in non-lame heifers (Bage et al., 2002a). Furthermore, those animals in the present study that did ovulate (100% healthy cows and 50 % of all lame cows) had similar progesterone profiles even on Day 5, suggesting that the low pregnancy rate in lame cows is due to failure of ovulation, not failure of luteal support during this early luteal phase previously high-lighted as critical to establish a successful pregnancy (Mann et al., 2001). The 50 % of lame cows had deficient luteal phases, reflecting the absence of ovulation of the synchronised dominant follicle. Noteworthy, however, is the presence of increased progesterone concentrations in 3/11 of the non ovulating lame animals. These are possibly the result of spontaneous luteinisation of the dominant follicle or ovulation after ultrasonography had stopped.

In a previous study, measuring oestradiol in daily milk samples (Walker et al., 2008), and in the present study (part one) when oestradiol concentrations were monitored twice daily in plasma, we were unable to detect any differences between oestradiol profiles in healthy and lame cows. This was probably because the measurements were too infrequent during

a follicular phase that lasts 2-3 days. However, oestradiol concentrations were lower in plasma samples taken more frequently (every 2 h) from lame non ovulating and non responding cows, although follicles were of similar size in all cows. The presence of a large follicular structure was associated with high oestradiol production in those animals with a higher LH pulse frequency; and only those same animals displayed full oestrous behaviour, had an LH surge and ovulated. It is not yet clear why half the lame cows had adequate LH pulses and consequently high oestradiol concentrations while 20 % lame cows did not (excluding the 30 % lame non responders that had low LH pulses and low oestradiol). Once an LH surge occurred, ovulation followed, with no differences in timing or concentration of LH between the lameness groups. This adds weight to the suggestion that once the thresholds of LH pulsatility and oestradiol required for the production of an LH surge were achieved, ovulation occurred. Oestradiol concentrations were higher in the healthy animals that displayed more intense oestrous behaviour than the lame non ovulating animals in agreement with other work (Lyimo et al., 2000b; Roelofs et al., 2004b).

Reduced intensity of oestrus due to lameness has been previously described (Walker et al., 2008) and this may also be explained by the decrease in progesterone priming which reduces responsiveness to oestradiol thus reducing intensity of oestrus expression (Fabre-Nys et al., 1991). The variability in the interval between onset of oestrus and ovulation reported by others (Saumande et al., 2005) may be a result of the effect of lameness as noted in the present study. At a practical level, quicker onset to start of oestrus and start of STBM in relation to ovulation may lead to incorrect artificial insemination times subsequently leading to poor fertility.

In conclusion, the present study has provided evidence that 20 % animals with a spontaneously occurring stressful condition (lameness), fail to express oestrus and ovulate what was an otherwise seemingly normal follicular structure. A further 30 % of lame animals were incapable of producing a functional follicle in response to exogenous hormonal stimulation. The failure to express oestrus and ovulate was undoubtedly associated with a reduction in LH pulse frequency, lower oestradiol concentrations or responsiveness to oestradiol, and the absence of an LH surge. Nevertheless, further work is required to determine why 50 % lame animals that had similar lower prior progesterone profiles did manage to maintain normal LH and oestradiol profiles, and ovulate, eventually producing a normal functioning corpus luteum.

Acknowledgements

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Influence of exogenous progesterone on follicular growth, ovulation, reproductive hormone concentrations and oestrus behaviour in lame dairy cows

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Abstract

Lameness and low milk progesterone prior to the ovarian follicular phase are associated with lowered intensity of oestrus and fewer cows that ovulate. The main objective of this study was to test the hypothesis that low progesterone reduces oestrus intensity and ovulation potential. This was achieved by supplementing lame cows with progesterone and determining the effect on follicle growth and ovulation, on endogenous progesterone and oestradiol profiles, and, on the intensity and timing of oestrus behaviour. Twenty nine cows, 30-80 days post-partum, were scored for lameness, and their follicular phases were synchronised by administration of gonadotrophin releasing hormone (GnRH) followed seven days later by prostaglandin F2alpha (PG). Two thirds of the lame cows received an intra-vaginal progesterone releasing device (CIDR) for a five day period starting three days after GnRH. Milk samples were collected throughout the study period, blood was sampled twice daily during the follicular phase and the ovaries were subjected to ultrasonography. Cows were observed for oestrus behaviour five times a day for a total of four hours each day for seven days following PG. The proportions of cows ovulating between the three groups of animals were similar: Non Lame, Lame + CIDR and Lame - CIDR (12/14 *versus* 7/10 *versus* 4/5; $P = 0.64$). For the same three groups, the following parameters were similar: mean follicular growth, maximum follicular diameter, time from PG to ovulation; progesterone concentration profiles throughout the study; and mean oestradiol concentrations during the period 36 h before ovulation. Similar proportions of

animals stood-to-be-mounted (STBM) in the Non Lame cows, Lame + CIDR and Lame - CIDR cows (12/14 *versus* 7/10 *versus* 4/5; $P = 0.64$). Total oestrus intensity was unaffected by lameness or the presence of a CIDR but was related to mean plasma oestradiol concentrations -36 to 0 h before ovulation ($P = 0.008$). Intervals from PG to onset of oestrus and to the first STBM were similar between Non Lame and Lame + CIDR cows ($P = 0.14$ and $P = 0.14$, respectively). In the 8 Non Lame STBM animals, vulval Sniffing first occurred 6 ± 2.7h before onset of STBM ($P = 0.058$). Chin Resting began 5.1 ± 0.9h before STBM ($P = 0.001$) and onset of rear mounting behaviour 4.3 ± 1.3h before onset of STBM ($P = 0.018$). The onset of Chin Resting occurred 1.4 ± 0.7h before the same animal was Chin Rested upon ($P = 0.082$). In the 8 Non Lame STBM cows, there were positive correlations between frequencies of the following behaviours: Sniff *versus* Sniffed; Sniffed *versus* Chin Rested; Sniffed *versus* STBM; and Chin Rested *versus* STBM ($P < 0.05$). In the 6 Lame + CIDR STBM cows, there were positive correlations between frequencies of Sniffed *versus* Rear mount; and Mounted not Stood *versus* STBM ($P < 0.05$).

In conclusion, progesterone supplementation did not affect endogenous progesterone and oestradiol profiles or follicular dynamics in lame cows. Total oestrus intensity was related to plasma oestradiol concentration and distinct associations have been identified between onset times and frequencies of individual oestrus behaviours.

N.B. Due to abnormal progesterone and oestradiol results, several animals were removed from the study after initial selection. This resulted in too few animals in the Lame – CIDR group which consequently affected statistical analysis. More animals are currently being studied to enable future publication.

Introduction

In the UK dairy industry, lameness has a negative impact on fertility (Lucey et al., 1986; Collick et al., 1989). This compromises welfare and profit, both directly and indirectly via effects on fertility as the incidence of lameness can be as high as 25% (Esslemont et al., 1996).

Our recent studies have shown that lame cows have lower progesterone concentrations in the latter part of the luteal phase, express less intense oestrous behaviour and are less likely to ovulate (Morris et al., 2008c; Morris et al., 2008b; Walker et al., 2008). It is also known that a reduction in late luteal phase progesterone priming results in reduced hypothalamic responsiveness to oestradiol (Gumen et al., 2005b). Additionally, fertility is affected by reduced progesterone concentrations in the period 4-7 days before insemination (Meisterling et al., 1987).

The present study was designed to test the hypothesis that low progesterone priming is responsible for the reduced oestrus behaviour and lowered likelihood of ovulating in lame cows. Follicular phases were synchronised with gonadotrophin releasing hormone (GnRH) followed seven days later by prostaglandin F2alpha (PG) (Morris et al., 2008c; Morris et al., 2008b; Morris et al., 2008a). A proportion of the lame animals received exogenous progesterone via an intra-vaginal releasing device (CIDR). Follicular growth rates, ovulation and hormone profiles were compared between non lame animals and lame cows with or without progesterone supplementation.

Oestrous behaviour is dependant on both oestradiol and progesterone (Allrich, 1994) and the duration of expression of secondary oestrous signs is shorter in animals that stand-to-

be-mounted (STBM) than those that do not (Yoshida et al., 2005). To further elucidate the relationship between different oestrus we assessed the total frequency and precise timing of individual oestrous behavioural signs especially in relation to each other, and between lame or non-lame animals.

Materials and Methods

The study was conducted on two commercial farms with 200 and 130 cows, respectively, from June to October 2007. The 31 multiparous lactating Holstein cows had an average milk yield of 8900 kg per lactation with milking (including study cows) starting at 06.00 h and 16.00 h each day. Animals were enrolled from the whole herd as those without any confounding clinical conditions except lameness. Body condition score (BCS) and somatic cell counts (SCC) were noted in order to account for the possible effect of these in subsequent statistical analyses. The cows entered the study 30 – 80 days post partum and at any one time, no more than eight cows were monitored. During the study periods, animals on one farm were out at grass with a supplementary Total Mixed Ration (TMR) fed indoors for one hour immediately after each milking. Pastures were of seasonal ryegrass, Italian ryegrass and white clover. Cows on the second farm were kept inside throughout and fed TMR *ad libitum*. Cows had been hoof trimmed at the end of the previous lactation. The study was performed under a UK Home Office licence for work on living animals with the approval of the University of Liverpool Ethical Review Process.

Individual cow SCCs from a pooled milk sample from all quarters of the udder were measured every four to six weeks by commercial companies employed by the individual

farms (National Milk Records Plc, Chippenham or Cattle Information Service, Watford). The SCCs of the study cows immediately prior to oestrous cycle synchronisation were used to define the prevailing status of the cow. Animals with clinical mastitis (presence of clots or watery milk, with or without inflamed udder) were excluded from the study. A cell count < 100,000 cells/ml was classified as low and a count ≥ 100,000 cells/ml was deemed a high SCC, reflecting either no or possible udder infection, respectively (Dohoo et al., 1993).

Body condition score was determined using a 1-5 scoring system (Chamberlain et al., 1996). Animals with BCS < 1.5 were classified as low BCS and those ≥ 1.5 were classified as moderate BCS.

Lameness was scored using a standardized 1-5 system (Sprecher et al., 1997c). An intra coefficient of variation of 0% was determined by multiple observations of three study animals on the same day. Both BCS and lameness scoring were performed weekly from three weeks before oestrous cycle synchronization for a total of five weeks in order to calculate a mean score for these chronic conditions. The lameness score of > 95% of individuals was the same, or one assessment was within 1 score different over the duration of the study. Thus, animals with a mean score < 1.5 were classified as Non lame and those with a mean score of ≥ 1.5 or more were classified as Lame.

Oestrous cycle synchronization: Cows received 100 µg buserelin (GnRH; 2.5 ml Receptal®; Intervet, Milton Keynes, UK) at morning milking followed 7 days later by 500 µg cloprostenol (PG; 2ml Estrumate®; Schering-Plough, Uxbridge, UK).

Progesterone supplementation: Progesterone supplementation was achieved by inserting an intravaginal CIDR® 1.94g progesterone; InterAg, Hamilton, NZ. Lame cows were randomly divided into two groups, one of which received CIDR (Lame + CIDR) compared to Lame - CIDR. Three days after GnRH injection, a CIDR was inserted for two days being replaced with a new CIDR which was removed immediately prior to PG injection on Day 0.

Milk sampling: Milk samples were taken on alternate days for three weeks prior to GnRH administration, then daily until the day after PG injection. From Days 2-7 after PG, milk collections were increased to twice daily, then reverted to daily for three weeks to monitor the subsequent progesterone profile. All samples were taken immediately before milking and promptly stored at -20 °C without preservative.

Ultrasonography: The ovaries of all animals were scanned *per rectum* with a Concept/ MCV Veterinary Ultrasound Scanner (Dynamic Imaging, Livingstone, Scotland) using a 7.5 MHz linear array probe twice daily from PG administration until ovulation, or until the appearance of a new follicular wave. Follicles were identified as non-echogenic structures with a defined wall between the antrum and normal ovarian tissue. Corpora lutea (CL) were identified as grainy echogenic structures with a distinct demarcation from the less echogenic normal ovarian stroma (Pierson et al., 1984). Diameters were calculated as the average of two perpendicular measurements. Dominant follicles were defined as those that achieved an internal diameter ≥ 10 mm in the absence of other actively growing follicles (Dobson et al., 2000b; Imwalle et al., 2002b). Ovulation was considered to have occurred when a follicle > 10 mm was absent 12 h later.

Hormone assays: Progesterone, analysed as 'pregnane metabolites', was measured in 50 µl whole milk samples after vortex mixing using an established EIA assay (Walker et al., 2008). Samples were compared with standards prepared using progesterone from Sigma - Aldrich, Poole, UK (Cat. # P0130). The minimum detectable amount was 0.015ng/ml; and the intra- and inter-assay coefficients of variation were 8.3 % and 13 %, respectively.

Oestradiol was measured in 0.5 ml plasma with an MAIA kit manufactured by Biodata S.p.A, Roma, Italy, using a previously described modification (Walker et al., 2008). Standards were from the MAIA kit using oestradiol in human serum matrix buffered with Tris and containing sodium azide, < 0.1% w/w and glycerol (2.5-10%). The minimum detectable amount was 0.2 pg/ml; the intra- and inter-assay coefficients of variation were 8 % and 13 %, respectively.

Visual observations of oestrus behaviour: All animals in the study were closely monitored for signs of behavioural oestrus. Briefly, the frequencies of seven different behavioural signs of oestrus were observed for 30-60 min five times per day for 7 days following PG injection. Cows were monitored at 5 a.m. before milking; 9 a.m. after milking; 12 noon; 3 p.m. before milking and at 9 p.m. for a total monitoring time of 4h. The signs of oestrus were further quantified using a modification of an accepted weighted scoring method (Van Eerdenburg et al., 1996) in which each behavioural sign of oestrus was attributed points based on the frequency of each observation in a 30-min observation period (behaviours and points shown in Table 1). When the sum of points in a consecutive 30-min observation period exceeded 100 points, an animal was considered to be in estrus, and no longer in estrus when <100 points for 2 or more consecutive periods. The onset or end of a behaviour was respectively defined as the first (minus 0.5 the

preceding interval) or last (plus 0.5 the following interval) time a particular behavioural sign was observed; duration was taken as the sum of intervals between the onset and end. Total oestrus intensity was defined as the total points received over a whole oestrus period. The onset and end of all oestrus behavioural signs were aligned to the first STBM.

Table 1. Behaviour scoring scheme: Each recorded observation of an oestrus sign was scored according to the weightings adapted from (Van Eerdenburg et al., 1996)

Behaviour	Points
(Vulval) Sniff	10
(Vulval) Sniffed	10
Chin Rest	15
Chin Rested (on)	15
Rear Mount (of another cow)	35
Mounted not Stood	10
(Stand to be mounted) STBM	100

Statistical analysis: Statistical differences were considered when $P < 0.05$ and reported as a tendency when $0.05 < P < 0.10$. All data were analysed using Minitab (Version 15; Minitab Inc. Pennsylvania, USA). Between Farm variation was accounted for in statistical analyses. As we have previously established a relationship between BCS or SCC and various reproductive parameters (Morris et al., 2008c; Morris et al., 2008a), both BCS and SCC were included in all statistical models but not studied further as explanatory variables. The associations between animals that ovulated or STBM, with lameness or CIDR treatment, were examined by χ-square analysis. Statistical methods included general linear model analysis of variance (GLM ANOVA) and paired Students *t* test. Linear regression analysis was used to identify the relationship between oestrus intensity and oestradiol concentration and Pearson's correlation was used to examine relationships between specific oestrus behaviours.

Results

A baseline milk progesterone value (0.17ng/ml) has been previously determined as the mean follicular phase concentration +2 SD in 21 non lame dairy cows (Morris et al., 2008c). Progesterone profiles in the present study revealed one animal that did not respond to oestrus synchronization because progesterone concentrations remained below baseline throughout the study. Another animal had a persistent, large follicular structure that was associated with an aberrant plasma oestradiol profile. Both these animals were removed from further analysis leaving data from 29 cows to be analysed.

There were 14 non-lame and 15 lame cows, and 10 of the latter group received a CIDR. Overall, 23 animals ovulated but there was no difference in proportions of cows that ovulated between Non Lame, Lame + CIDR and Lame - CIDR cows (12/14 *versus* 7/10 *versus* 4/5; $P = 0.64$, χ-Sq = 0.88).

Mean follicular growth rate, maximum follicular diameter and time from PG to ovulation were similar between the three groups ($P > 0.05$; Table 2.)

Table 2. Mean ± SEM follicular parameters in Non Lame or Lame cows with or without a CIDR, the ovaries of which were scanned by ultrasound twice daily following PG injection.

	Non Lame $n = 14$	Lame + CIDR $n = 10$	Lame - CIDR $n = 5$
Mean follicular growth rate (mm/day)	1.4 ± 0.4	1.4 ± 0.3	1.5 ± 0.6
Max. follicular diameter (mm)	18.6 ± 0.4	15.0 ± 1.1	18.2 ± 1.5
Numbers ovulating (%)	12 (86%)	7 (70%)	4 (80%)
Days to ovulation after PG	4.5 ± 0.4	4.3 ± 0.5	4.3 ± 0.6

Hormones: Milk progesterone concentrations were similar in the three groups during each of the specific time periods analysed, i.e. for 5 days prior to PG injection, the peri-ovulatory period, Day 5, Day 7 and during the mid luteal phase 12-17 days after ovulation ($P > 0.4$, GLM ANOVA; Table 3). Progesterone concentrations were greater 1 day following first CIDR insertion ($p = 0.03$, Pairwise t-test). In addition non CIDR cows showed no increase on the same days ($p = 0.05$)

Table 3. Milk progesterone concentrations (mean ± SEM; ng/ml) in Non Lame or Lame cows ± a CIDR.

Mean milk progesterone	Non Lame $n = 14$	Lame + CIDR $n = 10$	Lame - CIDR $n = 5$
For 5 days before PG	1.08 ± 0.16	1.06 ± 0.24	0.80 ± 0.20
Day -1 to +1 ovulation	0.09 ± 0.01	0.07 ± 0.01	0.05 ± 0.02
Day 5 after ovulation	0.37 ± 0.05	0.37 ± 0.10	0.27 ± 0.07
Day 7 after ovulation	0.59 ± 0.10	0.68 ± 0.19	0.57 ± 0.10
Days 12-17 after ovulation	0.93 ± 0.12	0.84 ± 0.18	1.12 ± 0.15

Mean oestradiol concentrations in plasma samples taken every 12h during the period -36 to 0 hours before ovulation did not vary between the three groups ($P = 0.812$, GLM ANOVA; Non Lame, 2.85 ± 0.32; Lame + CIDR, 2.19 ± 0.38; Lame - CIDR, 2.63 ± 0.76 pg/ml).

Visual observations of oestrus behaviour: Total oestrus intensity was unaffected by lameness or presence of a CIDR (data not shown; $P = 0.31$ and 0.53, respectively; GLM ANOVA with fixed factors of lameness and CIDR; including with or without ovulation in the model). However, mean plasma oestradiol concentrations between 36 and 12 hours before ovulation in animals sampled twice daily were related to total intensity ($R^2 = 23.2$, $P = 0.005$; Linear Regression Analysis). Total intensity was not associated with mean

progesterone concentration for 5 days prior to PG injection, at PG or in the peri-ovulatory period (-1 to +1 from ovulation; $P > 0.5$)

Of the 29 animals in the study, 21 were considered in oestrus (achieved > 100 points) but only 17 animals STBM. However, two of this latter group did not ovulate. Similar proportions of animals STBM in the Non Lame, Lame + CIDR and Lame - CIDR groups (9/14 *versus* 6/10 *versus* 2/5; $P = 0.63$, χ -Sq = 0.91).

In the 21 cows that were deemed to be in oestrus, all individual oestrus behaviours occurred in similar proportions of Non Lame and Lame + CIDR cows (Table 4, $P > 0.1$). However, there were insufficient animals in the Lame – CIDR group (n = 2) for further statistical analysis.

Table 4. Number of cows displaying individual oestrus behaviour signs in Non Lame and Lame ± CIDR cows which displayed oestrus (>100 points) when monitored for behaviour five times daily.

	Non Lame (n = 13)	**Lame + CIDR (n = 6)**	**Lame – CIDR (n = 2)**
Sniff	13	6	2
Sniffed	12	5	2
Chin Rest	12	6	2
Chin Rested	11	5	2
Rear Mount	10	4	2
Mounted not Stood	5	2	2
STBM	9	6	2

Variability of oestrus expression: There were wide ranges in the frequencies of the individual behaviours between the 19 animals that reached >100 points within the oestrus period: Sniff 3 - 63; Rear Mount 0 – 29; and STBM 0 – 33. The frequencies of individual oestrus behaviours were similar between Non Lame and Lame + CIDR cows ($P > 0.1$, Fig 1.)

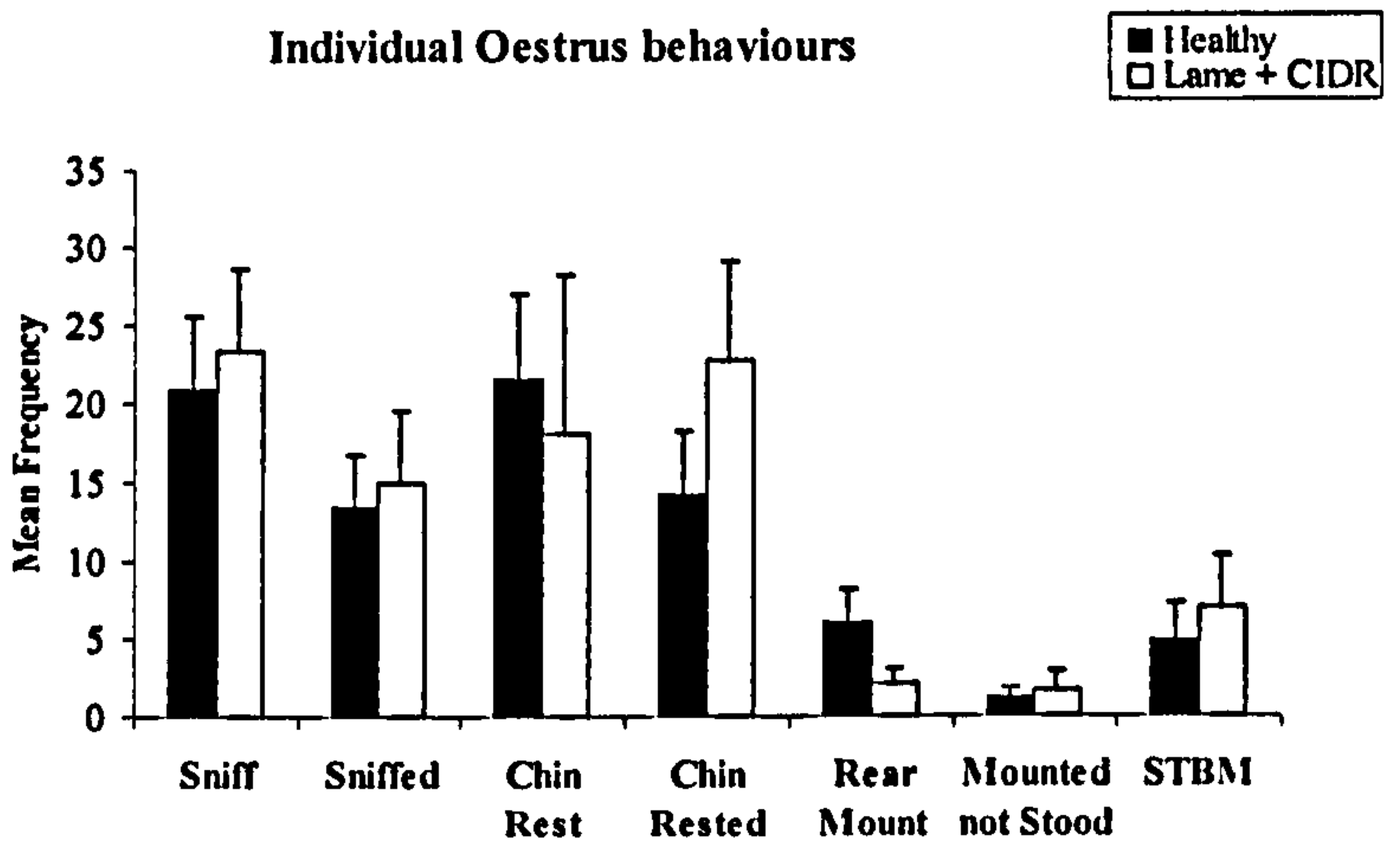


Fig 1. Frequency of individual oestrus behaviours in 13 Non Lame and six Lame + CIDR cows which displayed oestrus (>100 points) when monitored for behaviour five times daily.

Of the 15 animals that STBM in the two groups, 12 (80%) mounted the rear of another cow with no difference in the proportions of Non Lame (8/9) and Lame + CIDR (4/6) animals (χ -Sq = 1.111, $P = 0.292$). Similar proportions of Non Lame and Lame + CIDR cows were Mounted not Stood (6/9 *versus* 2/6; χ -Sq = 1.607, $P = 0.205$).

One animal in the Non Lame group displayed a 'split oestrus' (Stevenson et al., 1996) and hence was removed from further analysis leaving eight animals to be studied further. The intervals from PG to onset of oestrus and to the first STBM were similar between the Non Lame and Lame + CIDR cows respectively (Onset of oestrus: 66.8 ± 7.1h *versus* 77.2 ± 13.9h; $P = 0.14$; Fig. 2a and onset of STBM: 71.0 ± 7.0h *versus* 80.3 ± 14.6h; $P = 0.14$; Fig. 2b).

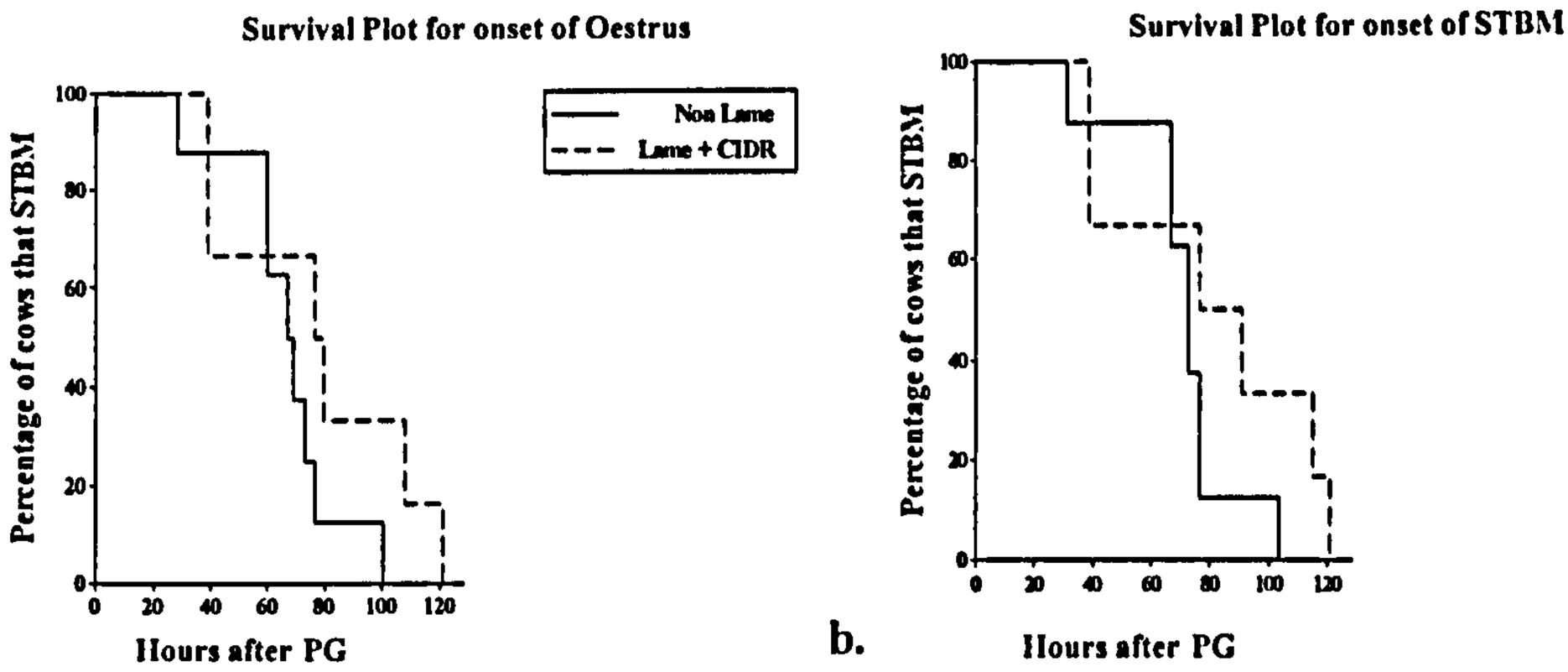
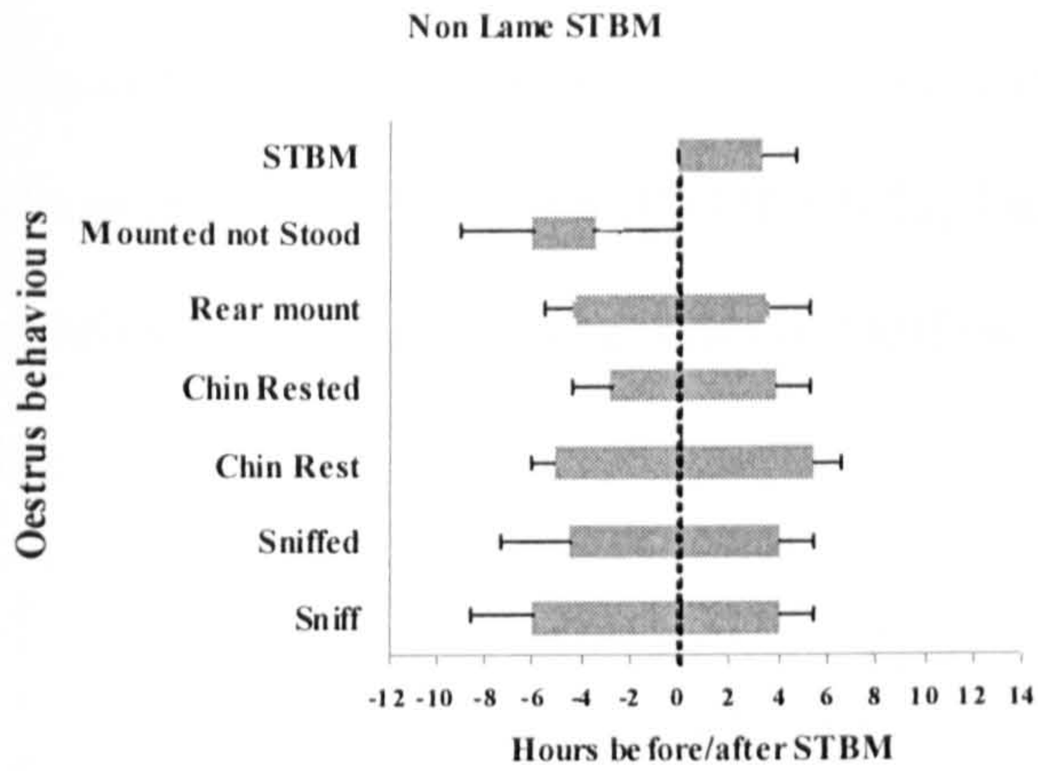


Fig 2. Survival plot generated by regression with life data for a) onset of oestrus and b) first STBM in eight Non Lame and six Lame + CIDR cows.

Relationship between times of onset of different oestrus behaviours: Using paired t-test analysis on data from the eight Non Lame STBM animals, onset of Sniff tended to occur 6.0 ± 2.7h before onset of STBM (T-Value = -2.27 $P = 0.058$; Fig 3a). Also, Chin Rest began 5.1 ± 0.9h before STBM (T-Value = -5.65, $P = 0.001$) and onset of Rear Mount was 4.3 ± 1.3h before onset of STBM (T-Value = -3.24, $P = 0.018$). The onset of Chin Rest tended to be 1.4 ± 0.7h before the same animal was Chin Rested upon (T-Value = -2.09, $P = 0.082$).

The onsets of all other individual oestrus behaviours were not different to all other behaviours within either the Non Lame STBM (n = 8) or the Lame + CIDR STBM cows (n = 6; $P > 0.05$; Fig 3).

a.



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b.

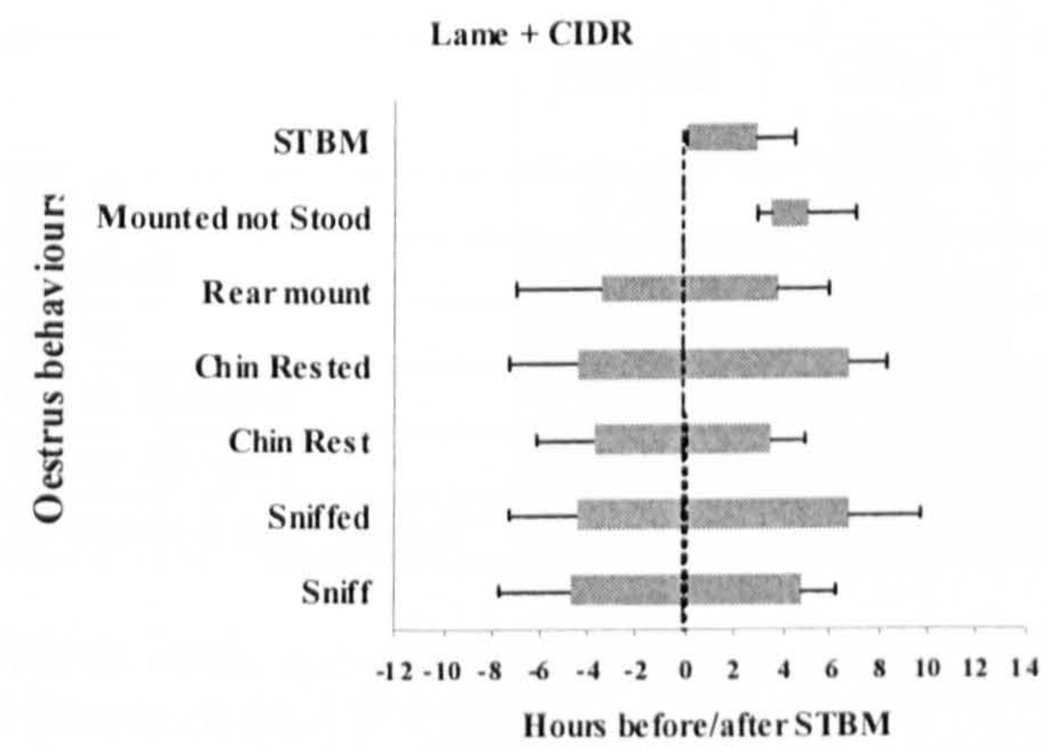

Figure 3. Mean (± SEM) onsets and ends of oestrus behaviours in a) eight Non Lame
325 STBM cows and b) six Lame + CIDR cows. The timings have been aligned to first STBM, indicated by the vertical dotted line.

In the eight Non Lame STBM cows, there were positive correlations between frequencies

330 of the following observed behaviours: Sniff *versus* Sniffed; Sniffed *versus* Chin Rested; Sniffed *versus* STBM; and Chin Rested *versus* STBM ($P < 0.05$; Table 5a; Pearson Ranked Correlation). The positive correlations of Sniff *versus* STBM; and Chin Rested *versus* Rear Mount tended to significance ($0.05 < P < 0.09$; Table 5a).

In the six Lame + CIDR STBM cows, there were positive correlations between frequencies of the following observed behaviours: Sniffed *versus* Rear-mount; and Mounted not Stood *versus* STBM ($P < 0.05$; Table 5a). There also tended to be a positive correlation between the frequencies of Sniff *versus* Rear Mount ($0.05 < P < 0.09$; Table 5b).

Table 5a. Rank correlation (r) between frequencies of paired individual oestrus behaviours in eight Non Lame STBM cows

	Sniffed	**Chin Rest**	**Chin Rested**	**Rear Mount**	**Mounted not Stood**	**STBM**
Sniff	0.72 [a]	0.45	0.59	0.46	0.04	0.68 [b]
Sniffed		-0.02	0.83 [a]	0.59	0.41	0.80 [a]
Chin			0.32	0.30	0.02	0.11
Chin Rested				0.69 [b]	0.53	0.84 [a]
Rear Mount					-0.15	0.43
Mounted not Stood						0.41

Within rows, correlations with superscripts ([a]) are different ($P < 0.05$); or ([b]) tend to be different ($0.05 < P < 0.09$).

Table 5b. Rank correlation (r) between frequencies of individual oestrus behaviour pairs in six Lame + CIDR STBM cows

	Sniffed	**Chin Rest**	**Chin Rested**	**Rear Mount**	**Mounted not Stood**	**STBM**
Sniff	0.49	-0.09	-0.58	0.79 [b]	-0.30	-0.33
Sniffed		0.61	0.16	0.85 [a]	0.34	0.28
Chin			0.46	0.44	0.44	0.70
Chin Rested				-0.05	0.22	0.46
Rear Mount					-0.10	0.00
Mounted not Stood						0.83 [a]

Within rows, correlations with superscripts ([a]) are different ($P < 0.05$); or ([b]) tend to be different ($0.05 < P < 0.09$).

Discussion

The proportions of cows that ovulated were similar in the Lame and Non Lame groups contrary to our earlier work (Morris et al., 2008b; Morris et al., 2008a). Also, the proportions of lame cows that ovulated, with or without progesterone supplementation, were similar. However, caution is urged with these results as the number of animals was very low. Furthermore, we were unable to support the hypothesis that lameness and/or presence of CIDR influenced milk progesterone concentration, plasma oestradiol concentration or oestrus intensity, possibly due to the low number of animals. However, the present study identified important relationships between the timings and frequencies of individual oestrus behaviours in Non Lame cows. Also, a positive correlation has been established between oestrus intensity and oestradiol concentrations. On the other hand, there was no significant relationship between oestrus intensity and progesterone concentrations in the previous luteal phase.

In the current study, growth rates and maximum follicular diameters of the dominant follicle were unaffected by lameness as in our previous studies (Morris et al., 2008b; Morris et al., 2008a) and in another study in non lame cows following progesterone supplementation (Cerri et al., 2007). Time to ovulation after PG was also similar between cows with and without CIDR in agreement with other work (Kyle et al., 1992). Thus, the current study confirms that interference with follicle growth is probably not one of the mechanisms by which lameness leads to dairy cattle sub fertility (Lucey et al., 1986; Collick et al., 1989) in contrast to other stressors (heat stress) in which follicle growth is compromised (Roth et al., 2000).

In other studies, progesterone supplementation increased plasma progesterone concentrations (Bicalho et al., 2007). In the present study, Non Lame animals and Lame + CIDR cows had numerically higher but not statistically different milk progesterone concentrations than Lame – CIDR cows. This concurs with other work comparing the difference between hormone concentrations in milk in CIDR treated and untreated cows (Chenault et al., 2003). Although, individual CIDRs release approximately 85mg/d progesterone (Rathbone et al., 2002), concentrations in serum and subsequently milk vary markedly (Macmillan et al., 1993), affected by the rate of progesterone metabolism and the size of individual cows (Cerri et al., 2007). In one study 34% of animals displayed vaginitis following removal of a PRID (Walsh et al., 2007) and with a similar expected percentage for CIDRs, it is suggested that levels of vaginitis may also have a varying effect on plasma progesterone concentrations. The wide variation may explain why a greater number of studies compare the presence or absence of progesterone supplementation with subsequent fertility rather than with absolute progesterone concentrations (Chebel et al., 2006; Stevenson et al., 2006; Bicalho et al., 2007). Progesterone concentrations throughout the five day period prior to PG were not lower in the Lame cows in the current study in contrast to our previous work (Morris et al., 2008b; Walker et al., 2008) probably due to the lower number of animals in the present study.

In the present study, plasma oestradiol concentrations were unaffected by lameness, as in a previous study (Morris et al., 2008b), or by the presence of a CIDR. There was a correlation of oestradiol concentration with intensity of oestrus signs in agreement with others (Lyimo et al., 2000a; Roelofs et al., 2004a) but contrary to our previous findings (Morris et al., 2008c). However, our previous results were based on a slightly different window (-36 to 0h *versus* -36 to -12h prior to ovulation in this study) and used a less

frequent sampling protocol (daily *versus* twice daily. Nevertheless, other works have found no relationship between plasma oestradiol concentration and oestrus intensity which might be explained by an individual oestradiol concentration threshold above which oestrus behaviours occur (Cook et al., 1986; Coe et al., 1989). Furthermore, as progesterone priming increases the quantity of oestradiol receptors in the mediobasal hypothalamus, increasing sensitivity to oestradiol (Blache et al., 1991; Blache et al., 1994), small differences in peripheral plasma oestradiol concentration between Lame and Non Lame animals may not be critical. In the current study, there was also no relationship between progesterone concentrations and oestrus intensity although previous work showed both a reduced progesterone concentration and lowered oestrus intensity in lame animals (Morris et al., 2008b). However, investigation of the effects of both oestradiol and progesterone on oestrus behaviour may be compromised by the preliminary nature of these results.

Progesterone supplementation in the present study did not increase the number of cows displaying oestrus similar to other work (Kyle et al., 1992) nor did it increase intensity in those animals which did show oestrus behaviour. Whereas another study, using injected progesterone, identified a decrease in the frequency of oestradiol-induced oestrus behaviours in ovariectomised cows as progesterone concentrations increased (Davidge et al., 1987). Despite the presence of animals which ovulated and failed to display STBM (silent heat) and those which did STBM but not ovulate, the proportion of Lame cows in the present study that STBM was similar to Non Lame animals, concurring with the proportion of animals ovulating within the same groups.

The similar intervals from PG to onset of oestrus and STBM in the present study were contrary to the early displays of oestrus behaviour in lame cows in a previous study (Morris et al., 2008b). The wide variation in the period from onset of oestrus to ovulation has already been documented in dairy cattle (Saumande et al., 2005). However, the similarity in onset times attributed to the presence of CIDR may be used as further evidence that low progesterone is a key factor leading to differences in timing of oestrus behaviour in lame dairy cattle. As previously mentioned, lame cows have low progesterone and display oestrus earlier than Non Lame animals (Morris et al., 2008b). In the present study, progesterone supplementation delayed the onset of oestrus behaviour to that of Non Lame animals.

In the present study, the wide variability in the frequency of each behaviour concurs with (Roelofs et al., 2005). Nevertheless, the similarity in frequency of individual behaviours between the Non Lame and Lame + CIDR cows suggests that progesterone supplementation has again corrected behavioural differences otherwise seen in lame *unsupplemented* animals. However, this hypothesis will only be confirmed with the extension of this study with more of these Lame – CIDR cows. We have identified only one other study in the English speaking literature in which the oestrus behaviours of sniffing and chin resting have each been considered as separate behaviours depending on which cow is the instigator or recipient of the action (Hurnik et al., 1987). That study in beef animals showed that peak frequency of the individual oestrus behaviours occurred within the first hour of the animal STBM contrasting with the present study in which all other behaviours began before the onset of STBM activity and ended after STBM activity was complete.

For the first time in Holstein dairy cattle, we have identified time relationships between the various individual oestrus behaviours. The three major active behaviours: Sniff, Chin Rest and Rear Mount all have a significant relationship with the time of STBM onset. This suggests that cows in oestrus actively seek out other animals with the future reward of being mounted themselves. Also, Chin Resting occurs ≈ 1.5h before being Chin Rested upon. All these observations support suggestions that oestrus behaviours are driven by the positive feedback of pheromone-stimulated dopaminergic reward (Fabre-Nys et al., 2003).

In contrast, the frequency patterns of individual oestrus behaviours revealed that the passive actions of being Sniffed and being Chin Rested in Non Lame cows were positively associated with the frequency of STMB. Therefore, those cows that are most attractive to other herd mates will subsequently stand still to receive most mounting attention. In the Lame + CIDR cows, the positive correlation between Mounted not Stood and STBM frequencies suggests that Lame animals are being mounted as much as they are refusing to be mounted unlike their Non Lame counterparts.

In conclusion, the present study showed that presence of a CIDR was a factor in correcting the too early onset of oestrus following PG. We also identified important time relationships and differences in frequencies between pairs of individual oestrus behaviours. However, there were too few animals in the study to confirm whether supplementation of lame cows with progesterone affected milk progesterone, plasma oestradiol concentration or oestrus behaviour intensity.

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General discussion and conclusions

One of the primary aims of this thesis was to investigate the effect of high somatic cell count (SCC), low body condition score (BCS) or lameness on ovarian follicular growth and ovulation.

Despite the presence of a large number of studies showing associations between these clinical conditions, we identified for the first time (Chapters 1& 3) that lameness reduced the proportion of animals that ovulated. In Chapter 3, there was also a suggestion that animals with low BCS were similarly affected although a confirmatory study with greater BCS variation is required. High SCC alone did not affect the number of cows that ovulated, although the combination of lameness and high SCC did further reduce the proportion that ovulated than either condition alone. Thus, there is a deleterious effect of *lameness* on ovulation failure, but also a synergistic effect on those cows which also have a high SCC.

In all Chapters, ovarian follicle growth and maximum diameter were *not* affected by high SCC, low BCS or lameness. This is very important because it indicates that the subfertility associated with these stressful conditions may be mediated by different mechanisms than other conditions such as heat stress (Roth et al., 2000). In Chapters 1-3, follicles that ovulated attained a larger diameter than those that did not ovulate. Even with a gradation of effect seen in Chapter 3, ovulation still occurs in approximately 50% of lame cows. This suggests the presence of a 'knife-edge' situation in which some lame cows can both grow and ovulate a dominant follicle in a similar manner to the non lame animal whereas follicles in the remaining lame animals fail to ovulate. Further research is

required to identify this critical difference which allows one lame cow to ovulate in contrast to another otherwise identical animal. This may be due to varying degrees of lameness, or differing perceptions of, or responses, to pain.

A second major aim of this thesis was to investigate hormonal involvement by examining progesterone, oestradiol and luteinizing hormone (LH) profiles, and comparing concentrations at designated times in healthy cows with those in cows with high SCC, low BCS or lameness.

In Chapter 3, progesterone concentrations in the period immediately before the synchronised follicular phase were lower in lame cows. This concurs with previous work by our group in unsynchronised animals (Walker et al., 2008) which suggests that reduced progesterone priming reduces the ability of a follicle to ovulate, possibly by affecting LH pulsatility and the LH surge (Caraty et al., 1999). This was confirmed by the lower LH pulse frequency in the lame non ovulating animals in Chapter 3. While previous investigations into problems affecting LH pulse frequency in ewes and cows have studied artificial stimuli such as transportation, acute hypoglycaemia and ACTH administration (Nanda et al., 1989; Dobson et al., 1999b; Dobson et al., 2000b), the present work is the first investigation into a naturally occurring chronic condition in dairy cattle. Failure to ovulate may also be a result of progesterone-induced low responsiveness to oestradiol (Gumen et al., 2005a) coupled with lower oestradiol concentrations observed in the non oestrus cows in Chapter 2, and in the non ovulating animals of Chapter 3.

The third objective of the thesis was the assessment of the impact of high SCC, low BCS and lameness on the display and timing of oestrus behaviour.

In Chapter 2, high SCC tended to reduce oestrus intensity and extend the interval from prostaglandin injection to the onset of oestrus. Similarly, in Chapter 3, lameness also reduced oestrus intensity but in contrast to high SCC, the interval to the onset of oestrus was shortened. Therefore, Chapter 3 provides evidence for a naturally-occurring phenomenon that concurs with other experimental observations suggesting that reduced progesterone priming has a negative effect on the timing and display of oestrus (Fabrenys et al., 1991). In practical terms, changes in timing of oestrus may further reduce fertility as there could be an increased asynchrony between artificial insemination and ovulation.

The final aim of this thesis was to test the hypothesis that low progesterone concentrations in lame cows were responsible for the previously observed low intensity of oestrus and aberrations in timings of oestrus and ovulation.

In Chapter 4, it was not possible to definitively conclude whether progesterone supplementation was responsible for; changes in milk progesterone or plasma oestradiol concentrations; or changes in ovarian follicle growth. Lame cows with a CIDR had an onset of oestrus and onset of STBM at similar times to Non Lame cows which suggests that the supplemental progesterone corrected the earlier differences seen in lame cows in Chapter 3. In Chapter 4, individual oestrus behaviours were examined in greater detail than previously in Non Lame dairy cattle. The relationships between onset time and frequencies of these newly documented categories have been established but enrolment of more cows is required to increase the robustness of these interactions and provide

confirmation regarding the mechanisms involved in the deleterious effect of lameness on dairy cow fertility.

Conclusion

Lameness and low BCS reduced the number of cows that ovulate. The situation was further exacerbated when lameness occurred concurrently with high SCC. Closely associated with this situation in lame cows was a reduction in progesterone-priming in the immediate preceding follicular phase, followed subsequently by a decrease in LH pulse frequency. Furthermore, in the animals that did ovulate, oestrus intensity was reduced by both lameness and high SCC, with lameness decreasing, and high SCC increasing, the interval to oestrus onset. Subsequent use of CIDRs ensured that lame animals displayed oestrus at the same time as Non Lame cows. It is essential that further investigations are undertaken to clarify and further identify the key mechanisms by which these very common conditions cause subfertility.

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