Stress and Oestrus in Dairy Cows

Thesis submitted in accordance with the requirements of the University of Liverpool for
the degree of Doctor of Philosophy by Susan Lorene Walker

September 2006

Declaration

Unless otherwise acknowledged, this thesis is all my own work, carried out in the Department of Veterinary Clinical Sciences, University of Liverpool, Veterinary Field Station, 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 for any other degree.

Sue Walker

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Acknowledgements

I would like to thank my supervisor Professor Hilary Dobson for her support and encouragement. You have been a wonderful role model. Thank you for giving me the opportunity to do my PhD. Thank you for teaching me the skills I will require to become an accomplished researcher. Thank you for always making me feel like I was your only student. Thank you for teaching me to be tougher. Thank you for always making time. I have truly enjoyed my time at Leahurst and it has been a privilege to have been part of your research team.

I would also like to thank everyone in the Wellcome Building, Dr Caroline Argo, Catherine Hayhurst, Dave Jones, Dr Michael Morris, Hilary Pursell, Jean Routly and Dr Rob Smith. This PhD was without doubt a team effort and I am forever grateful for everyone coming together and making time in your busy schedules to assist with this project. Together as a group, you have made me look good and I hope it has all been worth it.

Individually, thank you to Jean for assistance with the assays and all other things technical, Hilary P for your thoughtfulness and always making sure I looked like I was organized, Michael for reminding me there is more to life than work and Dave for your reliability and for making a cold rainy morning bearable. A special thank you to Rob, for always making time when there was none, to help with statistics and project ideas. I am very appreciative to everyone who spent endless hours, come rain or shine, walking, sitting and watching cows, the data you collected has great value.

I would like to thank Donald Tyson for supporting our research for 2 years and thank you to all the staff on the farm, both past and present, for their cooperation with the study, Frank Ashbury, Mel Chapman, Eddie Charnock, Martyn Jones and Ben Watson. I would like to extend a very special thank you to Paul Fox for his daily assistance and cooperation with the project. Thank for always being there to help. I have the utmost respect for you and the job you do.

Despite the many miles between us, thank you to all my friends, Bec, Elizabeth, Erica, JL, Mic and Nuch for your encouragement and understanding. Thank you to Janine Brown and Karen Goodrowe for your letters of support to help me get where I am today. As always, thank you to Coralie Munro for assistance with the EIA's. Thank you to Mary and Alan Finch for always being there for Mark and I. Thank you to my Aunt Judy for your encouragement and support. Thank you to my mother. Your support and words of wisdom were invaluable. I hope I have made you proud. Thank you for always listening.

Thank you to the following funding bodies for financial support; Natural Sciences and Engineering Research Council of Canada (NSERC), Universities UK and University of Liverpool.

Lastly, I would like to thank my husband, Mark Finch. Without a doubt I feel you have equal rights to this degree. How you managed to maintain two jobs with no sleep I will never know. The support, both emotionally and financially, you have given me is matched by no other. I don't know how you did it for 3 years and without a single complaint. I love you and will always be grateful.

Abstract

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The chronic stress of lameness is associated with poor fertility in the dairy cow. The objective of this study was to determine if lameness was associated with a reduction in oestrus intensity and to identify key factors involved.

Postpartum cows (>20 days postpartum) on a commercial dairy farm were scored for lameness, assessed for social and daily time budgets and observed for primary and secondary signs of oestrus using a weighted scoring system to determine oestrus intensity. Cows came into oestrus naturally or as a result of a clinical fertility treatment or an oestrous synchronization regime.

Lame cows expressed oestrus with lower intensity ($p<0.05$) and in brief, lameness had an impact on general mounting activities (performing and receiving mounting) and several secondary behavioural signs of oestrus including, chin resting, sniffing and restlessness. Following progesterone exposure, lameness had no effect on the incidence of oestrus. Overall, lameness had no effect on the total duration of oestrus ($p>0.05$); however, lame cows were mounted by fellow herd-mates for a shorter duration compared to nonlame cows ($p<0.05$). The reduced intensity of oestrus was associated with a low progesterone profile prior to the observed oestrus ($p<0.05$) but not with abnormal oestradiol or cortisol profiles ($p>0.05$). Lame cows were more likely to have a low body condition score ($p<0.05$) and fewer cows with a low body condition score were observed in oestrus ($p<0.05$). Time budget analysis revealed that lame cows spent less time 'elevated' on their feet and more time lying down ($p<0.05$). This was reflected in lame cows spending less time expressing oestrus ($p<0.10$), walking ($p<0.05$) and standing ($p<0.05$). Lameness also had an impact on the pattern of expression of oestrus during the day as lame cows were less likely to be observed in oestrus during the morning hours compared to nonlame cows ($p<0.05$). There was no difference between lame and nonlame cows in the proportion of time spent grazing, drinking or ruminating but lame cows had a lower bite rate than nonlame cows. There was no association between social rank and lameness ($p>0.05$); however, cows of a higher social rank tended to be closer to the front of the group for both milking and leaving the field and lame cows were near the rear of the group as they left the field later ($p<0.05$) and entered the milking parlour later ($p<0.05$) compared to nonlame cows. Lame and nonlame cows had similar resting cortisol values from day 20-80 postpartum ($p>0.05$). Lame cows did not cope as well (i.e. were more fearful) to an acute stressor compared to nonlame cows. Lame cows had a lower behavioural response and greater cortisol response to an acute stressor ($p<0.05$) and control over the environment (i.e. high side-consistency in the parlour) was not behavioural trait associated with lameness ($p>0.05$).

In conclusion, this thesis provides evidence for alterations in endocrinology and behaviour in lame cows to highlight the cost and detriment to welfare and fertility that lameness imposes on dairy cattle.

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List of abbreviations

corticotrophin-releasing hormone	CRH
arginine vasopressin	AVP
adrenocorticotrophic hormone	ACTH
luteinizing hormone	LH
gonadotrophin releasing hormone	GnRH
dominance value	DV
standing to be mounted	STBM

Literature Review

Literature Review

The Oestrous Cycle

The domestic cow (*Bos taurus*) is polyestrous with recurring cycles averaging 21 days with a range of 18-24 days. There are four stages of the oestrous cycle, pro-oestrus, oestrus, metoestrus and dioestrus (Noakes, 1997). During pro-oestrus there is an increase in dominant follicular growth that is characterised by a progressive increase in oestradiol concentrations, and there are behavioural signs that indicate the approach of oestrus. Oestrus is characterized by sexual receptivity and intense sexual desire and is coincident with peak plasma concentrations of oestradiol (Coe & Allrich, 1989; Glencross et al., 1981; Erb & Morrison, 1958; Lopez et al., 2002). The female will stand immobilised to be served by a bull or be involved in homosexual behaviour where a herd-mate mimics the mounting behaviour of the bull. When an animal demonstrates sexual proceptivity/receptivity it is considered to be in oestrus or 'in heat'. Metoestrus immediately follows behavioural oestrus and it is the stage in which the dominant follicle matures, ovulates following a surge in LH and a corpus luteum begins to develop. Ovulation is generally considered to occur on day 1 of the oestrous cycle. Following metoestrus, dioestrus is dominated by presence the corpus luteum, which is fully formed by day 7 and actively produces progesterone until approximately day 18. Following regression of the corpus luteum, the reproductive tract ceases to be dominated by progesterone, and by approximately day 21 the cow will display oestrus.

Behavioural signs of oestrus

The principal and most commonly used indicator of oestrus is an immobilization response in which a heifer or cow stands immobile during mounting by a herd-mate or bull, i.e. standing to be mounted (Allrich, 1993). It is behaviour rarely performed outside the oestrous phase of the cycle. Although standing to be mounted is regarded as the definitive sign of oestrus it is certainly not a perfect means to determine the onset and termination of oestrus. Observing standing behaviour presents a challenge as it can represent less than 1 % of the total duration of oestrus (Senger, 1990). There is also considerable individual variation between the number of standing mounts exhibited by an individual during a single oestrous period (Esslemont et al., 1980). Using continuous visual observations, ranges of 3 to 140 mounts (Esslemont & Bryant, 1976) and 3 to 225 mounts (Coe & Allrich, 1989) have been reported. The number of cows that are in oestrus at the same time influences the occurrence of standing heat. (Van Vliet & Van Eerdenburg, 1996), reported that 77% of standing heats occurred when more cows were in oestrus at the same time. Additionally, it has been reported that only a percentage of cows show standing heat (Hall et al., 1959; Pennington et al., 1986; Mai et al., 2002;

Hurnik et al., 1975; Van Eerdenburg et al., 2002). (Lyimo et al., 2000) observed standing heat in only 53% of cows. (Hurnik et al., 1975) stated that the proportion of oestrous cows that did not display standing behaviour were 35, 26 and 12% in consecutive postpartum heats. (Van Vliet & Van Eerdenburg, 1996) with 12 observations of 30 minutes a day observed standing heat in only 37 % of oestrous events. The authors also noted, based on the results from other studies, the occurrence of standing heat can range anywhere from 38 to 90 % (Hall et al., 1959; Williamson et al., 1972; Thatcher & Wilcox, 1973; O'Farrell, 1982; Fonseca et al., 1983; Hackett & McAllister, 1984; Britt et al., 1986; Heersche & Nebel, 1994; Lyimo et al., 2000). Therefore, although standing to be mounted is a definitive behavioural sign of oestrus additional oestrus-related behaviours should be observed.

Attempted mounts performed by an individual may also be recorded. Performing the mounting (mounting rear) or disoriented mounting (mounting head or side of the animal) is a relatively good indication of oestrus. It has been suggested that mounting the head side of the a cow can be highly discriminative of the cow being in oestrus (Van Eerdenburg et al., 1996). However, unlike 'standing heat', performing a mounting activity can occur outside oestrus (Van Eerdenburg et al., 1996; Van Vliet & Van Eerdenburg, 1996). (Hurnik et al., 1975) reported that 19% of cows not in oestrus exhibited mounting behaviour. When an individual receives mounting but does not stand can also occur frequently during oestrus; however, it is not as discriminative as other mounting activities as it can occur equally in or out of oestrus (Van Eerdenburg et al., 1996).

Although mounting activity (initiating and receiving) is indicative of oestrus, mounting activity may not always be present during oestrus. (Van Vliet & Van Eerdenburg, 1996) reported that 14% of cows in oestrus (based on progesterone concentrations) where only mounted once and 46% of the cows were not mounted at all. Therefore, there are other signs of oestrus that can also be observed. Secondary behavioural signs of oestrus can include restlessness (increased activity), cajoling, head bunting, chin resting, flehmen response from sniffing vagina or urine of other cows, bellowing when isolated, soliciting, scuffed tail head and dirty flanks. These secondary signs of oestrus have a gradual onset and termination (Allrich, 1993; Esslemont et al., 1980). Approximately 12 hours before standing heat is displayed, secondary oestrous signs progressively appear and increase in frequency, following a period of standing heat the secondary signs gradually decrease in frequency and cease approximately 12 hours later. The majority consider these secondary behaviours and physiological signs as belonging to the pro and met-oestrus periods and suggest that they should be used carefully when making breeding decisions however, some feel that these secondary signs are very useful as standing behaviour does not occur in enough cows to be a definitive symptom (Van Eerdenburg et al., 1996; Van Vliet & Van Eerdenburg, 1996; Lyimo et al., 2000).

Relationship between oestrus, ovulation, luteinizing hormone and oestradiol

Behavioural oestrus is on average 12 to 18 hours in duration (Allrich, 1993). However, there is variation within the literature (Table. 1) and it is difficult to compare the duration of oestrus as many factors differ between experiments including, behaviours used to characterise the onset and cessation of oestrus, individual variation (parity, genetics, temperate/tropical) and herd size. It has been suggested that the effective range of the duration oestrus may be more like 3 to 28 hours (Allrich, 1993).

Table 1. Average duration and range of oestrus using different methods of observation

Reference	Method	Mean Time (Hours)	Range
(Humik et al., 1975)	Visual (24 h/day)	7.5 to 10.1	-
(Lyimo et al., 2000)	Visual (30 min every 3 h)	20.3±10.4 (SD)	6 h to 33h
(Coe & Allrich, 1989)	Visual (24 h/day)	14.9±0.7 (SE) 17.2±0.7 (SE)	2.4 h to 27.5h
(Roelofs et al., 2004)	Visual (30 min every 3 h)	13.4±0.9 (SE)	6 h to 21h
(Dransfield et al., 1998)	HeatWatch	7.1±5.4 (SD)	33 mins to 35.8 h
(Van Vliet & Van Eerdenburg, 1996)	Visual (30 min every 2 h)	13.7±6.7 (SD)	-
(Britt et al., 1986)	Visual (8 h intervals)	13.8±0.6 (SE) 9.4±0.8 (SE)	-
(Esslemont & Bryant, 1976)	Visual (24 h/day)	14.9 4.7 (SD)	-
(Wishart, 1972)	Visual	14.7±1.6	10 h to 18 h
(Xu et al., 1998)	HeatWatch	8.6±0.46 (SE)	1.0 h to 21.3h
(Cavalieri et al., 2003)	HeatWatch Visual (24 h/day)	10.9 14.4	10 h to 11.9h 13.3h to15.6h
(Lopez et al., 2002)	HeatWatch	3.6±0.8	0.2 h to 12h
(Stevenson et al., 1996)	HeatWatch	14±0.8 (SEM)	2.6 h to 26.2h
(Walker et al., 1996)	HeatWatch	9.5±6.9 (SD)	-
(At-Taras & Spahr, 2001)	HeatWatch	5.83±0.78 (SE) 5.57±1.02 (SE)	-

The onset of behavioural oestrus coincides with increasing oestradiol concentrations (Lopez et al., 2002; Dobson et al., 1975; Coe & Allrich, 1989; Glencross et al., 1981). Based on 3-hour intervals, maximum oestrous behaviour (based on a scoring system) coincides with maximum oestradiol concentrations (Roelofs et al., 2004). The surge in LH induced by oestradiol, also occurs around the onset of oestrous behaviours (Allrich, 1994; Chenault et al., 1975; Glencross et al., 1981; Lemon et al., 1975; Rajamahendran & Taylor, 1991) and (Roelofs et al., 2004) demonstrated that maximum behaviours and maximum oestradiol concentrations coincide with the LH surge. Similarly, (Walton et al., 1987) demonstrated that the onset in the surge in LH always followed the onset of standing behaviour by 1 to 2 hours. The average interval between peak plasma oestradiol concentrations and ovulation is 22.3 ± 3.9 hours (Mosher et al., 1990) and 30.7 ± 6.3 hours (Lopez et al., 2002).

The mean duration from the onset of standing behaviour until ovulation is 24.2 ± 3.9 hours, using mount detectors and ultrasound once daily to confirm ovulation (Lopez et al., 2002). Using the same heat detection method (Walker et al., 1996) reported a comparable interval of 27.6 ± 5.4 hours. (Roelofs et al., 2005b) also reported a similar range from standing oestrus to ovulation of 26.4 ± 5.2 hours, using visual observations (8 x daily) and ultrasound scans every 3 hours. Additionally, (Roelofs et al., 2005b) reported that the onset of mounting was the best predictor of ovulation occurring 28.7 ± 5.3 hours before ovulation. A more variable interval from standing oestrus to ovulation of 37.7 ± 11.0 hours (range 18 to 60 hours) based on daily visual observations of oestrous behaviours and ultrasound twice daily has been reported (Augusto et al., 1997).

The average interval from the end of oestrus to ovulation is ~ 12.0 hours with a large range of 0 to 24 hours (Trimberger, 1948; Hall et al., 1959; Wishart, 1972; Augusto et al., 1997; Roelofs et al., 2005b). (Walton et al., 1987) reported that there is no useful relationship between the end of oestrus and ovulation due to the large variation exhibited between individuals in total duration of oestrus.

Oestrus uncoupled with ovulation

There are instances where oestrous behaviour may not be coupled with ovulation. Puberty can be characterised as the first oestrus with ovulation, however, some heifers may exhibit behavioural oestrus but this will not be followed by ovulation and the subsequent formation of the corpus luteum this is termed a non-pubertal oestrus (Allrich, 1994; Nelsen et al., 1985; Rutter & Randel, 1986). In other situations conversely ovulation may occur however, but there is no expression of behavioural oestrus. Following parturition a cow enters postpartum anoestrus, this anoestrus ends with the first postpartum ovulation. This first ovulation is often not associated with behavioural oestrus and is termed 'silent' ovulation or 'silent' oestrus. The percent of cows which exhibit silent ovulation varies throughout the literature ranging from 50 to 94% (Allrich, 1994; King et al., 1976; Kyle et al., 1992; Savio et al., 1990; Schams et al., 1977). In the majority of cows subsequent ovulations are then associated with expression of oestrus. Interestingly, it also has been observed that a small percentage (~6%) of pregnant cows will display oestrus (Erb & Morrison, 1958; Thomas & Dobson, 1989; Williamson et al., 1972).

Methods of Oestrous Detection

Visual observation

Visual observation is the most traditional and commonly used form of oestrus detection. A review by (Rorie et al., 2002) states that visual observation is an accurate method of detecting animals in oestrus and is ~50% to 70% efficient. A good detection rate from visual observation depends on the skill of the observer and the time of day, repetition and frequency of the observations. Deciding when to perform observations is influenced by management practices. Sexual activities can be shifted by distractions such as milking, feeding and grazing and therefore influence the number of recorded oestrous events (Van Vliet & Van Eerdenburg, 1996; Pennington et al., 1986; Esslemont & Bryant, 1976; Hurnik et al., 1975; Williamson et al., 1972). Additionally, oestrous behaviours have been reported to be more frequent during the nocturnal period and early morning (Hackett & McAllister, 1984; Van Vliet & Van Eerdenburg, 1996). Under natural conditions or continuous lighting a higher incidence of mounting activity was observed during nocturnal hours (Hurnik et al., 1975; Williamson et al., 1972). However, some have found no diurnal pattern associated with the onset of oestrus (Esslemont & Bryant, 1976). Continuous (Pennington et al., 1986; Amyot & Hurnik, 1987) or almost continuous (Esslemont et al., 1980) visual observations reveal that displays of oestrus occur evenly throughout a 24 hour period. This has recently been confirmed using electronic devices (see below) that allow for 24 hour monitoring (Dransfield et al., 1998; Xu et al., 1998). The conflicting data concerning diurnal and nocturnal oestrual activities may be related to management practices, as already mentioned, and/or the duration and frequency of recorded oestrous events. The frequency at which behaviours are recorded will influence the efficiency of detection, as signs of oestrus are not continuously displayed. This has been demonstrated by (Van Vliet & Van Eerdenburg, 1996) where time of day, frequency and duration of observation periods were compared. Decreasing the duration of the observation period from 30 to 20 minutes resulted in a decrease of >20 % in detection rate. Additionally, a heat detection rate of 70 % or greater was achieved with only two or three observation periods of 30 minutes per day.

Mounting activity

Continuous visual observations are time consuming, tedious and require diligent attention. Limiting factors may include the space or area over which observations can be performed and staff required. To minimize the amount of physical time spent observing the animals and to lower labour costs, simple techniques have been employed to aid oestrus detection. Techniques such as tail painting when paint is applied to the base of the tail and sacrum. When a cow stands to be mounted the paint is removed by the rubbing action of being mounted. This is a relatively cheap and effective method of oestrus detection. Efficiency ranges from 73% to 96% (Ball et al., 1983; Elmore et al., 1986; Mai et al., 2002; Macmillan & Curnow, 1977). Another simple device is the chin ball device. Streaks of marking fluid run parallel to the vertebrae along the back of the cow after being mounted. They are attached to individuals who are used as teaser animals such as androgenised cows or teaser bulls (Halsey, 1978; Kiser et al., 1977; Mai et al., 2002). (Mai et al., 2002) reported a 77% efficiency in heat detection using a chin ball device. It should be noted that when using bulls, general safety and health problems such as the spread of venereal disease should be concerns for management (Donaldson, 1968). Although relatively efficient, false positives have been reported when using both tail paint and the chin ball device (Mai et al., 2002). (Kerr & McCaughey, 1984) reported 30.8% false positive result for tail painting. False positives may have been due to licking of the aids, overcrowding, shedding of winter coats, accidental rubbing and/or cows in oestrus mounting other cows (Mai et al., 2002).

Another oestrous detection aid based on mounting activity are mount detectors. The detectors, attached to the sacral region, are pressure sensitive and can be either visually or electronically read. Visual devices turn red in colour (the pressure from mounting cows releases a red dye changing the dome from white to red; Kamar™,) or indicate mounting activity with a flashing a light (DEC ShowHeat®, IVM Technologies, France; MountCount®, DDx Inc., Denver, CO). Electronic devices (HeatWatch®, DDx Inc., Denver, CO) coupled to an external recording device monitor an individual's mounting activity in a continuous and comparative fashion. Activation of a pressure sensor by weight of a mounting herd-mate produces a radio wave transmission, which is coupled to a radio receiver, and stored information is downloaded to a computer. Threshold values selected by the manufacturer decide whether or not a cow is in oestrus.

The efficiency and effectiveness of using electronic mount detectors has been the focus of several studies. Timing of artificial insemination using electronic mount devices has resulted in pregnancy ranging from 45% to 95% have been reported (Rorie et al., 2002; Stevenson et al., 1996; Rae et al., 1999; Nebel et al., 1995). (Rorie et al., 2002) reported that the oestrous detection and pregnancy rates were similar between the different types of commonly available mount detectors. Studies report the efficiency of oestrous detection to be 70% to 100% with very high accuracy (Lopez et al., 2002; Smith et al., 1993; Cavalieri et al., 2003; Stevenson et al., 1996; At-Taras & Spahr, 2001; Xu et al., 1998; Mai et al., 2002). However, some false negative results have been reported. Cows observed to be standing did not have mounts recorded by the system. In this case, either the sensor had been displaced or fallen off during mounting or mounting was from the side rather than directly on the tail head (At-Taras & Spahr, 2001). (Saumande, 2002) reported a 12.8% occurrence of false positive results using the DEC ShowHeat®. False positive may have occurred because the sensitivity of the pressure system was set too low. High numbers of devices have been reported lost and/or require replacement, this suggests a more effective and practical method of securing sensors on cows is required (At-Taras & Spahr, 2001; Saumande, 2002; Rorie et al., 2002). Despite technical concerns, electronic mount detectors have been shown to be comparable (Dinsmore & Cattell, 1993; Xu et al., 1998; Cavalieri et al., 2003) if not superior to visual observations (Stevenson et al., 1996; At-Taras & Spahr, 2001; Smith et al., 1993; Mai et al., 2002; Lopez et al., 2002) (Table 2).

Table 2. Efficiency of different methods of oestrous detection

Reference	**Confirmation of Ovulation/ Oestrus**	**Synchronized vs. Spontaneous**	**Visual Method**	**Criteria**	**Visual Efficiency (%)**	**Heat Mount Detector Type**	**Heat Mount Detector Efficiency (%)**
(Lopez et al., 2002)	progesterone	Synchronized	Visual (2x daily for 30 mins	Standing to be mounted	75.0	HeatWatch	100
(Stevenson et al., 1996)	Confirmed by HMD/visual (?)	Synchronized	Visual (2 x daily for min 45 mins)	Standing to be mounted	73.0	HeatWatch	100
(Cavalieri et al., 2003)	progesterone	Synchronized	Visual (Continuous)	Mounting activity	97.5	HeatWatch	93.8
(Xu et al., 1998)	progesterone	Spontaneous	Visual (2x daily for 20 mins) plus Tail paint	Mounting activity	98.4	HeatWatch	91.7
(At-Taras & Spahr, 2001)	pedometers/ HMD/milk production/time since last oestrus/ opportunistic rectal palpation	Spontaneous	Visual	Mounting activity	54.4	HeatWatch	86.8
(Mai et al., 2002)	progesterone	Synchronized	Visual (2 x daily 1 hr)	Standing to be mounted	52.2	HeatWatch	70.6
(Rae et al., 1999)	Confirmed by HMD/visual (?)	Synchronized	Visual plus Tail Chalking	Standing to be mounted	53.1	HeatWatch	65.1
(Saumande, 2002)	progesterone	Spontaneous	Visual (4 x daily 10-15 mins)	Mounting activity	68.8	ShowHeat	35.4

Pedometry

Oestrus detection may be automated by utilizing electronic technology. Pedometry-aided detection of oestrus measures physical activity and this is related to stage of the oestrous cycle. There is an increase in activity associated with the onset of oestrus in cows (Hurnik et al., 1975). Pedometers are small devices attached to the leg of the cow and count the number of steps taken. They contain data storage and analytical capabilities and past activity can be compared with ongoing or present activity. This allows for an increase in efficiency as comparisons of activity can be made within an individual. The device may be read using a manual receiver or coupled to a computer via a remote receiver. Cows equipped with pedometers have higher physical activity during oestrus compared to other stages of the oestrous cycle (Farris, 1954; Kiddy, 1977; Liu & Spahr, 1993; Maatje et al., 1997; Lewis & Newman, 1984; Roelofs et al., 2005a). The expression of certain oestrous behaviours are also correlated to an increase in pedometer readings (Pennington et al., 1986; Van Vliet & Van Eerdenburg, 1996) and there is a strong relationship between time of insemination following an increase in such activity and high pregnancy rates (Maatje et al., 1997). (Roelofs et al., 2005a) has demonstrated a strong relationship between pedometer readings and ovulation allowing for a more precise insemination time. Some question the practicality of pedometers, as they may require frequent replacement or result in false positive readings, reducing the accuracy of oestrus detection (Pulvermacher & Wiersma, 1991; Williams et al., 1981; Senger, 1994). In a review by (Firk et al., 2002) increases in pedometry measurements, based on a variety of threshold limits, detect 68% to 100% of cows in oestrus. In general, measuring activity as a predictor of oestrus and ovulation is an efficient and relatively cost-effective method requiring minimal human labour.

Electrical Resistance

Measurements of electrical impedance within the vagina or vaginal mucus can be used as an indicator of oestrus. During oestrus swelling of the vulva is a classic sign indicative of oestrus. Tissue swelling is the result of changes in tissue hydration, which is reflected in changes in electrical resistance (ohms) in the reproductive tissues (Ezov et al., 1990). The electrical resistance is highest (or electrical conductivity is lowest) during the luteal phase and declines during the follicular phase of the oestrous cycle. The lowest resistance coincides with the LH surge a few hours after the oestradiol peak and onset of oestrus (Lewis et al., 1989; Senger, 1994; Schams et al., 1977). The interval between lowest resistance and time of ovulation is between 32 and 24 hours (Aboul-Ela et al., 1983; Leidl & Stolla, 1976; Schams et al., 1977; Schams & Butz, 1972). By measuring and plotting changes in resistance overtime it is possible to estimate the timing of the onset of oestrus. However, there are deficiencies with measurements of electrical impedance, which include high cost of the device and high labour requirements, as external probes require periodically insertion into the vagina for readings. Additionally, there is considerable between-animal variation, therefore, an individual's baseline needs to be established prior to oestrus in order to detect any changes that occur during oestrus (Dransfield et al., 1998; Elving et al., 1983). There is also variation within an individual and resistance may be influenced by non-oestrus related parameters. Cysts, ulcerous inflammation or improper positioning of the probe in the vagina can result in undesirable high rates of false positives and false negatives (Elving et al., 1983; Lehrer et al., 1995; Boyd, 1984; Leidl & Stolla, 1976). The drawbacks observed with electrical impedance limit the practicality of this approach as a means of reliable oestrous detection.

Hormone Monitoring

Oestrous detection may be aided by through the measurement of hormones in plasma or milk. Progesterone concentrations can confirm the date of oestrus and presumed ovulation (Van Vliet & Van Eerdenburg, 1996; Lourens et al., 2002; Heres et al., 2000). Progesterone concentrations can give valuable information regarding the presence or absence of a functional corpus luteum, however, it cannot be used to positively identify a cow in oestrus (Allrich, 1993). Progesterone concentrations increase in the presence of a functional corpus luteum, thus a high progesterone result indicates a non-oestrous state. A low progesterone result however can indicate that the animal is either in oestrus, will be in oestrus within 2 – 2.5 days, was in oestrus within the last 2 – 2.5 days or is in postpartum anoestrus. Low progesterone levels may also indicate follicular cysts on the ovaries (Allrich, 1993). Therefore progesterone analysis is limited in its use in oestrous detection. Nor are progesterone concentrations an accurate predictor of ovulation as there is a large variation (range 2 days) in the timing between a decrease in progesterone and ovulation (Roelofs et al., 2006).

Oestradiol concentrations gradually increase and decrease during oestrus and peak concentrations are associated with the initiation of oestrous behaviours (Chenault et al., 1975; Lyimo et al., 2000; Lopez et al., 2002). To observe peak concentrations daily or twice daily samples are required (Lopez et al., 2002). Monitoring hormone concentrations requires laboratory facilities and reagents therefore it can be impracticable for routine use. However, portable assays are available but they are expensive and tend to give qualitative not quantitative results (which would not be suitable in the case of oestradiol). New in-line milk biochemical sensors are been developed to monitor hormone concentrations however they are expensive and are still being tested for practical use. Therefore, with the available current technology, hormone monitoring has a limited practical use in oestrous detection, however, it does provide a retrospective confirmation of oestrus and ovulation.

Temperature

During the oestrous cycle significant changes in body temperature occur (Firk et al., 2002). Temperatures are low prior to oestrus then increase on the day of oestrus (Lewis & Newman, 1984; Wrenn et al., 1958). Increases from 0.1 to 0.5 °C can be observed during oestrus (Firk et al., 2002). Measurements of temperature can be performed via the rectum, vagina, by implants or indirectly in the milk. Milk temperature measurements can be recorded automatically during the milking process. There is a strong correlation between milk and body temperature (Roth et al., 1987). The practical application of temperature measurement as an oestrous detection method has been disputed as changes in body temperature may be due to inflammatory reactions (Boyd, 1984), activity levels (Metz et al., 1987) or ambient temperatures (Lewis & Newman, 1984). Additionally, rectal and vaginal measurements of temperature are time consuming and are not practicable for commercial farms. However, implants and milk measurements can monitor temperature automatically and continuously. In the case of implants environmental temperatures and atmospheric influences can also be eliminated (Firk et al., 2002). (Mosher et al., 1990) concluded using intravaginal radiotelemetry the interval between a rise in temperature and time to ovulation was consistent and suggested temperature may be as reliable a predictor of ovulation or other periovulatory events.

Additional Aids

Additional events that have been recommended as indicators of oestrus include, clear vulva mucus or "bulling string", ferning pattern in cervical mucus, red swollen vulva, detection of oestrus specific odours (pheromones) and a decrease in milk production (Van Vliet & Van Eerdenburg, 1996; Cook et al., 1986; Gt. Brit. Ministry of Agriculture Fisheries and Food. Agricultural Development and Advisory Service, 1984; Noakes, 1997; Thomas & Dobson, 1989; Williamson et al., 1972; Firk et al., 2002; Hawk et al., 1984). Comparatively, these signs tend to be non-specific and in some cases are considered questionable; therefore, they should be used with caution as a reliable means of method of oestrous detection.

Summary

An overall method to improve the efficiency and accuracy of oestrous detection is to employ more than one method (Lehrer et al., 1992; Senger, 1994; Firk et al., 2002). (Pennington et al., 1986) reported that efficiency is increased, and false positives are reduced, when visual observations are combined with activity measurements. (Williams et al., 1981) also demonstrated an increase in efficiency of oestrous detection by utilizing two or more techniques. The individual efficiency of oestrus detection for, twice daily visual observations, heat mount detectors, and pedometers ranged from 67% to 74%, whereas the combined detection rates for visual observations plus mount detectors and visual observations plus pedometers, increased to 84% and 93%, respectively. (Senger, 1994) suggested that the coupling of three automated oestrus detection aids (mounting detectors, activity measurements and electrical impedance measurements) into one implanted device would result in an 'ideal system'. Such an approach would reduce problems associated with any one method and result in a highly efficient method of oestrous detection. In a review by (Allrich, 1993) it was suggested that oestrous detection aids should only supplement the information gained by visual observations and not replace it. It was stated that oestrous detection aids can be misused and may provide incorrect information and in some cases there is poor agreement of the oestrous characteristics established through visual observation and those detected by mounting detectors. It was concluded that although both methods may be equally efficient at detecting oestrus the measured characteristics using the automated mounting detector were underestimating the magnitude of oestrous characteristics (Cavalieri et al., 2003). It has also been reported 50% of cows in oestrus do not stand to be mounted (which is the gold standard) and standing to be mounted represents less than 1% of the total duration of oestrus (Van Eerdenburg et al., 2002; Senger, 1990; Lyimo et al., 2000); therefore, the sole use of aids based on mounting activity may be limiting detection efficiency. Consequently a combination of techniques that include monitoring mounting activity and other secondary signs of oestrus (pedometry and/or visual observation of other oestrus-related behaviours) should result in maximum oestrous detection efficiency and accuracy.

Endocrine Control of Oestrus

The neuroendocrine mechanisms that control oestrus can influence the onset and duration of oestrus, additionally they may possibly govern the intensity of activity that is associated with oestrus. In general, pituitary gonadotrophins, which induce the final follicular maturation, result in increased levels of oestradiol. Oestradiol, in the relative absence of progesterone, acts on the hypothalamus to induce oestrous behaviour (Allrich, 1994). However, all endocrine mechanisms responsible for modulating oestrous behaviour in cattle are not yet completely understood.

Oestradiol

Early evidence for oestradiol as the primary oestrus-inducing hormone was though studies using an exogenous source of oestradiol alone to induced oestrus in ovariectomized cows (Asdell et al., 1945; Melampy & Rakes, 1958). Oestrus has been induced by oestradiol in ovariectomized cattle (Carrick & Shelton, 1969; Katz et al., 1980; Lefebvre & Block, 1992), horses (Asa et al., 1984), sheep (Fabre-Nys & Martin, 1991b; Fabre-Nys et al., 1993) and pigs (Ford, 1985). Additionally, it was reported in spite of oestrus-inducing concentrations of oestradiol, immunization against oestradiol has resulted in inhibition of the expression in cattle (Martin et al., 1978) and sheep (Fairclough et al., 1976). As immunization essentially neutralizes endogenous oestradiol this strongly suggests that oestradiol is the primary hormone responsible for inducing oestrous behaviour.

Oestrus-inducing concentrations of oestradiol are considered to be an "all or none" phenomenon (Allrich, 1994; Cook et al., 1986). Once a threshold of oestradiol concentration is reached which induces oestrus, additional amounts have no further stimulatory effects on the expression of oestrous behaviours. This has been demonstrated in both ovariectomized (Cook et al., 1986; Ray, 1965) and intact cattle (Coe & Allrich, 1989; Glencross et al., 1981; Walton et al., 1987). This has also been demonstrated in superovulated cattle (Coe & Allrich, 1989). Superovulation increases circulating concentrations of many ovarian steroids including oestradiol (McGowan et al., 1985; Saumande & Lopez-Sebastian, 1982). (Coe & Allrich, 1989) demonstrated despite peak serum concentrations of oestradiol surrounding oestrus in superovulated cattle (49.0 ± 3.1 pg/ml) being fourfold higher than non-superovulated cattle (12.9 ± 3.0 pg/ml), and the rate of increase of oestradiol during the 24 hours prior to peak values greater in superovulated animals, there was no difference between the groups in the proportion of heifers induced into oestrus or the expression of standing or mounts behaviours. Additionally, during the non-superovulated cycle, peak serum concentrations and oestrous behaviours were not correlated (Coe & Allrich, 1989). (Walton et al., 1987) also failed to detect a relationship between serum oestradiol concentrations and oestrous behaviours. Similarly, (Cook et al., 1986) reported the frequency of the expression of various oestrous behaviours, including mounting, chin resting, sniffing and licking are similar between ovariectomized cows that receive low doses of the synthetic oestrogen (oestradiol benzoate) and those that received markedly higher doses. Together, this supports the idea that oestrus-inducing ability of oestradiol is not displayed in a dose-response relationship. However, using a weighted scoring system to score oestrous intensity, (Lyimo et al., 2000) and (Roelofs et al., 2004) both found a positive relationship between the intensity of oestrous expression and maximum oestradiol concentration. Although in the latter study it was suggested that this relationship mainly existed when oestradiol concentrations are low.

Although oestrus-inducing concentrations of oestradiol appears to be an "all or none" phenomenon (Allrich, 1994) the threshold required to induce oestrus in an individual may be different from the mean. The minimum absolute dose of oestradiol benzoate needed to induce oestrus in the majority (<80%) of ovariectomized cows is within the range of 400 to 600 µg (Carrick & Shelton, 1969; Cook et al., 1986; Ray, 1965; Allrich et al., 1989). However, lower doses of oestradiol benzoate, 125 µg and 250 µg, are able to induce 20 and 60 % of cattle into oestrus, respectively (Cook et al., 1986). Increasing doses of oestradiol benzoate 300, 600, 1200, 2400 and 4800 µg induced 40, 90, 90, 100 and 90 % of ovariectomized cattle into oestrus, respectively. Therefore, although there is an average dose at which the majority of cows will be induced into oestrus there are a few individuals that are induced into oestrus at lower dosages suggesting an individual variation for threshold levels (Allrich, 1994; Cook et al., 1986).

It has been suggested that higher dose of exogenous oestradiol are followed by shorter times to oestrus (Carrick & Shelton, 1969). Following prostaglandin treatment in superovulated heifers, there is increased rate of serum oestradiol concentrations. This results in an increase above threshold values sooner and an early induction of oestrus as compared with the non-superovulated heifers (Coe & Allrich, 1989; Barnes et al., 1982). However, (Cook et al., 1986) observed that increasing dosages of exogenous oestradiol does not hasten the initiation of oestrus in ovariectomized cows and the interval from injection of oestradiol benzoate to the onset of oestrus was similar over a wide range of dosages (125 µg to 4800 µg).

It has been reported in ovariectomized gilts increasing dosages of oestradiol induces longer periods of oestrus (Signoret, 1967). However in cattle, increasing amounts of exogenous oestradiol do not increase or alter the duration of oestrus in ovariectomized cows (Cook et al., 1986; Glencross et al., 1981; Asdell et al., 1945). Additionally, despite markedly elevated serum oestradiol concentration in superovulated heifers the duration of oestrus was only 2.3 hours longer than non-superovulated animals (Coe & Allrich, 1989). This suggests that once oestrus has been initiated by oestradiol the duration of oestrus is independent of dosage.

Oestrus is induced through the action of oestradiol on the hypothalamus, in the relative absence of progesterone (Allrich, 1994). The neurons in the brain that induce oestrous behaviours are not necessarily the same as those involved in the GnRH/LH surge that results in ovulation, and may vary between species (Blache et al., 1991). In rodents, oestradiol triggers female sexual behaviour by acting on the mediobasal hypothalamus whereas the LH surge is at least partially controlled by more anterior structures. Insertion of oestradiol microimplants into the medial preoptic area (but not the mediobasal hypothalamus) will evoke an LH surge in ovariectomized rats (Goodman, 1978) and microimplants of an oestrogen receptor antagonist into the medial preoptic area eliminate the LH surge (Petersen et al., 1989). In the ewe, oestradiol triggers sexual behaviour also at the level of the hypothalamus (Blache et al., 1991; Clegg et al., 1958). Similar to rodents, the placement of oestradiol microimplants into the mediobasal hypothalamus (but not the anterior hypothalamus or preoptic area) provokes oestrous behaviours but different to rodents, also elicits an LH surge (Blache et al., 1991; Caraty et al., 1998). The central sites of oestradiol action remain poorly defined in cattle.

Progesterone

Progesterone concentrations are normally very low during proestrus and oestrus (Kaneko et al., 1991; Lemon et al., 1975; Thibier & Saumande, 1975; Walters & Schallenberger, 1984; Chenault et al., 1975). These low concentrations of progesterone in the peri-oestrus period are a prerequisite for the expression of oestrus. Despite oestrus inducing oestradiol concentrations during the oestrus period, elevated progesterone concentrations have inhibitory effects on oestrous behaviours, the LH surge and ovulation (Davidge et al., 1987; Lee et al., 1988; Christian & Casida, 1948; Duchens et al., 1995a; Rajamahendran et al., 1979; Imwalle et al., 2002; Carrick & Shelton, 1969). Interestingly, the administration of progesterone (10 mg) along with oestrus-inducing levels of oestradiol benzoate (500µg) in ovariectomized heifers did not lower the percentage of cattle that stood to be mounted (Allrich et al., 1989). It has been suggested that the inhibiting effects of progesterone may be an "all or none" occurrence, once progesterone concentrations have increased beyond a threshold, oestrus is inhibited (Allrich, 1994). However, the degree with which oestrous behaviour is impaired may be dependant on progesterone concentration. High levels or sub-luteal of progesterone completely suppress oestrous behaviours (Sirois & Fortune, 1990). Supra-basal levels of progesterone also suppress oestrous behaviour but to varying degrees. Heifers treated with high, medium or low supra-basal progesterone implants have decreased oestrus-related behaviours in a linear fashion. Relatively high supra-basal concentrations suppressed standing behaviour but not secondary signs of oestrus, medium elevated concentrations resulted in a variable degree of standing behaviour and heifers treated with low concentrations did not differ from untreated controls (Duchens et al., 1995b; Duchens et al., 1995a; Duchens et al., 1994).

In cattle, there appears to be disagreement in literature with regards to the role of prior exposure to progesterone on the oestrus-inducing action of oestradiol. Research has demonstrated that prior progesterone exposure may (Allrich, 1994; Stevenson et al., 1989; Melampy et al., 1957; Carrick & Shelton, 1969; Bell et al., 1983) or may not (Davidge et al., 1987; Kyle et al., 1992; Vailes et al., 1992) facilitate the actions of oestradiol. In the ewe, prior progesterone priming is not only essential for the display of oestrus but increases the intensity of oestrous expression (Caraty et al., 2002). Additionally, the presence of progesterone during the luteal phase in the ewe increases the number of oestradiol receptors in the mediobasal hypothalamus (area responsible for sexual behaviours) and increases sensitivity to oestradiol and the resultant GnRH surge (Blache et al., 1994; Blache et al., 1991).

In ovariectomized cows treatment with increasing concentrations of progesterone prior to oestrus resulted in a linear decrease of primary and secondary oestrous behaviours (Davidge et al., 1987). However, during that study progesterone values were high during oestradiol treatment and it is widely accepted that oestrus intensity is reduced or inhibited if progesterone values are elevated coincident with the expected onset of oestrus (Allrich, 1994). The administration of progesterone (2mg intravaginal releasing device) 10 days postpartum for 5 days did not increase the proportion of cows that expressed oestrus (Kyle et al., 1992). Similarly, in prostaglandin treated heifers, greater and longer duration of progesterone exposure prior to luteolysis had no effect on the number or duration of standing events. Unfortunately, secondary oestrous behaviours were not recorded (Stevenson et al., 1998). Contrary to this and similar to sheep, some studies suggest that progesterone priming in cattle regulates the occurrence of oestrous behaviours (Carrick & Shelton, 1969; Stevenson et al., 1989; Bell et al., 1983; Vailes et al., 1992). (Bell et al., 1983) demonstrated that cows that exhibited oestrus at the first postpartum ovulation had transient increases in progesterone prior to ovulation, whereas an increase in progesterone was not seen in cows that failed to exhibit oestrus, (Stevenson et al., 1989) also demonstrated that pre-treatment with progesterone increases the efficiency of oestrous expression from 54% to 71% in prostaglandin treated cows. In oestradiol treated ovariectomized cows, prior treatment with progesterone for 5 days resulted in more mounts, chin resting and sniffing the vulva of fellow herd-mates, although this was not statistically different (Vailes et al., 1992).

Similar to the ewe, progesterone can restore sensitivity to oestradiol in heifers that have been induced into a refractory state (i.e. following pregnancy). Some cows experience a 'silent' ovulation or oestrus during the first postpartum ovulation. The high concentrations of oestradiol present near the end of pregnancy (Hoffmann et al., 1976; Stellflug et al., 1978) are thought to induce a state of refractoriness at the level of the hypothalamus to oestrus-inducing concentrations of oestradiol (Allrich, 1994). It is believed that progesterone secreted during the first postpartum luteal phase overcomes this refractory state responsible for the silent oestrus, thus allowing subsequent ovulations to be associated with the behavioural expression of oestrus (Allrich, 1994). Ovariectomized heifers given large does (10mg) of oestradiol over several weeks become refractory to small does (400μg) of oestradiol but pre-treatment with progesterone removes the refractory state and results in a larger proportion of heifers exhibiting oestrus (Carrick & Shelton, 1969). Progesterone exposure (10mg/day) for 5 days is adequate to restore sensitivity to oestradiol (Carrick & Shelton, 1969). Additionally, although prior exposure to progesterone did not affect the onset of oestrus or oestrus duration, progesterone exposure along with oestradiol remained necessary for the expression of oestrus (Carrick & Shelton, 1969).

Androgens

In ovariectomized cattle, the administration of testosterone (1,2 or 20 mg) does not result in the behavioural expression of oestrus (Nessan & King, 1981). (Allrich et al., 1989) demonstrated that the administration of testosterone (12.5 mg) in oestradiol treated ovariectomized cows did not facilitate the actions of oestradiol. Testosterone given in large does (100 to 400 mg) can induce oestrous behaviour, however, the behavioural response is diminished compared to oestradiol treated cows (Katz et al., 1980). It has also been demonstrated that the androgens, androstenedione and dihyrotestosterone, are unable to induce oestrus in ovariectomized cattle (Katz et al., 1980). In the ovariectomized ewes, androgens do not play a major role in the control of oestrous behaviours (Fabre-Nys & Martin, 1991a). If any, the actions of testosterone are most likely related to conversion to oestradiol in the hypothalamus.

Gonadotrophin releasing hormone

In cattle, the administration of a single GnRH injection (400-500 μg) to ovariectomized cattle along with low or high levels of oestradiol benzoate does not potentiate the actions of oestradiol as there are no differences in the duration of oestrus, the interval to oestrus from oestradiol injection or in the expression of oestrous behaviours (Allrich et al., 1989; Cook et al., 1986). In both sheep and cattle, the commencement of the GnRH surge coincides with the commencement of the LH surge, but the surge duration of GnRH lasts longer than LH (Fabre-Nys et al., 1993; Yoshioka et al., 2001; Caraty et al., 2002). Elevation of GnRH (~35-48 hours) is mirrored by the duration of oestrous behaviours in sheep (Caraty et al., 2002). In cattle, the duration of standing oestrous behaviour also mirrors the elevated duration of GnRH in cerebrospinal fluid (Yoshioka et al., 2001). This points to a role for GnRH in oestrous behaviours in cattle. Indeed in sheep, (Caraty et al., 2002) have demonstrated that GnRH is involved in the control of receptive behaviours. These authors suggest that oestradiol is responsible for the initiation of oestrus behaviours while GnRH determines oestrus duration.

In rodents, brain injections of GnRH can stimulate sexual behaviours (Pfaff et al., 1994; Boehm et al., 2005). Injections of GnRH in ovariectomized rats primed with low doses of oestrogen induce sexual behaviours (McCann et al., 1983). Additionally, antisera to GnRH and inhibitory analogues of GnRH can inhibit mating behaviour in rats (McCann et al., 1983). It is possible that GnRH may facilitate the actions of oestradiol by acting on the neurons responsible for sexual behaviour (Moss et al., 1979). GnRH has also been shown to facilitate oestrous behaviours in monkeys, horses, birds, reptiles and fish (McDonnell et al., 1989; Kendrick & Dixson, 1985; Cheng, 1977; Smith & Mason, 1997; Volkoff & Peter, 1999).

Oxytocin

Female sexual behaviours are also associated with oxytocin release (Kendrick et al., 1988). In rodents there is evidence that oxytocin can influence reproductive behaviours (Insel et al., 1997). Administration of exogenous oxytocin to rats has an important function within the central nervous system for regulating reproductive behaviours, such as increased contact with the male and increase in receptive lordosis behaviour (Arletti & Bertolini, 1985; Gorzalka & Lester, 1987; Schumacher et al., 1989; Caldwell et al., 1986). It has also been suggested that endogenous oxytocin may influence the initiation of sexual receptivity but not the expression of the behaviour itself. The cerebroventricular administration of an oxytocin antagonist before the onset of receptivity results in a dose-dependant decrease in lordosis, but administration after post sexual receptivity was ineffective for decreasing lordosis (Witt & Insel, 1991). It should be noted the effect oxytocin has on sexual receptivity in rat have not been demonstrated in other species (Insel et al., 1997). Although, it has been suggested that in sheep elevated oxytocin in the hypothalamus decreases the duration of sexual receptivity (Kendrick et al., 1993; Kendrick & Keverne, 1992).

Disruption of Oestrus

Stress and reproduction: general overview

Endocrine control of reproduction in females is mediated by interactions within the hypothalamic pituitary-ovarian axis. This involves the sequential release of gonadotrophin-releasing hormone from the hypothalamus, follicle stimulating hormone and LH from the anterior pituitary, and progesterone and oestrogen from the ovary. The ovarian steroids through their positive or negative feedback on the higher centres of the brain, hypothalamus or pituitary, regulate the secretion of gonadotrophins and maintain normal reproductive function.

The 'stress' response is mediated via the hypothalamic-pituitary-adrenal axis, which involves the sequential release of CRH and AVP from the hypothalamus, ACTH from the anterior pituitary and glucocorticoids, such as cortisol, from the adrenal gland. Again positive and negative feedback mechanisms operate on the higher brain, hypothalamus and pituitary gland, to maintain normal homeostasis of the body. It was first suggested by (Selye, 1939) that stress affects reproduction through an interaction between hormones of the hypothalamic pituitary-ovarian axis and those of the hypothalamic-pituitary-adrenal axis. More recently, similar areas and neurotransmitters within the hypothalamus have been shown to control stress and reproduction, therefore, it is understandable that activation of one system (stress) may impact precise function of the other (reproduction) (Dobson et al., 2003).

Stress can be defined as a disruption in homeostasis (Pacak & Palkovits, 2001; Rivier & Rivest, 1991). Stress is revealed by the inability of an animal to cope with its environment and a phenomenon that is revealed by a failure to reach genetic potential (Dobson & Smith, 2000). The stimuli that disrupt homeostasis are termed stressors and can be physical, psychological or physiological. An acute stressor is a stress that lasts briefly (seconds up to a few hours) whereas a chronic stressor is continuous and last much longer (days, weeks or months). The impact of a stressor may depend on the nature of the stressor (acute *versus* chronic), duration, intensity, predictability/control and the steroid milieu of the individual when the stressor occurs as well as the sex or species involved (Tilbrook et al., 2000; Collu et al., 1984; Pacak & Palkovits, 2001). In cattle, there are many potential stressors that have been identified, negative energy balance, high milk yield, postpartum diseases, regrouping, inflammation and infections, lameness, social factors, overcrowding, poor housing, transport or heat stress (Borsberry & Dobson, 1989; Hassall et al., 1993; Bouissou & Boissy, 2005; Collier et al., 2006; Butler, 2000; Nanda et al., 1990; Hasegawa et al., 1997).

Stress may disrupt the function of hypothalamus-pituitary-ovarian axis at each level (Rivier & Rivest, 1991; Dobson et al., 2003). The impact of disruption may depend on the nature of the stressor and/or individual, however, the hormonal mechanisms with effects on fertility are probably common, irrespective of stressor involved (Dobson & Smith, 2000). Reproduction in the female is dependant on a carefully synchronized sequence of endocrine and behaviour events. If these events are delayed or disrupted (i.e. by 'stress') fertility may be compromised. Females are particularly vulnerable to the affects of acute stress during the pre-ovulatory period when dramatic shifts in hormone concentrations occur. Disruption at this period could also disturb oestrous behaviours, ovulation, fertilization and possible establishment and maintenance of pregnancy.

One major impact of acute stress during the periovulatory period is the disruption of hypothalamic function. In general, stress-induced adrenal stimulation delays or inhibits the GnRH surge (Dobson et al., 2003). This results in abnormal ovarian function or delay or inhibition of the LH surge and thus ovulation. Normal pulsatile patterns (amplitude and/or frequency) of GnRH-LH are reduced by exposure to acute stressors such as transport (Smith & Dobson, 2002; Dobson, 1987; Dobson et al., 1999), high-doses of insulin (Dobson & Smith, 2000), restraint (Stoebel & Moberg, 1982b), handling (Martin et al., 1981) or isolation (Tilbrook et al., 1999). Other sensitive periods when acute stress may have detrimental effects are luteolysis or implantation, however, acute stress during other stages of the reproductive cycle do not appear to be as vulnerable (Moberg, 1985; Liptrap, 1993). In rats, prolonged or chronic stress results in excessive glucocorticoids secretion which can decrease GnRH and gonadotrophin secretion (Sapolsky, 1992; Sapolsky et al., 2000). The frequency of GnRH pulses from the hypothalamus dictates the rate of ovarian follicle growth thus endocrine changes may impair or reduce follicular activity (Dobson & Smith, 2000). During the follicular phase there is an increase in oestradiol concentrations in response to follicular growth. The increase in oestradiol is responsible for the correct timing of oestrous behaviour and ovulation, should a stressor block the expression of oestrus or delay ovulation and lengthen the oestrous cycle, this results in a decrease in oocyte and embryo quality and fertility.

The measurement of glucocorticoid concentrations is often used as a measure of 'stress' as it is a reflection of activation of the hypothalamic-pituitary-adrenal axis and so called 'proof' of stress. An elevation in glucocorticoid is a normal response to a stressor and is vital in maintaining normal function (i.e. stimulate gluconeogenesis and provide energy for 'fight or flight'). The amplitude of the hormone response may correlate with the severity of the stimulus (Smith et al., 2003a; Garcia et al., 2000). Glucocorticoids have been shown to inhibit gonadotrophin secretion in a variety of species ranging from rodents (Baldwin, 1979; Baldwin & Sawyer, 1974) to ruminants (Stoebel & Moberg, 1982a; Barb et al., 1982; Li & Wagner, 1983) and primates (Sapolsky et al., 2000). Glucocorticoids mainly have an effect at the level of the hypothalamus and pituitary to inhibit gonadotrophin release. However, there is also some *in vitro* evidence that glucocorticoids may have a direct effect on the ovary by suppressing granulosa cell differentiation, the expression of LH receptors or the secretion of oestrogen by inhibiting aromatase activity (Moberg, 1985). It should be noted that the increased secretion of glucocorticoids is not always associated with a decrease in gonadotrophins, there may be also species differences in the extent to which glucocorticoids inhibit the secretion of gonadotrophins (Tilbrook et al., 2000; Turner et al., 2005). Also the sex and/or hormonal milieu of an individual may impact on glucocorticoid suppression of gonadal responsiveness to gonadotrophins (Sapolsky et al., 2000). The effect of glucocorticoids on gonadotrophin secretion may also be dependent in the health (i.e. stress *versus* nonstressed) of an individual. (Matsuwaki et al., 2004) suggest that a stimulatory role of glucocorticoids during infection in rodents as activation of the hypothalamic-pituitary-adrenal axis plays a role in maintaining normal LH pulses. In ruminants, (Karsch et al., 2002) also suggest different pathways for disruption of ovarian activity during immune/inflammatory stress *versus* healthy animals.

Importantly, after an initial large release of glucocorticoids, prolonged stimulation leads to a normal gradual reduction in glucocorticoid concentrations as a result of negative feedback. Although glucocorticoid concentrations have decreased, the stress signal at higher brain levels may still be present and the animal still be experiencing the 'stressor' as aversive (Smith & Dobson, 2002). Therefore, interpretation of cortisol as a measure of welfare of animals should be used with caution.

Oestrus Disruption

The mechanism by which 'stress' alters the behavioural expression of oestrus in cattle remains to be established. To understand the mechanisms of how stress disrupts oestrus, researchers often administer various hormones from neuroendocrine systems to mimic the stress response. Injections of CRH inhibit sexual behaviour in the rat (Rivier & Vale, 1984). During proestrus in intact and ovariectomized cows, single injections of very high does of ACTH (320 IU) decreases the duration of oestrus, delays the onset of oestrus and decreases the proportion of cattle in oestrus, respectively (Hein & Allrich, 1992). Similarly, very high repeated doses of ACTH (100 IU) during proestrus delay the onset of oestrus (Stoebel & Moberg, 1982a). Infusion of cortisol for 90 hours to proestrus cattle inhibits oestrous behaviours (Stoebel & Moberg, 1982a). A single dexamethasone injection (a synthetic glucocorticoid; 4mg) decreases the incidence of oestrus in oestradiol-treated ovariectomized cows (Allrich et al., 1989; Allrich et al., 1984; Cook et al., 1987); however, it does not delay the onset of oestrus, duration of oestrus or the frequency of oestrus-related behaviours (Allrich et al., 1984). Similarly, (Cook et al., 1987) were unable to influence the incidence of oestrus, onset of oestrus or number of oestrus-related behaviours in ovariectomized progesterone-primed oestradiol-treated cows with single injections of exogenous cortisol (40-200mg). Exogenous glucocorticoids also moderate oestrous behaviours in other species (Asa & Ginther, 1982; Ford & Christenson, 1981). In ovariectomized progesterone-primed oestradiol-treated ewes, dexamethasone delays or inhibits oestrus (Ehnert and Moberg 1991). Multiple injections of ACTH in intact ewes delays the onset to oestrus (Doney et al., 1976). Although in most cases synthetic stress hormones appear to impair oestrous behaviours, this may not represent a 'physiological' response to stress and should be interpreted with caution (Turner et al., 2005).

One of the most significant effects of stress is at the level of GnRH-LH pulsatility (Smith et al., 2003b). The interruption of LH pulsatility (by GnRH antagonists or by stressors) lowers oestradiol production (Dobson & Smith, 1998; Oussaid et al., 1999). As oestrous behaviours are oestradiol dependant this could result in the inhibition, depression and/or delay of oestrus. Additionally, GnRH could be involved in oestrous behaviours, therefore diminished GnRH pulsatility could result in a depression of oestrous behaviours (Caraty et al., 2002). Of interest a gonadotrophin inhibiting hormone (GnIH) has been identified in birds, hamsters and fish (Tsutsui et al., 2000; Ukena & Tsutsui, 2005; Ikemoto & Park, 2005; Kriegsfeld et al., 2006). In birds, GnIH has been shown to decrease sexual behaviours (Bentley et al., 2006).

Cow related factors that influence oestrous detection

It is well noted that failure to detect oestrus in cows is due partly to human observation skills; however, poor identification of oestrual cows may be due to inherent characteristics of the cow herself.

Individual variation

It has been demonstrated that oestrous activity can vary both within and between dairy cows (Van Vliet & Van Eerdenburg, 1996; Hurnik et al., 1975).Within an individual, variation in the intensity of oestrous behaviours may depend on when oestrus was observed (early versus late postpartum, dioestrus, during pregnancy). Age may also influence oestrus expression (Ron et al., 1984; Stevenson et al., 1983). The intensity of oestrus expression can also vary with time postpartum. Mounting activity is lowest early in the postpartum period and increases thereafter (Hurnik et al., 1975). Additionally, individuals may show secondary signs of heat when not in oestrus (Esslemont et al., 1980; Hurnik et al., 1975; Kilgour et al., 1977; Mylrea & Beilharz, 1964). It has been reported that 19% of cows not in oestrus will mount other cows, thus leading to possible incorrect identification of oestrual cows (Hurnik et al., 1975). Additionally, 6 % of pregnant cows display signs of oestrus (Erb & Morrison, 1958; Thomas & Dobson, 1989; Williamson et al., 1972). Differences in oestrous behaviour between individuals can be related to genetic differences (Hackett & McAllister, 1984). In a mixed group of purebred Charolais and Charolais/Braham crosses, 40% of mounts were made by the purebred Charolais on the Charolais/Braham crossed animals, whereas only 8% of mounts were made by the Charolais/Braham crossed animals on the purebred Charolais.

Dominance, social status, cortisol and coping ability

A relationship between social status and reproductive success has been demonstrated in primates, rodents and pigs (Molteno & Bennett, 2000; Wasser, 1996; Pedersen et al., 2003; French, 1997). Social status may relate to an individual's neuroendocrine and/or fear responses to stress and, therefore, reveal an ability to cope in a stressful environment (Zayan & Dantzer, 1990). Coping is a behavioural reaction that aims to reduce the effect of aversive stimuli (Wechsler, 1995) and coping successfully in a social environment requires adopting certain behavioural strategies (Prelle et al., 2004). In general, adrenocortical activity can be related to several factors including reactions to aggression (Hessing et al., 1994; Koolhaas et al., 1999; Ruis et al., 2000b) or an individual's disposition to learn and innovate (Rushen, 1986; Pfeffer et al., 2002). However, basal adrenocortical activity has also been implicated as a fundamental characteristic of an individual that is related to the individual's overall behavioural coping strategy. For example, the terms 'passive' or 'active' coping style to stressful situations are commonly used (Koolhaas et al., 1999). In rodents, birds and pigs these coping styles refer to a psychobiological response when an active (e.g. confrontation, fight, escape) behavioural response is associated with low corticoid concentrations and high corticoid concentrations are associated with a passive (e.g. quiescence, immobility, low levels of aggression) behavioural response (Jones & Satterlee, 1996; Beuving et al., 1989; De Boer et al., 1990b; Mendl & Deag, 1995; Koolhaas et al., 1999; Korte et al., 1997). Therefore, when environmental stressors are too demanding (i.e. during social stress) and an individual cannot cope, activation of the hypothalamic-pituitary-adrenal axis and suppression of reproductive behaviours (i.e. oestrus) is a likely outcome.

In some species, subdominant status is associated with an increase in adrenocorticoid activity (Zayan & Dantzer, 1990; Mendl et al., 1992). However, among various species of primates, the relationships between social rank and patterns of stress response are not consistent. In subordinate female common marmosets, cortisol concentrations are ~50% lower than dominants, whereas in subordinate female talapoin monkeys basal cortisol concentrations are nearly 50% higher than their dominant counterparts (Abbott et al., 2003). The authors conclude that different primate societies (i.e. frequency of stressor and/or social support) can predict whether subordinate or dominant animals will exhibit elevated basal cortisol concentrations. In cattle, the adrenal glands of subordinates are significantly bigger compared to glands from dominant individuals (Bouissou, 1985). However, no correlation has been found between rank and blood cortisol concentrations (Adeyemo & Heath, 1982; Arave et al., 1977). Similarily, in beef-suckler cows there is no relationship between dominance value and faecal corticoid concentrations (Mülleder et al., 2003). Although no relationship between dominance and cortisol has been demonstrated in cattle, similar to primates, cortisol concentrations can vary when individuals are grouped based on differing social or coping strategies. In a study by (Mülleder et al., 2003) three coping strategies were identified, one dominant type coping style (*'agonistic dominant'*) and two subdominant styles (*'non agonistic' and 'agonistic challenging'*). Subdominant groups coped differently with their lower social status, *'non agonistic'* participated in more non-agonistic social behaviour (licking and head play) and avoiding agonistic interactions, where as *'agonistic challenging'* were involved in more agonistic interactions. Faecal cortisol concentrations were greatest in subdominant *non agonistic*, *agonistic dominant* had intermediate values and subdominant *agonistic challenging* individuals had the lowest concentrations. Significant differences in cortisol concentration were only demonstrated between the two subdominant groups. The authors concluded that, although differences exist between the subdominant groups, social 'rank' was not related to cortisol production.

In cattle, some studies suggest that there is no relationship between characteristics of oestrus and social dominance. In a herd of ovarian cycle synchronized Braham cows (*Bos indicus*) there was no relationship between social rank and mounting behaviour (Orihuela & Galina, 1997). (Kabuga et al., 1992) stated that neither agonistic interactions received or dominance values were correlated to mounts received or oestrus duration. When individuals were introduced in one-to-one encounters with an induced oestrus cow, there was no difference in mounting activity exhibited between dominant and subordinate cows (Alexander et al., 1984). There is also evidence that individuals may prefer some partners over others (Cecim & Hausler, 1988). Mounting may occur in preferential directions between pairs of animals irrespective of their rank, therefore ignoring social status of an individual during oestrus (Hafez & Bouissou, 1975). Oestrus affects the frequency distribution of agonistic interactions, that double during the oestrous period (Hurnik et al., 1975). A relationship between rank and sexual motivation has also been demonstrated in other species (pigs) (Pedersen et al., 2003). Similarly, (Hurnik et al., 1975) observed a positive correlation between the number of observed mounts and the rank of victimization, when higher ranking cows more often initiate activity and mounting others. (Wagnon et al., 1966) also observed top ranking cows monopolising oestrus cows and preventing other cows from mounting. (Kabuga et al., 1992) observed that high status cows initiated more mounts than low status cows. Furthermore, (Weibold et al., 1983) suggested that there is a relationship between rank and oestrous characteristics. Middle ranking cows expressed oestrus with less intensity, as they exhibited fewer mounts and less standing behaviour, than high or low ranking individuals. Interestingly, studies indicate that the highest and lowest ranking individuals in a group maintain relatively constant positions over time, whereas middle ranking individuals sometimes change their social position (Kondo & Hurnik, 1990; Hook et al., 1965; Oberosler et al., 1982). (Arave & Albright, 1976) noted that dominance is least stable for middle ranking cows. Additionally, middle ranking cows have problems adjusting socially to new group members (Krohn & Konggaard, 1980), suggesting that poorer social stability results in poorer expression of oestrus.

Cues from the Environment that Influence Oestrus

Pheromones

In mammals, pheromones are air-borne chemical substances or 'signals' that are excreted externally and cause a specific reaction in the recipient. Pheromones can act singly or in combination with other stimuli including auditory, visual or tactile cues (Rekwot et al., 2001). Pheromones transmit a range of information including territoriality, anti-predation, social group behaviours and reproductive status (Brown & Macdonald, 1987; Rekwot et al., 2001). Reproduction pheromones are multifunctional, serving not only to attract but also to induce sexual behaviours in others (Izard, 1983). Many ungulates routinely investigate or 'test' the urine or anogenital region by licking, nosing, sniffing or massaging the vulva of an oestrual female (Hafez & Bouissou, 1975; Rekwot et al., 2001). Normally this is followed by a flehmen behavioural response whereby an individual lifts the head, opens the mouth and curls the upper lip. Flehmen behaviour is related to the vomeronasal organ, a bilateral blind sac, which in cattle, sheep and goats opens to the nasopalatine canal. Flehmen is involved in the transport of olfactory signals from the oral cavity to the sensory epithelium of the vomeronasal organ which contains receptors for pheromones (Estes, 1972; Jacobs et al., 1980; Rekwot et al., 2001). Signals derived from the vomeronasal organ are ultimately targeted to the brain (Dudley et al., 1996). The effect of pheromones on reproduction are mediated by GnRH neurones (Boehm et al., 2005). Behavioural studies, along with immunocytochemical detection of immediate early genes and neuropeptides, reveal that GnRH containing neurones are activated by stimulation of the vomeronasal organ and some of the activated GnRH neurones project to the hypothalamus where they are believed to induce sexual responsiveness (Dudley et al., 1996). In rodents, the electrical stimulation of the vomeronasal organ induces Fos expression in medial preoptic GnRH neurones (Boehm et al., 2005). In marmoset monkeys, urinary pheromone cues from dominant females have been implicated in the inhibition of gonadotrophs and absence of ovulation in low ranking females (Barrett et al., 1990). In cattle, pheromones from the bull hasten the onset of puberty in heifers (Izard & Vandenbergh, 1982a; Roberson et al., 1991; Rekwot et al., 2000a) and exposure to bulls or testosterone-treated cows decreases the duration of postpartum anoestrus and stimulates oestrous behaviour (Burns & Spitzer, 1992; Rekwot et al., 2000b; Fernandez et al., 1993; Izard, 1983; Zalesky et al., 1984; Alberio et al., 1987). Pheromones from females are involved in synchronizing oestrus in herd-mates.

The application of cervical mucus to cows following prostaglandin treatment results in a more uniform synchronization of oestrus (Izard & Vandenbergh, 1982b).

Biological assays have been designed to characterize pheromone activity by using the flehmen response in bulls (Alberio et al., 1987) or by using dogs or rats that have been trained to detect cycle-dependant odours in urine or vaginal secretions (Kiddy & Mitchell, 1981; Kiddy et al., 1978; Ladewig & Hart, 1981; Dehnhard & Claus, 1988). Maximal responses to the bioassay corresponded to observed day of oestrus. Ovarian hormones are the likely source that initiated the quantitative and/or qualitative changes in oestrus-related odours (Hawk et al., 1984; Kumar et al., 2000). The endocrine dependence of some urinary volatile compounds has been reported in rodents (Boyer et al., 1989; Jemiolo et al., 1987; Schwende et al., 1984; Andreolini et al., 1987), wolves (Raymer et al., 1984) and bears (Dehnhard et al.). In cattle, the presence of the ovary appears to be essential for the synthesis of oestrus-inducing pheromones as urine samples collected from oestradiol-treated ovariectomized cows, with clear oestrous symptoms, did not induce pheromone activity (measured with a rat bioassay) in the urine (Dehnhard et al., 1991).

In cattle, through stimulation of sexual behaviours, oestrus-related pheromones have been detected in various body fluids including urine, faeces, milk, plasma, saliva, perineal skin gland secretions, vaginal/cervical mucus (Kiddy et al., 1978; Blazquez et al., 1988; Sankar & Archunan, 2004; Izard & Vandenbergh, 1982b). Although urine is a primary source for oestrous pheromones, vaginal mucus, compared to saliva, faeces and milk, stimulates the maximum exhibition of flehmen by bulls (Sankar & Archunan, 2004). In cattle, there have been attempts to characterise compounds in urine, milk and vaginal mucus in relation to oestrus (Bendall, 2001; Klemm et al., 1987; Rivard & Klemm, 1989; Ma et al., 1995; Abbott et al., 2003; Kumar et al., 2000; Weidong et al., 1997). Using gas chromatography and mass spectrometry (Weidong et al., 1997) found 36 volatile compounds in milk that exhibited quantitative differences during the oestrous cycle. (Kumar et al., 2000), also using gas chromatography and mass spectrometry, identified two volatile compounds in urine that were unique to the ovulatory phase of the oestrous cycle. However, the functional role of these compounds needs to be confirmed by effects on sexual behaviour in bulls. Acetaldehyde was also identified as a volatile component of bovine vaginal secretions and showed a successive increase and decrease zero to three days before oestrus, with another unidentified compound being unique to proestrus (Ma et al., 1995). After dialyzation or separation on ion-exchange resins, (Nishimura et al., 1991) suggested mounting-inducing pheromones in mucus were neutral substances of relatively low molecular weight.

Table 3. Potential candidates for sexually active pheromones quoted in the literature

Putative pheromone	Reference
2-heptanone	(Klemm et al., 1987)
1-methoxy-3-pentene	
1-phenylethanone	
1-phenylpentanone	
heptyl-3-one	
2-propanone-1,3-diol-1-amine	
1-pyperyl-2-ethanone	
heptyl methyl ester	
1-phenylheptane	
3-methylamine	(Sankar & Archunan, 2004)
acetic acid	
propionic acid	
2-propyl phthalate	(Kumar et al., 2000)
1-iodo-undecane	
acetaldehyde	(Lane & Wathes, 1998)

Pheromones are also often associated with carrier proteins, that transport the pheromone into the environment, influence bioavailability and can affect activity of the recepient sensory organ. To date, knowledge of the functional relationship between pheromones and large proteinaceous compounds is limited (Baxi et al., 2006).

Season and temperature

High environmental temperature affect oestrus. Cows exposed to high temperature and humidity exhibit oestrus of a diminished intensity (Bond & McDowell, 1972; Gangwar et al., 1965). Hot weather decreases the duration of standing heat (3.0 h in hot weather and 6.7 h in cool weather), but it does not affect the duration of a mount or the total number of mounts (At-Taras & Spahr, 2001). During summer there was a reduction in the total duration of oestrus (7.3 hours compared to 9.7 hours in winter) (Xu et al., 1998). Additionally, during warmer weather there is a reduction in the number of mounts observed (13.6 versus 8.5 per oestrus). Tropical storms also suppress oestrous activity (Hurnik, 1987). Alternatively, (Walker et al., 1996) and (Rodtian et al., 1996) found no effect on duration of mounting activity following an increase in temperature.

Management practices

The practice of inducing and or synchronizing animals into oestrous obviously influences the rate of oestrus detection. The number of animals in oestrus at the same time influences oestrous activity as it allows for the opportunity to 'share' oestrous behaviours (Hurnik et al., 1975; Helmer & Britt, 1985). When there are two or more cows close to oestrus or in oestrus, a sexually active group is formed. The more individuals participating, the greater the mounting activity (Orihuela & Galina, 1997). The majority of standing heats (77%) are observed when there are a greater number of cows in oestrus at the same time (Van Vliet & Van Eerdenburg, 1996). Increasing the number of interacting females in oestrus at one time from 1 to 4 increased the number of standing events from 11.2 to 49.8, respectively (Hurnik et al., 1975). Additionally, mounting duration lasts longer (7.4 seconds) when more cows are in oestrus than when oestrus is observed with only one partner (4.6 seconds)(Hurnik, 1987). It has also been demonstrated that non-synchronized cows remaining in a herd with synchronized cows show oestrus concomitantly with the synchronized cows, thus demonstrating a clustering behaviour (Galina et al., 1996).

Housing influences oestrous expression. (Pennington et al., 1986) found that the quality of the floor surface influences mounting activities. The unshaded drylot and feed manger areas provide the best footing and are least crowded, accounting for 88 % the total oestrous behaviours. (Britt et al., 1986) noted that the durations of oestrus, mounting and standing to be mounted activity are greater on dirt rather than on concrete. Oestrous detection is sometimes based on increased movement of cows. Housing conditions such as tied stall or loose housing may impact oestrous expression (Hackett et al., 1984). Based on pedometry, cows in oestrus are about four times as active as cows not in oestrus in a free stall setting and 2.7 times more active when held in a comfort stall (Kiddy, 1977; Senger, 1994). Additionally, the available area in which to display oestrous behaviours influences the oestrus intensity (Metz & Mekking, 1984).

Poor oestrous expression has been attributed to the required daily movement of cows for milking (Orihuela & Galina, 1997). Furthermore, (Vaca et al., 1985) reported a reduced oestrous expression when the environment is changed, as when moving from pasture to pen housing.

In dairy cattle, re-grouping individuals on a regular basis based on physiological status (lactation day, pregnancy or dry) may have a negative impact on oestrus expression through social stress. In cattle, familiarity among herd-mates reduces stress (Boissy & Le Neindre, 1990). Although some studies suggest that cattle adapt to repeated groupings (Kondo et al., 1984; Veissier et al., 2000), the introduction of unfamiliar individuals results in increased aggression and social stress which could impact oestrous expression (Gonzáles & Galindo, 2000; Hasegawa et al., 1997; Mench et al., 1990; Bøe & Færevik, 2003). In a review by (Bøe & Færevik, 2003), it states that this is more apparent in low ranking individuals and for introduced animals than for resident animals in established groups. Problems may also be higher for individuals that lack early social experience, as group-reared calves are more socially confident and show less fear than isolated-reared calves (Bøe & Færevik, 2003). However, the introduction of novel animals can also cause sexual interest, as evident by increased mounting by bulls (Almquist & Hale, 1956). Based on one to one introductions, the introduction of a novel cow stimulated more mounting and flehmen behaviours than the introduction of a familiar cow (Alexander et al., 1984).

Health and clinical diseases

How an animal 'copes' with its environment is complex and comprises a multitude of factors including the inherent characteristics of an individual (genetics, temperament) and determinants of the environment (social dynamics, management practices, housing, husbandry and nutrition). Failure to cope with challenges presented by the environment (i.e., poor welfare) is manifested in those animals that succumb to clinical production diseases or health problems, such as poor body condition score, ovarian cysts, retained fetal membranes, endometritis, dystocia, milk fever, mastitis and lameness.

Periparturient diseases are associated with poor fertility and different diseases with different incidences have different effects on a cow's subsequent fertility (Borsberry & Dobson, 1989; Fourichon et al., 1999). In a paper by (Fourichon et al., 1999) which reviewed 70 papers published from 1960 to 1997, clinical ketosis, dystocia and retained fetal membranes are associated with a 2-3 days increase the calving to first insemination interval and a 6-12 day increase in calving to pregnancy interval. Metritis and cystic ovaries increased the calving to first insemination and calving to pregnancy intervals by 7-11 days and 19-30 days, respectively. Milk fever, stillbirth, displaced abomasun and mastitis appeared to have no effect on reproduction. However, (Borsberry & Dobson, 1989) reported that milk fever did adversely affect the calving to pregnancy and calving to first insemination intervals. Milk fever, retained fetal membranes and endometritis and mastitis are also associated with a delay in the commencement of luteal activity postpartum (Morrow et al., 1966; Huszenicza et al., 2005). The cause of reduced fertility could be related to delayed uterine involution or impaired endocrine initiation of ovarian cyclicity postpartum due to stressful conditions (Dobson & Alam, 1987; Morrow et al., 1966; Borsberry & Dobson, 1989).

Similar to other periparturient diseases, lameness is associated with poor reproductive performance as seen through an increase in several reproductive parameters (Table 4).

Table 4. Reproductive parameters associated with lameness compared to normal herd-mates

Reference	Calving to 1st insemination interval (days longer)	Days to 1st ovulatory oestrus (lame/not lame)	Calving to pregnancy interval (days longer)	Number of inseminations per pregnancy (lame/not lame)	1st service to pregnancy interval (days longer)	Pregnancy at 1st insemination (lame/not lame)	Days pp CLA (lame/ not lame)
(Collick et al., 1989)	4	-	14	2.14 (1.72)	-	46% (56 %)	-
(Barkema et al., 1994)	2.9 to 4.6	-	-	-	3.4	No difference	-
(Hernandez et al., 2001)	8	-	40	5 (3)	-	-	-
(Hernandez et al., 2005)	-	-	30-66		longer		
(Garbarino et al., 2004)	-	-	-	-	-	-	32-36 (29)
(Lucey et al., 1986)	17	-	30	-	-	-	-
(Melendez et al., 2003)	No difference	-	-	higher	-	18 % (43%)	-
(Petersson et al., 2006)	-	84.4 (60.4)	-	-	-	-	49.8 (33.4)

The annual incidence of lameness on UK farms varies from farm to farm but the incidence has been reported from 6% in the 1960's, up to 25 % in the 1980's to late 1990's and more recently up to 50% (Leech et al., 1960; Collick et al., 1989; Prentice & Neal, 1972; Whitaker et al., 1983; Arkins, 1981a; Ward, 2001). Lameness tends to affect cows early in lactation, mainly within the first 3 months of lactation (Prentice & Neal, 1972; Collick et al., 1989; Eddy, 1980; Whitaker et al., 1983; Arkins, 1981b). Oestrus is less likely to be observed in cows that suffered lameness before insemination (Peeler et al., 1994). Similarly, (Petersson et al., 2006) reported that the interval to the first ovulatory oestrus is later in lame cows. Mild lameness was not associated with the intensity of oestrous expression (Gomez et al., 2003). However, (Sood & Nanda, 2006) observed fewer standing events in mildly lame cows. It has been suggested that poor oestrous detection in lame cows is the likely factor in reducing fertility levels than any putative effects of lameness (Lucey et al., 1986). However, reproductive efficiency decreases with a number of postpartum diseases, so may not just be the physical mechanical limitations of lameness that depress reproductive efficiency.

Methods of Determining Stress & Stress-Related Behaviours

Definitions

There are behavioural differences not only between species but also within individuals of the same species (Plomin, 1990). Even when individuals are reared under the same conditions there can be extensive individual variability in behaviour (Boissy, 1995). Early studies focused on similarities between individuals rather than differences; however, today, individual differences in behaviour may be regarded as the rule rather than the exception (Huntingford, 1984; Manteca & Deag, 1993a; Bekoff, 1977). Understanding the origins and implications of individual differences is important as it may enable prediction of behavioural responses based on those expressed in one situation compared to another and possibly allow early identification of individuals that may have long-term difficulty adapting to environmental challenges (Van Reenen et al., 2004).

In humans and domestic animals the ability to demonstrate inter-individual variability consistently over time and in response to environmental change indicates the basic dimensions of personality (Zuckerman, 1991; Boissy, 1995). One aspect of an individual's personality is temperament. Temperament is related to how an individual perceives and reacts to change or a potentially alarming or challenging situation (Boissy, 1995; Manteca & Deag, 1993a). Temperament is an intervening variable, which temporally modulates a large range of fundamental behaviours, i.e. social, reproductive, feeding or adaptive behaviours.

Fear and anxiety are behavioural motivators defined as emotional states induced by the detection of actual danger (fear) or potential danger (anxiety) threatening the well-being of an individual (Boissy, 1995). Individual differences in response to a stressful stimuli are not random but rather governed by stable underlying biological characteristics or traits (Koolhaas et al., 1997; Van Reenen et al., 2004). Fearfulness, sometimes termed emotional or behavioural reactivity, is a feature of temperament, predisposing an individual to respond in a similar fashion to a variety of potentially challenging situations (Boissy & Bouissou, 1995). Fearfulness can therefore be considered a personality or temperament trait which defines the susceptibility of an individual to react to a variety of potentially alarming situations (Boissy, 1995).

Measuring fear

Fear in animals can be assessed objectively in experimental conditions by investigating the nature and physical properties of the fear-eliciting stimulus and the magnitude of the corresponding psychobiological response. Psychobiological responses comprise behavioural changes, which counteract the effects of the stimulus, and neuroendocrine adjustments that are needed to maintain homeostasis.

Fearfulness, as a personality or temperament trait, may also be quantified using observers' ratings to provide useful information about subtle aspects of an individual's behaviour. An observer can assess an individual's overall behavioural 'style' or emotional 'tone', therefore, measuring characteristics that can not be assessed using conventional recording methods (Manteca & Deag, 1993a).

Stimuli and behavioural tests for studying fear

The most fundamental fear-eliciting stimuli are related to persistent dangers that exist in the ecology or evolutionary history of a species. Fears of potential predators or physical fears such as fear of heights or fear of darkness all have a definite survival value as the life expectancy of an individual is clearly increased if it can react to avoid sources of danger. These fundamental fear-producing stimuli can be learned through interaction with conspecifics (social learning) or from a conditioned process as in an experimental setting. Heifers can be conditioned to be frightened by a visual object, originally non-threatening, through association with an electric shock (Boissy, 1990). Assessing fear can include exposure to visual, acoustic or olfactory stimuli from both conspecifics and predators (Boissy et al., 1998; Terlouw et al., 1998). Fear of predators can be examined experimentally using odours, as many species will avoid faecal odours of predators. Cattle will not feed from bins that have been contaminated with bear, coyote, cougar or fox faecal odours (Pfister et al., 1990). Additionally, visual, auditory and/or olfactory signals may be exchanged between conspecifics affecting behaviours in response to aversive situations. In various species, when exposed to fear-eliciting stimuli, individuals show a stronger response when alone than when tested in the presence of partners (Coe et al., 1982; Jones & Merry, 1988; Taylor, 1981). In gregarious species like cattle, social isolation is a known fear-eliciting stimulus (Hopster & Blokhuis, 1994). The reduced reactivity to a stressful event in the presence of partners is called 'social buffering' and it is most distinct in gregarious species (Epley, 1974). In cattle, the effect does not depend on physical contact, as the presence of the conspecifics separated by a wire mesh screen has been shown to reduce the animal's reaction to an aversive event (Boissy & Le Neindre, 1990). Reactions to a fear-eliciting situation also are affected by the 'stress state' of conspecifics. In a study by (Boissy et al., 1998) heifers were able to perceive, possibly through olfactory cues in the urine, the stressed state of conspecifics and became more fearful as a result.

Another fear producing stimuli is novelty. Exposure of an animal to novelty is one of the most potent experimental conditions to cause a negative emotional response (Boissy, 1995). Assessing neophobia involves the introduction of an unfamiliar or novel object into a familiar environment. In cattle, researchers have exposed individuals to traffic cones, brightly painted and striped objects, rotating lights, buzzers, umbrella's and even tambourines (Boissy & Bouissou, 1995; Plusquellec & Bouissou, 2001; Boissy &

Bouissou, 1994; Van Reenen et al., 2004). The frequency and duration of behaviours such as time spent near and distance from an object, sniffing an object, the latency to approach an object, walking, immobility, vocalizations and defecations are then recorded. The avoidance responses in a 'novel object' test are thought to reflect the fear of novelty (Van Reenen et al., 2004; Boissy & Bouissou, 1995).

In addition to the 'novel object' test, other methods of assessing fear include motivation tests whereby an individual is placed in a conflict situation between a motivation (i.e. food or social) and avoidance of a fear-eliciting event. An example of food motivation test would be to place a fasted individual in an unfamiliar room and offer food in a familiar bucket and record total feeding time. A surprise motivational test would involve an individual who is habituated to feed from a bucket but is then 'surprised' with a blast of air or sudden opening of an umbrella (Boissy & Bouissou, 1995). An example of a social motivation test would be the motivation to clear a frightening obstacle (an electric fence) to rejoin herd-mates (Boissy & Bouissou, 1995; Plusquellec & Bouissou, 2001).

Another commonly used fear-eliciting test in cattle involves human contact. Similar to the novel object test, individuals can be lead into a familiar area and exposed to a stationary 'novel' human (Van Reenen et al., 2004). Ease of handling or measurements of docility can been examined whereby, in the presence or absence of humans, individuals are able to move in a small arena and are challenged with a combination of non-restrained and restrained tests (Plusquellec & Bouissou, 2001; Le Neindre et al., 1995; Gauly et al., 2001). Additional fear-eliciting tests may involve placing an individual in a crush or squeeze cage or a separation test whereby individuals are social isolated from herd-mates (Boissy & Le Neindre, 1997; Grignard et al., 2001; Gauly et al., 2001).

A classically reported test to induce fear is the exposure of an individual to a novel environment (i.e. open-field test). The open field test is an experimental situation that provides a number of threatening situations including novelty, absence of shelter and identifiable landmarks and absence of conspecifics. It often consists of placing an individual in a novel area, which is marked by a number of squares, for a few minutes and recording aspects of behaviour best thought to indicate fear (i.e. amount of activity, defecation and vocalization). It is most extensively used in rodents however it has been applied to domestic pigs (Mormède et al., 1984), sheep (Moberg et al., 1980) and cattle (Kilgour, 1975; Dantzer et al., 1983a; Kovalcikova & Kovalcik, 1982; Boissy & Bouissou, 1995; De Passillé et al., 1995; Boivin et al., 1992). In cattle, despite widespread use, interpretation of results from the open-field test are controversial (Rushen, 2000; Le Neindre, 1989). For example, some interpret the degree of activity as a sign of nervousness (Warnick et al., 1977), while others refer to it as the level of 'locomotor' motivation (Dantzer et al., 1983a; Dellmeier et al., 1990). According to (Rushen, 2000) both are probably right as the behavioural responses that an animal makes to any given stressor are a reflection of different motivations. In a study by (De Passillé et al., 1995), factor analysis was used to tease apart different behavioural motivations in the open field test. It was concluded that using seven variables (sniffing/licking, walking, running, jumping, standing immobile, vocalization and defecation) three 'factors' effectively suggested independent sources of motivation. The factors were labelled as 'fear' (vocalization and defecation), 'exploration' (sniffing/licking) and 'locomotion' (running and jumping). All three factors correlated with the total amount of activity of the calf, therefore, a calf that was very active was either exhibiting a high level of fear, exploration, locomotor motivation or a perhaps combination of all three. Thus, it then becomes clear that just measuring total activity and as a reflection of fearfulness is dangerous as other sources of motivation may account for the animals' behaviour (De Passillé et al., 1995; Rushen, 2000).

As demonstrated above, it is not valid to measure a single variable that is thought to represent a certain emotional state such as fear. Therefore, correlations are often employed to assess the effectiveness of methods used to investigate 'fear'. Several behavioural and physiological responses (or variables) can be monitored in several different challenging situations (as mentioned above) and correlations drawn within and between tests.

One correlation analysis frequently used in ethology to reveal motivational structures underlying behaviour is principal component analysis (PCA). This statistical technique transforms an original set of variables into a substantially smaller set of uncorrelated individual variables called components. These components capture the majority of variation in the original set of variables. In other words, a number of variables can be shown to influence the same component and thus a large number of variables can now be explained in terms of a smaller number of underlying variables. Within each component every variable is measured by it's 'loading' on the component (i.e. the greater it's correlation to the component the more influence it has). For example if the variables, fearful of conspecifics, fear of people and 'tense' all had significant loading on a component while all other variables had very low loadings the component could be label 'tense-fearful' (Dunteman, 1989). Differences in component scores can then be compared between different test groups (i.e. lame vs. nonlame individuals).
To achieve an objective evaluation of emotional reactivity associated with fear, one can measure the response after experimentally varying the degree of threat by changing the event from a mild disturbance to a more threatening physical or psychological event (Désiré et al., 2002). Changing the event may involve changing the physical characteristics of the fear-eliciting stimuli as presentation, movement, intensity, duration, suddenness or proximity can influence its impact. For example, for cattle a rotating light placed on top of a bucket with a continuous buzzer underneath appeared to be more frightening than a plain traffic cone as a significantly higher percentage of cows would investigate the cone rather than the rotating light (Plusquellec & Bouissou, 2001).

Grading the characteristics of the same threatening event can also be used to objectively evaluate the emotional reactivity associated with fear (Boissy, 1995). For example, an open-field test vocalization/defecation rates were associated with the factor 'fear', the degree of novelty of the enclosure was altered, by adding the factors: 1) experience with the enclosure and 2) addition of a novel object. The amount of vocalization/defecation by calves was higher for younger calves and was increased by adding a novel object and allowing the calves to become familiar with the enclosure reduced the occurrence of these behaviours (De Passillé et al., 1995).

As stated earlier, fearfulness as a personality or temperament trait can dictate the general susceptibility of an individual to react to a variety of potentially alarming situations (Boissy, 1995). This suggests that an individual is predisposed to respond similarly over time and to a variety of potentially challenging situations (Boissy & Bouissou, 1995). Not only is there evidence that individuals are consistent in their response to different challenges but responses are consistent over time. Cattle exposed to repeated social isolation demonstrate high repeatability in variables measured within individuals (Hopster & Blokhuis, 1994). Additionally, agitation behaviour is persistent over a number of restraint and handling sessions in cattle (Grandin, 1993). In heifers, flight reactivity to humans remains fairly constant up to seven months of age (Kerr & Wood-Gush, 1987). When tested at several ages goats will show similar reactivity responses towards humans (Lyons et al., 1988). This indicates a general response pattern characteristic for an individual animal. Additionally, studies in several species report of consistency of individual differences in behavioural reactivity across different challenge situations (Jones & Waddington, 1992; Grignard et al., 2001; Van Reenen et al., 2004; Boissy & Bouissou, 1995; Hessing et al., 1994; Le Scolan et al., 1997; Thodberg et al., 1999; Grandin, 1993). This supports the idea that the responsiveness of an individual to a challenge could be mediated by a single underlying characteristic or trait such as fearfulness.

In contrast, it should be noted, however, that some studies report inconsistencies in individual reactivity (Van Reenen et al., 2004; Spoolder et al., 1996; Pollard et al., 1994). It has been suggested that an animal that is fearful in one situation may not experience an emotional state of fear in another situation or they may be differentially expressed (i.e. by vocalization, by locomotion or avoidance/approach) (Van Reenen et al., 2004; Romos & Mormède, 1998). This suggests that different tests or different aspects of the same test may measure different types of fearfulness (Romos & Mormède, 1998; File, 2001; Wilson, 1998).

Behavioural Responses

The most obvious indicator resulting from a fear-producing stimulus is a behavioural response. Behavioural indices of emotional reactivity are commonly used, as they are quick, non-invasive and technically easier to obtain than physiological measures. Additionally, they are considered to reveal a more direct interpretation of an individuals' feelings or emotions (Rushen, 2000). For example, in cattle, individuals that are more agitated during handling in an auction ring are also more startled by sudden sounds and movements, therefore; behavioural reactivity can be used as an indicator of the individual's temperament (Lanier et al., 2000).

The first reaction to an aversive event is body orientation. The animal orientates sense organs to the stimulus so that it can effectively locate and evaluate the source of the stimulus. Orientation responses are usually followed by a startle response and then defence or flight reactions (Broom & Johnson, 1993). Startle responses comprise postural changes preparing the individual for "flight or fight". Additional fear indicating responses include expressive movements (facial expression), vocalization (alarm calls) and defecation. Responses can be contradictory; for example, active defence (attack or threat) or avoidance behaviours (flight, hiding or escape) and immobility are all behaviours that can be observed in response to an aversive event. Conversely, behaviours suggesting low levels of fear can be expressed. For example, in novel object tests, time spent sniffing the object is considered to represent low levels of fear (Boissy & Bouissou, 1995) or time spent sniffing/licking in an open-field experiment are thought to be associated exploratory behaviours not a fear response (De Passillé et al., 1995). The intensity, duration, frequency and or patterning of all these behavioural responses can be measured as an index of disturbance (Broom & Johnson, 1993).

The delay before normal behaviour and activities resume can also be a useful measure (Broom & Johnson, 1993). Fear is an intervening variable which can alter a range of fundamental behaviours (i.e. feeding, sexual, aggressive and exploratory) (Boissy, 1995). It has been suggested that high levels of fear may result in a disturbed or total inhibition of activities, low levels of fear may increase the activity in which the individual was engaged and intermediate degrees of fear lead may lead to a conflict situation between the expression of fear and activity levels (Boissy, 1995).

Behavioural indicators of stress may be correlated with physiological responses and, therefore, can be used to predict the effect of stress on the biological functioning of the animal. However, using behaviour alone as an indicator of fear may be unsatisfactory as it may not necessarily always reflect the emotional state of the individual (Hinde, 1985). This may be due to the interpretation of behavioural responses to an aversive situation. (Rushen, 2000) states that the control of behavioural responses to stress are complex and to validate their use as indices of stress, the underlying causes of the behaviour, including both the motivation and neurophysiological basis of the behaviour must be understood. Additionally, physiological indicators may be more sensitive indicators of change in an individual's emotional state (Broom, 1988; Moen et al., 1978).

Neuroendocrine responses

The major neuroendocrine systems that are sensitive to environmental challenges are the sympathetic-adrenal-medullary system and the hypothalamic-pituitary-adrenocortical (hypothalamic-pituitary-adrenal axis) axis. Measurable neuroendocrine responses or biological responses to fear are linked to the responses of these two systems. Numerous studies have demonstrated the activation of the hypothalamic-pituitary-adrenal axis in which a variety of aversive events cause a release of CRH and AVP from centres in the brain stimulating the release of ACTH from the pituitary, which in turn causes activation of the adrenal cortex to release glucocorticoids. The sympathetic-adrenal-medullary system is responsible for the release of catecholamines (adrenaline and noradrenaline). Catecholamines can be adrenal in origin (released from adrenal medullar), sympathetic in origin (Dodd & Role, 1991) or released from a dense neuronal network in subcortical areas in the brain (Le Moal & Simon, 1991). Together, these systems prepare the body for an active "fight or flight" response to an acute threat through metabolic and cardiovascular adjustments. Additional endocrine systems that are altered under stressful conditions involve gonadal steroids, hypothalamo-pituitary neuropeptides (prolactin, corticotrophin releasing factor, oxytocin, and vasopressin) and another group of peptides localized in the brain, pituitary and adrenal medulla (opioids; β-endorphin and enkephins)(Boissy, 1995).

Most research concentrates on the pituitary-adrenal system. Many stimuli or conditions elicit an increase in corticosteroids and ACTH. Glucocorticoids can be measured in plasma, however the action of taking a sample often evokes a response in itself. In most species the delay before glucocorticoids are released is 2 minutes, therefore the effects of the treatment can be tested if the first sample is taken within 2 minutes (Broom & Johnson, 1993). In cattle, sampling can also be done through venous catherization or non-invasively (glucocorticoids only) in urine, faecal, saliva or milk samples (Morrow et al., 2002; Negrao et al., 2004; Verkerk et al., 1998; Palme et al., 1996). Catecholamines are more difficult to measure as they have a short half-life and are released in relatively low concentrations. Thus the activation of the autonomic nervous system can be indirectly evaluated by the effects on certain vegetative functions such as frequency of defecation, changes in cutaneous resistance or cardiovascular measure such as heart rate or blood pressure.

Relationship between behavioural and neuroendocrine responses

Behavioural patterns are intimately related to physiological and neuroendocrine responses observed in fear-eliciting situations (Boissy, 1995; Dantzer & Mormède, 1983). For example, in rhesus monkeys an individual's heart rate increases upon hearing a standard noise and is strongly correlated with behavioural indices of fearfulness and changes in cortisol concentrations, suggesting that differences could be predicted from physiological or behavioural measures (Suomi, 1983). Similarly, there is a correlation between heart rate and emotionality score in horses (McCann et al., 1988). In rats subjected to electric shock, behavioural responses are related to the increase in catacholamine concentrations (McCarty & Kopin, 1978). Social isolation of heifers induces a behavioural struggling reaction and increases in vocalization, heart rate and cortisol concentrations (Boissy & Le Neindre, 1997). Furthermore, both behavioural and cortisol responses of beef calves to separation and tethering decreases over repeated exposure (Gauly et al., 2002).

Neuroendocrine responses affecting behaviour

The correlations that are found between an animals behaviour and physiological responses to stress are not surprising considering both responses can be controlled by the same neuroendocrine system. For example, secretion of CRH from the hypothalamus after the introduction to a novel environment or intracerebroventricular (i.c.v) injection of CRH in rats are both associated with a marked increase in locomotor behaviour (a behavioural change associated with stress) and increases in blood pressure and heart rate (Morimoto et al., 1993). This suggests that the central secretion of CRH as a result of the stress elicits both physiological and behavioural responses. There is a defined neurocircuitry which underlies the effects of CRH on the hypothalamic-pituitary-adrenal axis (Herman & Cullinan, 1997; Dobson et al., 2003). However, little is known of the system by which CRH secretion is affecting behavioural responses to stress or its relation with the animal's emotional reaction (Rushen, 2000). Pituitary-adrenal hormones have been shown to modulate behaviours. Injections of dexamethasone and ACTH increase the reaction rate to a fear-producing signal (a sound previously associated with inescapable electric shocks) in pigs (Mormède & Dantzer, 1978). Gonadal steroids can influence behavioural responses of reactivity. Testosterone-treated heifers and chickens exposed to various fear-eliciting tests exhibit lower fear reactions than controls (Boissy & Bouissou, 1994; Archer, 1973b; Archer, 1973a). Opioid peptides are involved in various endocrine systems (including the hypothalamic-pituitary-adrenal axis) and underlie the occurrence of stress-induced analgesia (Przewlocki, 1993). In response to restraint, naloxone (an opioid antagonist), increases hypothalamic-pituitary-adrenal axis responses and vocalization in pigs (Rushen & Ladewig, 1991). Similarly, abnormal stereotyped behaviours of tethered sows are decreased following an single naloxone injection (Cronin et al., 1985).

Behaviour affecting neuroendocrine responses

The relationship between behaviour and neuroendocrine responses may not be just unidirectional, i.e. neuroendocrine changes produce behavioural changes. Endocrine status can be modified if the subject engages in specific behaviours (Rushen, 2000; Leshner, 1978).

In conflict or frustration situations displacement activities may be expressed (i.e. preening, fur plucking or pacing movements). These activities are irrelevant and out of context with the animals current motivational state. It is suggested that displacement activities enable subjects to cope with aversive conditions by decreasing arousal or switching attention to something other than the aversive stimulus (Hinde, 1970). In pigs, stereotyped chain-pulling during the interval between food deliveries reduces plasma concentrations of corticosteroids compared to individuals having no chain available. This suggests that the displacement activity of chain pulling is an effective means to dissipate any tension or anxiety (Dantzer & Mormède, 1981).

The emotional state of the animal can affect a neuroendocrine response. Naloxone injections in cattle normally result in an increase in cortisol concentrations, however, in stressed individuals (isolated or lame cattle) there is no effect on cortisol concentration (Rushen et al., 1999; Nanda et al., 1992). Chronically stressed cattle, induced by 3-weeks of isolation or deprivation of lying down, were considered to be frustrated and had similar baseline cortisol and ACTH concentrations, however; compared to control individuals, the stressed individuals had an increase in cortisol when exposed to a novel environment (Munksgaard & Simonsen, 1996). Additionally, rats that are given inescapable electric shocks differ in ACTH responses depending on whether they are tested in pairs or singly. Rats tested in pairs physically attack one another (known as shock-induced fighting) where as singly tested rats display freezing behaviour. Despite the acts of aggression, the physical stimulus of electric shocks in rats tested in pairs exhibit lower ACTH concentrations than those tested singly. It is suggested that the fighting behaviour is an effective way for the animals to cope with the shock situation as it results in reduced hormonal activation that would otherwise result from exposure to the physical stressor (Conner & Vernikos-Danellis, 1971).

Consequently, neuroendocrine and behavioural responses to a threat are not stereotypical responses and are intimately related to one another. Neuroendocrine responses can be influenced by psychological factors and behavioural strategies but on the other hand behavioural responses can be influenced by the neuroendocrine state of an individual.

Neuroendocrine and behaviour responses that are not correlated

Behavioural patterns and physiological responses may be influenced by different mechanisms. In rats tested in a open field experiment the duration of freezing behaviour is independent of increased heart rate (Candland et al., 1967) and corticosteroid plasma concentrations (Restrepo & Armario, 1987). In hand or dam-reared goats there is no consistent relationship between observed heart rate and changes in behaviour when exposed to a human or other goats (Lyons, 1989; Lyons & Price, 1987). Similarly in rats, i.c.v injections of a CRH antagonist will reduce the amount of time rats spend burying an electric probe (a behaviour commonly observed following an electric shock from the probe) but had no affect on the shock-induced concentrations of corticosterone or ACTH (Korte et al., 1994). Thus, although CRH may be mediating both the behavioural and hypothalamic-pituitary-adrenal axis responses, it may be acting on different populations of receptors in the brain (Korte et al., 1994). In isolated heifers, although naloxone increased the incidence of vocalization there was a limited effect on heart rate, cortisol concentration or response to a laser (used to test pain sensitivity). It was concluded that naloxone-sensitive opioid receptors were not involved in these responses. This suggests that the physiological and behavioural responses were being controlled by different neuroendocrine systems (Rushen et al., 1999).

A neuroendocrine response to stress may also affect different behaviours differently. For example, CRH may affect various fear-related behaviours differently. In rats injections of CRH over 10 days results in increased locomotor activity for 2 days. In the same study, fear of entering open arms in a maze (presumably due to a fear of falling) persisted for 7 days, thus lasting several days longer than the effect of CRH on locomotor behaviour (Buwalda et al., 1997).

Factors that affect the pattern of behavioural and neuroendocrine responses

Although a certain amount of consistency exists in an individual's emotional reactivity, fear-related behavioural and neuroendocrine responses depend on the interaction between individual reactivity (dependent on genetics, early development, experiences and neuroendocrine status) and its environment (i.e. its experimental conditions).

Individual reactivity is shaped by genetic background (species, sex, strain), experiences (early experiences and learning processes), the state of the neuroendocrine system and age. In cattle, two dairy breeds of cows selected for (Hérens) or against (Brune des Alpes) fighting and dominance ability had different reactivity to fear-eliciting novelty tests (Plusquellec & Bouissou, 2001). Futhermore, Salers and Friesians differ in their reaction (urinating, defecating and vocalization) to a novel environment (Le Neindre, 1989). Gender may also influence reactivity to a fear-eliciting stimulus. In cattle, female calves are less docile than males in their behavioural reactions to separation and restraint tests (Gauly et al., 2001). It was also suggested that this may change with age and sexual maturity as reproductive hormonal status may have an influence on behaviour (Gauly et al., 2001). Although in cattle, temperament, as a fear reaction to being touched, appears to develop at an early age (0-8 weeks) and remain stable thereafter (Kerr & Wood-Gush, 1987). However, this can be influenced by an individual's early life experiences, which lead to marked differences in adulthood emotional reactivity. Mother-reared cattle are more active than non-mothered individuals when exposed to a novel environment (Le Neindre, 1989). Early environmental influences are thought to act during sensitive periods of brain development. For example, rats and fowl that received environmental enrichment and regular positive handling in infancy are generally less reactive later in life (Jones & Waddington, 1992; Chevins, 1990). Additionally, early environment enrichment and handling have an effect on neuroanatomical development, increasing the number of hippocampal glucocorticoid receptors, which can influence both behavioural and endocrine regulation (Meaney et al., 1989; Renner & Rosenzweig, 1987). Past environmental influences also affect behavioural and physiological responses. Previous positive handling and gentle interactions with humans reduces stress reactions in cows, including a lower heart rate and less restless behaviour, during rectal palpation or sham artificial insemination (Waiblinger et al., 2004). Learning about a new situation leads to an increase in cortisol and will therefore, complicate the interpretation of the results (Rushen, 1986). Isolated-reared lambs tend not to explore a novel environment as much as mother-reared lambs (Moberg & Wood, 1982). However, there was no difference in the cortisol response. Individuals that behaved in a more inactive manner when challenged tend to have higher concentrations of glucocorticoids (Dantzer et al., 1983b). The active animals that were exploring in the study by (Moberg & Wood, 1982) were probably learning more, thus leading to in an increase in cortisol concentrations and, therefore, differences between the two groups could have been masked (Manteca & Deag, 1993c). Behavioural reactions to an aversive situation may also be affected by an individual's neuroendocrine status. Reaction to an aversive event can be influenced by the interaction between the availability of hormones and the functional properties of their receptors, both in the periphery and in the brain. Adrenalectomy decreases novelty-induced locomotion in rats (Veldhuis et al., 1982) and following a corticosterone infusion, tonic immobility (a fear-related response in hens) can be prolonged (Jones et al., 1988).

The experimental conditions such as the social context, control and predictability and properties of the stressors (size, lighting, colour, substrate, novelty and procedure) affect the emotional reactivity of an individual. Behavioural responses performed during an aversive event help the animal deal with the particular situation, thus the types of responses are often specific for a particular situation. Flight or fight behaviours can vary in intensity in relation to the extent to which an individual was disturbed (Broom &

Johnson, 1993). Similarly, behavioural responses vary dependant on whether the stimulus is either present (fear) or potential (anxiety) (Boissy, 1995; Rushen, 2000). The magnitude of a fear-related response can depend on the physical (i.e. heat, electric shock, acoustical or olfactory) and/or psychological properties (i.e. uncertainty, conflict, frustration or novelty) of the stimulus. Corticosteroid concentrations following agonistic interactions in pigs are not only dependent on the presence or absence of fighting (where fighting results in elevated concentrations of corticosteroid) but corticosteroid concentrations are more pronounced in subordinate vs. dominant individuals (Dantzer & Mormède, 1983). This suggests that the hypothalamic-pituitary-adrenal axis is sensitive to the psychological component of the stressor. In cattle exposure to acute heat or cold abruptly increases the concentration of cortisol eventually stabilizing at final levels below (hot/high temperatures) or above (cold/low temperatures) basal concentrations. Gradual exposure to changes in temperatures (hot or cold) results in a gradual changes to final concentrations. The abrupt initial change in cortisol concentrations after acute exposure reflects the emotional reaction to the situation while the gradual changes reflect the reaction to the physical properties of the stressor (heat and cold) (Dantzer & Mormède, 1983). Aspects like control and predictability play a major role in shaping the magnitude and quality of neuroendocrine responses (Wiepkema & Schouten, 1988). The degree of control that an animal can exercise over a threatening environment may also determine the pattern of the neuroendocrine response. In rodents, elevated plasma corticosterone in response to novelty ceases once the animal has free range to a familiar environment (Barnett & Cowan, 1976; Misslin & Crigrang, 1986). The predictability of an act may also reduce its impact on the neuroendocrine response.

In mice and rats, repeated exposure to novelty provokes a lower activation of the pituitary adrenal axis if the exposure is regular (Shanks et al., 1990; Muir & Pfister, 1987). Similarly, social context can alter an individual's response to stressful events ("social buffering"). Rats, fowl and monkeys exposed to a novel environment or fear-producing stimuli show stronger behavioural and physiological response when they are tested alone than when they are tested in the presence of conspecifics (Taylor, 1981; Coe et al., 1982; Jones & Merry, 1988; Hennessy et al., 1982). In heifers, the mere presence of conspecifics is sufficient to lessen the threatening effect of an unexpected event (Boissy & Le Neindre, 1990). In a review by (Dantzer & Mormède, 1983) the pituitary-adrenal response is thought more related to the emotional state and/or perception by the subject than the physical properties of the stressful situation. Exposure of monkeys to treatments such as heat, cold, fasting or exercise do not activate the pituitary-adrenal axis if emotional arousal is avoided (Mason, 1971).

Observers ratings

In addition to physiological and behavioural responses, fearfulness, as a personality or temperament trait, may also be assessed using observer's ratings. Observer rating assessment is widely used in the study of animal temperament and personality in a variety of species including domestic cats (Fever et al., 1986), goats (Lyons, 1989), donkeys (French, 1991), pigs (Wemelsfelder et al., 2001), rhesus monkeys (Stevenson-Hinde et al., 1980), brown bears (Fagen & Fagen, 1996), cheetahs (Wielebnowski, 1999) and black rhinos (Carlstead et al., 1999).
Ratings are a retrospective qualitative assessment of behaviour providing useful information about subtle aspects of an individual's behaviour describing an overall behavioural 'style' or emotional 'tone'. The ratings are based on the integration of several pieces of information by an observer over long periods of time, including incidental behavioural events, subtle details of movement and posture, and the context in which behaviours occur. In general, observer ratings do not replace direct recording methods, however they do provide useful information about subtle aspects of an individual's personality or style that cannot be easily obtained using standard behavioural recording methods. In fact, observer ratings may provide a higher level of description, as they capture the overall pattern of an individual's behaviour in a wide variety of situations (Martin & Bateson, 1986). Thus they measure characteristics like 'tense' or 'aggressive' that could not be assessed using conventional recording methods (Manteca & Deag, 1993a; Wemelsfelder et al., 2001).

In most studies, the observer ratings use a form, which lists all the individuals to be assessed, with a linear scale drawn for each individual animal. One form is used for each behavioural 'component' to be measured. A cross is marked on each scale at a position corresponding to the observers overall assessment of how much the individual expressed that item. The distance to the mark on the scale is then used as the respective numerical score for that individual. Observer ratings require two or more observers to measure reliability to ensure measurements are repeatable and consistent and they both must have detailed knowledge of the subjects (Martin & Bateson, 1986). Ratings are generally validated by comparing data with quantitative scores based on the direct observation of behaviour or the results of behaviour tests (i.e. fear-eliciting novelty tests)(Stevenson-Hinde et al., 1980; Fever et al., 1986; Lyons, 1989; Wielebnowski, 1999).

Fear and stress related diseases

The study of emotional reactivity or fearfulness is one approach to investigate the processes that underlie the variability in vulnerability to stress and stress-related diseases. It is of considerable benefit should a set of fearfulness traits be identified that predict the likelihood of developing stress and stress-related pathologies (Boissy, 1995). Studies suggest that the development of stress-related diseases can be influenced by the susceptibility to be more easily frightened. For example, in poultry and rats, the more intense the fear reactions are, the greater the psychobiological consequences for a chronic stressor (Jones, 1989; Tejedor del Real et al., 1991).

Methods of Determining Social Relationships in Cattle

Social interactions

Social interactions can be divided into agonistic (aggressive acts) and non-agonistic (allogrooming and sexual behaviour (Bouissou et al., 2001). The dominance-submissive relationships are formed though agonistic encounters between individuals. Most agonistic encounters occur in the sequence; approach, threat, and physical contact (or fighting) resulting in a win/lose situation. The approach can be either deliberate, where a cow actively seeks out another individual, or passive such as in a chance meeting. The threat can be non-physical when there is spontaneous withdrawal of the subordinate (lose) without any visible signs of agonistic behaviour from the dominant individual (win). If a threatened animal is slow to submit or fails to notice the threat it becomes physical resulting in a butt or forceful pushing (win) when one animal lowers it head and turns away (lose). The threat may also be unsuccessful in producing a submissive response, which may lead to fighting. However, fights are rare and relationships are generally maintained from threats and avoidance reactions without physical contact.

Social group

Ancestral cattle (*Bos primigenius*) lived in social units and the composition of these groups changed with the seasons. Cows and calves formed groups of ~20-30 animals, based on maternal relationship and mature males were either solitary or in groups of ~2-10, only joining the females for the mating season (Buchenauer, 1999; Bouissou et al., 2001). Different breeds of feral-living domestic cattle (*Bos taurus* and *Bos indicus*) show similar social structures (Buchenauer, 1999; Daycard, 1990; Hall, 1986).

The social group of domesticated cattle varies. In developing countries cattlemen drive the herds looking for food, guarding the cattle as they move. In the modern western dairy industry, calves are isolated from their mothers soon after birth (within 1-3 days). The young females are artificially reared together in small groups until they join the rest of the herd after calving. Young males are used as veal calves or are raised in feedlots. Groups of cows are often assembled based on production and physiological status (dry, lactating); males are not usually free to be with the herd. In suckling herds, cows will raise their calves until 4-9 months of age; graze with their young during the summer months and usually there is one bull running with the herd. When males are not used for breeding they are gathered into feedlots or fattening units.

Dominance hierarchy

The social system of cattle is characterized by a dominance hierarchy. In free-range herds of cattle, adult males dominate all adult females, who in turn dominate all juveniles. However, at ~ 1 ½ years young males begin to move up the ranks, fighting with adult females, by 2 ½ years they dominate all females and then join the adult male rank order (Schloeth, 1961).

The structure of a dominance hierarchy is linear, linear-tending or complex. In general, the smaller the group and/or the more heterogeneous (age, weight and sex) the group the simpler the hierarchy (i.e. linear, linear-tending) (Hafez & Bouissou, 1975). For example, small groups (under 10) are usually linear whereas in groups of ~50, no single individual is dominant over all other members of the herd, suggesting a complex hierarchy as no cow is completely free of inhibition from at least one other herd member (Beilharz & Zeeb, 1982). In cattle under 'natural' conditions, there is a clear relationship between age and dominance and a more linear order is often observed. Whereas in managed conditions, where individuals are often re-grouped based on parameters of physiology and/or production, this relationship may be disturbed resulting in what are termed triangular relationships. In this case, a normally dominant cow may become subordinate to a young heifer she meets for the first time yet she remains dominant over many other cows with whom she has previously been grouped (Wierenga, 1990). This ultimately results in a complex hierarchy formation. Based on feral herds, bulls tend to have a more strictly linear hierarchy than cows, however, this relationship is less stable from year to year (Hall et al., 1988; Bouissou et al., 2001).

In wild ungulates, social organization is a based on a matriarchal group, juveniles are integrated into the hierarchy based on their age and mother's social position (Bouissou et al., 2001). In domestic cattle, where the animal's natural way of living is modified, the social status of the young does not appear to be determined by the mother (Bouissou, 1985; Le Neindre, 1984). The development of dominance/submissive relationships are learned through agonistic interactions and are dependent on the social context the calf was raised. For example, mother-reared calves establish dominance relationships earlier (4-5 months) than artificially-reared calves (9-10 months; (Hafez & Bouissou, 1975; Bouissou, 1985).

In general, it is accepted that cattle quickly establish social hierarchies. In feral conditions, new individuals are rarely introduced; however, in a commercial setting new individuals are frequently added to a group. When a strange individual is introduced, true fighting rarely occurs; rather subtle gestures (movements of the head or threat posture) are sufficient to establish positioning in the hierarchy (Bouissou, 1974). Mixing and re-grouping cattle results in a tenfold increase in agonistic interactions during the hours following mixing (Bouissou et al., 2001). It is estimated that within 10 minutes, 66% of dominance-submissive relationships are established between pairs and within the first hour 94% of relationships are established (Hafez & Bouissou, 1975). (Brakel & Leis, 1976) noted that, within the first hour after re-grouping, aggressive encounters were double the number occurring 2 days after re-grouping. Similarly, (Kondo & Hurnik, 1990) noted that the greatest number of encounters occur within the 2-hour period after assembling a 'new' group of individuals.

Prior social experience in meeting strangers, leads to a reduction in time required to establish dominance. Experienced animals are quicker to acknowledge the superiority of others after the first bunt or threat; therefore, dominance is more rapidly established and more often without fighting in socially experienced individuals. Additionally, relationships are more stable between experienced animals as compared to those between inexperienced animals (Bouissou, 1975).

As with feral cattle, dominance relationships between domesticated adult females are known to be extremely stable, persisting for several years. Although, changes in rank have been observed. This usually occurs with frontal head to head fighting lasting for several minutes (Wierenga, 1990). Changes in rank, or rank reversals between individuals are rare (less than 10 %) and are often difficult to explain (Beilharz & Mylrea, 1963; Reinhardt & Reinhardt, 1975; Sambraus, 1975).

Although, relationships between individuals are relatively stable, an individuals rank in the social hierarchy may shift dependant on group composition. An animal may have to re-test their relative individual dominance in a newly formed group. When a stranger is introduced to an established group, its new rank may not be related to its rank in a previous group (Hafez & Bouissou, 1975). Strangers introduced to a new group generally acquire a relatively low rank, regardless of age or weight (Schein & Fohrman, 1955; Sambraus, 1969). Strangers will place themselves in the hierarchy without disturbing the already established dominance relationships that exist between other members in the group (Schein & Fohrman, 1955).

Factors that influence social relationships

There are several factors that influence dominance relationships in cattle such as recognition, sex, age, genetics, fear, emotionality and physical characteristics.

The foundation of social hierarchies is the mutual recognition between individuals. It is thought that a cow can recognize 50 - 70 other individuals (Sambraus, 1978; Fraser & Broom, 1997). When group size becomes too large, individuals may have difficulty memorizing the social status of peers resulting in an increase in aggressive interactions (Stricklin et al., 1980a; Hurnik, 1982). Recognition allows for preferential relationships in groups of cattle. For example, heifers reared together engage in more non-agonistic interactions, are less aggressive among themselves, remain close together during feeding and resting and are more tolerant in a food-competitive situation than with heifers they were not reared with (Bouissou & Hövels, 1976). Additionally, there is a preference for particular allogrooming partners, which are often animals of similar age, rank, familiarity or are related (Bouissou, 1985; Reinhardt, 1981; Sato et al., 1993; Wood, 1977). Allogrooming is thought to have a calming function as it lowers heart rate (Sato & Kuroda, 1993). These preferential relationships ultimately influence status in the social hierarchy.

Early experience influences social rank in adulthood. For example, group-reared calves are dominant over individually-reared and isolated calves (Warnick et al., 1977; Broom & Leaver, 1978). Foster-reared Saler heifers are more dominant than bucket-reared heifers (Le Neindre & Sourd, 1984).

Once a dominance-submissive relationship is formed between individuals, as a result of learning and experience, the relationship persists over long periods of time (Beilharz & Zeeb, 1982; Sambraus & Osterkorn, 1973). The relationship endures regardless of physical changes. For example, the presence of horns is important during the establishment period of the hierarchy (Bouissou, 1972) but once the relationships are stable, dehorning does not modify social status (Hafez & Bouissou, 1975).

Investigators have reported positive correlations between dominance and body measures (Guhl & Atkeson, 1959; Beilharz et al., 1966; Dickson et al., 1970; Schein & Fohrman, 1955; Reinhardt & Reinhardt, 1975; Alexander et al., 1984). However, (Sambraus & Osterkorn, 1973) noted that, although rank was correlated with body weight, it was more so with age. Older cows, which were no longer superior in strength to some younger cows, maintain status by threat alone, no physical strength for fighting was needed. Therefore, rank is maintained by factors other than body weight alone. Additionally, correlations observed between rank and weight may simply be a consequence of dominance rank.

Dominance is correlated with age (Schein & Fohrman, 1955; Dickson et al., 1967; Sambraus & Osterkorn, 1973). Individuals can gain in dominance up to ages ~ 8-10 after which they may show a progressive decline (Reinhardt & Reinhardt, 1975; Beilharz & Zeeb, 1982) or remain at this level. (Sambraus, 1975). It should be noted that age is associated with factors such as seniority, weight and experience, which could be influencing rank.

Generally, dominance relationships are maintained by non-physical threats; therefore suggesting senses such as vision and olfaction are important in the maintenance of relationships. However, when animals were blindfolded in a competitive food situation dominance was still expressed in these non-visual conditions (Bouissou, 1971). Furthermore, 80% of dominance relationships are maintained in anosmic blindfolded cows (Mansard & Bouissou, 1980). However, physical separation with visual contact eliminates dominant/submissive behaviours (Bouissou, 1971).

Hormones can influence dominant status. Long-term (100 days) androgen treatment in heifers, 3 to 6 months of age, were dominat to non-treated controls when tested 3-5 months later (Bouissou & Gaudioso, 1982). Treatment with oestradiol benzoate over 90 or 180 days, completely changed the social order of established herds (Bouissou, 1978; Bouissou, 1990). Treated animals become dominant over non-treated individuals. Rank reversals occurred between 22-44 days or 24-155 days post treatment with exogenous androgen or oestradiol, respectively (Bouissou, 1990; Bouissou & Gaudioso, 1982). The rank reversals were long lasting as they persisted at least 3 months after the cessation of treatment (Bouissou, 1990). Interesting, changes in rank are not a result of increased aggressiveness but rather a reduction in fear levels explained by a lower reactivity to aggression from others (Bouissou, 1990). Neither oestrus, ovariectomy or pregnancy modifies rank (Bouissou, 1985). Cows in oestrus will, however, temporarily ignore rank position and approach superiors in the hierarchy despite being repeatedly threatened or chased (Hurnik et al., 1975; Esslemont et al., 1980; Bouissou, 1985; Schein & Fohrman, 1955; Wagnon, 1965), possibly through a reduction in fear. This indiscriminate behaviour does not, however, modify dominance relationships between individuals (Bouissou, 1990).

There is a strong genetic base for dominance. Monozygous twins, reared under different conditions (isolation or group-rearing) had very similar dominance ranks (correlation of 0.93) when transferred to large groups of heifers (Purcell & Arave, 1991). Additionally, exchanging identical twins between two groups does not modify the groups social structures (Purcell & Arave, 1991). Additionally, dominance differences exist among breeds. For example, (Wagnon et al., 1966) demonstrated that Angus tend to dominate Shorthorns, which in turn dominate Herefords. Similarly, in a mixed herd of Angus and Hereford cows, Angus cows were dominant to Hereford cows (Stricklin et al., 1980b). (Brakel & Leis, 1976) who introduced cows into established herds of different breeds found that Brown Swiss were more dominant to Holstein that were more dominant to Guernsey that were more dominant to Jersey cows.

Temperament, including emotional reactivity or fearfulness, is one of the most important factors in determining social position (Bouissou et al., 2001; Plusquellec et al., 2001). Fear-eliciting tests in heifers demonstrate that the less fearful an individual was at 6 or 18 months the more dominant they become at the age of 30 months, thus demonstrating the importance of fear in dominance relationships in cattle. When anosmic, blindfolded and unfamiliar cows first meet; some animals consistently withdraw as soon as they sense the presence of another individual, thus becoming subordinate. This suggests that some individuals are innately fearful and this plays a large role in the establishment of dominance relationships (Bouissou, 1985). Moreover, in a series of controlled experiments, the ability to achieve dominance was the result of a lower fear of conspecifics, as well as a lower general reactivity (Bouissou, 1978; Boissy & Bouissou, 1994; Bouissou & Gaudioso, 1982; Bouissou, 1990).

Leadership is the ability of an animal to influence movements and activities of herd-mates. Leaders may react faster than others to environmental change stimulating the movement of others. Interesting, leadership shows little correlation with dominance

(McPhee et al., 1964; Dietrich et al., 1965; Syme & Syme, 1979; Reinhardt, 1983). Individual differences in fearfulness may be important in determining leadership, with the most confident individuals leading the group (Reinhardt, 1983; Manteca & Deag, 1993b). During voluntary group movements, middle ranking cows are at the front, then high-ranking cows and low-ranking cows follow in the rear (Arave & Albright, 1981; Kilgour & Scott, 1959). However, during forced movement (i.e. into the parlour) a positive correlation between dominance and milking order has been observed, when more dominant cows enter the parlour first (Phillips & Rind, 2002; Dickson et al., 1970; Reinhardt, 1973; Soffie et al., 1976). Contrary to this, (Beilharz & Mylrea, 1963) state that during forced movement subordinate cows are in front.

Determining social relationships

Dominance can be defined as the behaviour of one individual inhibiting the behaviour of another, the social hierarchy of a group and/or rank of an individual encompasses all such relationships (Beilharz & Zeeb, 1982; Albright & Arave, 1997). Dominance relationships in cattle have been studied extensively and various methods have been proposed to assess dominance (i.e. a dominance value; DV). The calculation of a DV is valuable as it summarizes the position of an animal in a group as a single parameter and enables correlations to be made between DV and other factors (i.e. age, weight (Boyd & Silk, 1983). The DV is also helpful when the dominance structure is complex. This technique gives each individual a DV and indicates a semi-quantitative difference between individuals.

Measuring dominance often involves recording agonistic encounters as a win or lose situation (Beilharz & Mylrea, 1963). From these interactions, there are several different methods that have been used to calculate DV's; however, they can be classed into two categories; 1) DV's that are based on the absolute number of animals another dominates or 2) DV's that are based on numbers of encounters.

DV calculations based on absolute numbers of animals dominated focuses on the 'dominance relationship' between pairs of animals. There is a positive correlation between aggression (measured by the number of agonistic acts) and relative numbers of animals subordinate to a dominant animal (Reinhardt & Reinhardt, 1975; Wagnon, 1965; Wierenga, 1990). Therefore, DV can be calculated as the ratio of the number of subordinate animals to the total number of dominant relationships of that animal (Sambraus, 1975; Wierenga, 1990; Reinhardt & Reinhardt, 1975; Hurnik et al., 1975). It represents the (relative) number of animals that are subordinate to that animal. The DV itself gives no information about the total number of displacements that an animal enters only the 'final result' (Wierenga, 1990). The 'dominance relationship' is analysed for each pair of cows based on the recorded number of displacements (agonistic interactions). The dominant cow in the pair is the cow that 'displaces' the other cow. A dominance relationship can be classified as 'known' if one cow displaces another cow at least twice as frequently (Sambraus, 1975). To calculate DV's using this methodology all dominance relationships between pairs in the group should be known.

Generally speaking DV calculations that rely on numbers of encounters consider factors such as 1) number of displacements (wins) by an individual, 2) number of times that individual is displaced (lose), 3) total number of encounters (wins and loses) by that individual, 4) difference between the number of wins and loses of different individuals, 5) difference in the total number of encounters of different individuals and/or, 6) total number of encounters of the whole group. There have been several different methods of calculating DV based on encounters. For example DV can be calculated as the average proportion of wins a cow has with all cows she interacts with, it can be adjusted for the number of comparison animals, or the DV can be based on function (least-squared) differences between pairs of cows (Beilharz et al., 1966; Kaiser, 1959). Another example is to calculate the DV based on the sum of interactions initiated by the observed cow with the sum of all interactions initiated by another animal to which the observed cow was exposed (Beilharz & Zeeb, 1982; Bartos, 1986), also adjustments for unequal numbers of encounters can be calculated (Friend & Polan, 1978; Friend et al., 1977). The DV can also be calculated as a ratio of encounters won by an individual to total number of encounters (Arave & Albright, 1975). (Galindo & Broom, 2000) calculated the DV (termed 'indices of displacement') as the number of times a cow displaces other individuals over the number of times she displaces another cow plus the number of times she is displaced. Another method reported by (Orihuela & Galina, 1997) based DV (termed 'social index') on paired comparisons (Donaldson, 1967; Donaldson, 1970; Kaiser, 1959) in which the total number of encounters won by an individual is divided by the total number of encounters recorded for the whole herd.

Often once a DV has been calculated, individuals can be ranked (i.e. 1 to 20 in a herd of 20 animals) and the social hierarchy can be determined. To achieve this every member of the group must, therefore, be considered to construct the social hierarchy. Alternatively, the social hierarchy can be constructed by recording animal interactions in two-way tables with wins horizontally and losses vertically. Rows can be rearranged (minimizing the numbers in marked cells left of the diagonal) to give a straight-line estimate of 'rank' order (Schein & Fohrman, 1955). It should be noted that although informative, rank should be used with caution. Rank does not take into consideration the relative difference between DV of two individuals making it difficult to use parametrical statistical techniques to relate dominance to other factors of interest (Boyd & Silk, 1983). Recording interactions in a two-way table as proposed by (Schein & Fohrman, 1955) is an easy method for tallying the outcome of encounters. This can then be used to calculate individual DV's and/or rank and determine the social hierarchy of the group (Arave & Albright, 1975; Kondo & Hurnik, 1990; Orihuela & Galina, 1997; Wierenga, 1990). However, all relationships in a group must be recorded which may prove difficult in large herds or in experimental situations where subject individuals are maintained within a large herd. Therefore, DV's based on numbers of interactions allows for focal sampling on an individual. DV can be calculated on an individual basis, based on their interactions with other members of the group (Galindo & Broom, 2000; Arave & Albright, 1975).

It is worth noting that dominance relationships may not always be unilateral (Wierenga, 1990). In 41% of pairs both members displace each other; these are termed contradictory displacements. In 99% of these cases, the number of times both animals displaced each other differed (i.e. one animal had provoked at least one more displacement). It was concluded that one pair-member usually provoked considerably more displacements (three or more) while the other pair-member provoked few or none. Therefore, the pair-member who provoked the higher number of displacements was considered to be the dominant animal. Some authors accept the existence of contradictory displacements (Reinhardt & Reinhardt, 1975; Wagnon, 1965; Sambraus, 1969) while others consider these displacements to be mistakes and suggest a method to correct the DV (Beilharz & Zeeb, 1982). (Wierenga, 1990) demonstrated that, despite contradictory displacements, dominance relationships remain stable over a number of years suggesting that they are not random occurrences. Secondly, although ~40 % of pairs of individuals will show contradictory displacements, the actual number of displacements only represents 1-4% of the total number of interactions observed within the group (Sambraus, 1969; Wagnon, 1965; Wierenga, 1990). Lastly, the subordinate animal performed few displacements than the dominant animal, again suggesting that this is not random. This is also seen in other species where the subordinate animal sometimes displaces the dominate one but the dominates are responsibly for 90% of all aggressive acts initiated (Jackson, 1988).

Agonistic interactions or dominance can be observed in 'non-competitive' situations (i.e. free movement in a pasture, *ad libitum* access to food and water). However, an alternative method for summarizing social relationships can be carried out in competitive situations, decreasing the observation time required to determine DV's. (Mülleder et al., 2003) demonstrated a ~4 fold increase in the number of interactions recorded during a competition test as compared with an undisturbed situation. Successful competition can be defined as the demonstration of priority of access (e.g. food, water, lying places). This can be measured by observing behavioural interactions where resources are limited (at food or water troughs) or where two animals cannot have access to the resource simultaneously (food in a small bucket) (Andersson, 1987; Landeata-Hernandez et al., 2002). The latter is termed 'competitive orders' and has been criticized by some, stating that the competitive order does not always reflect dominance relationships (Craig, 1986; Syme, 1974). Others have shown that a strong correlation relationship exists between dominance values and a priority of access to food (Beilharz & Zeeb, 1982; Albright & Arave, 1997; Sereni & Bouissou, 1978).

Alternatively, agonistic acts can be monitored where resources are limited (i.e. at a food trough). Control of the resource itself is not being measured but rather the agonistic interactions that the situation elicits. These interactions are based on aggression. Aggression (i.e. fighting or threatening) leads to non-aggressive inhibition of behaviour (i.e. dominance (Albright & Arave, 1997). Competition at the feed troughs can be related to dominance, as dominant cows tend to spend more time eating than cows of a lower rank (Bouissou, 1970a; Friend et al., 1977; Friend & Polan, 1974; Manson & Appleby, 1990; Mülleder et al., 2003; Olofsson, 1999). Additionally, competition increases, by limiting feeding space, there is a increase in the correlation between intake and dominance value (Friend et al., 1977; Bouissou, 1985). Furthermore, there is a relationship between positioning and dominance at the feed trough, the greater the difference in rank the further apart individuals place themselves (Manson & Appleby, 1990). Therefore, competitive agonistic interactions at a feed trough can be correlated to dominance values in cattle (Bouissou, 1970b).

Methods of recording

There is large variation in the literature concerning the amount of time or frequency required to assess dominance; DV are based on total times and frequencies ranging from 6 - 2000 hours of observation and 25 minutes per day to 24 h continuous recording, respectively (Table. 5). Additionally, observations were recorded over varying periods; 7 days up to 150 days.

Table 5. Range of observation times and frequency used to assess dominance in cattle

Total observation time	**Frequency**	**Period of time observations made**	**Authors**
6 h	1 h ~3 times per week	14 days	(Arave & Albright, 1975)
8 h	~2 h per day	32 days	(Kondo & Hurnik, 1990)*
20 h	1 h per day for 20 days	~ 30 days	(Bouissou, 1990)*
32 h	4 h every day	8 days	(Galindo & Broom, 2002)
48 h	Continuous 24h periods	2 days	(Olofsson, 1999)
96h	3 h every day	32 days	(Phillips & Rind, 2002)
168 h	Continuous 24h periods	7 days	(Kabuga et al., 1992)
193 h	6 Continuous 24h periods with random observations	~100 days	(Wierenga, 1990) *
208 h	6 h per day	~ 90 days	(Mülleder et al., 2003)
375 h	~ 2h every other day	~150 days	(Orihuela & Galina, 1997)
2000 h	25 mins per day	80 days	(Hurnik et al., 1975)

* Minimum amount of time recorded

Additionally, there are no guidelines as to how many interactions must be recorded within a pair and whether every individual must interact with every other animal in the group to give an accurate estimation of DV. Based on results from (Wierenga, 1990), in a group of 20 individuals, every pair of cows would have to interact twice to give a reliable estimate of DV (Albright & Arave, 1997). Additionally, (Hafez & Bouissou, 1975) suggested that to adequately determine dominance in a group of individuals, it is essential to consider the relationship of each animal to every other animal in that group, as dominance relationships in a large herd may be broken down into one or more groups of individuals, and any individual may simultaneously be a member of one or more of the groups. However, (Bernstein, 1981) suggested that in large herds, in which smaller groups exist, individuals may not know their dominance relationship with every member of the group and therefore they have no overall 'picture' of ktheir social rank, making it difficult to determine the hierarchy. However, in a group of 41 heifers (with a possibility of 820 interactions), (Beilharz & Mylrea, 1963) arbitrarily set the minimum number of encounters to 10 per animal (equates to 410 interactions) and found that an individual's DV estimated during a one-day observation period was highly correlated (> 0.9) with cumulative DV over a 4-day period. Therefore to calculate a reliable and repeatable DV, every individual does not need to interact with every other individual in the group. Additionally, the probability of achieving the goal of every individual interacting with every other individual in a reasonably short time is low unless the group is very small (Albright & Arave, 1997; Beilharz & Mylrea, 1963).

Aims of the Thesis

Aims of the thesis

Lameness is a painful and stressful condition and is therefore a welfare issue for cattle (Greenough et al., 1997). Additionally, lameness has important economic implications for the dairy industry, as lameness is associated with poor reproductive performance (Collick et al., 1989; Ward, 2001). Poor reproduction performance could be related to lame cows expressing a less intense oestrus and therefore decreasing the chances of detection, resulting in a missed or poorly timed insemination and decreased reproductive efficiency. *Therefore one of the aims of the present study was to examine the relationship between lameness and intensity of various behavioural signs of oestrus and the incidence of oestrus on a commercial dairy farm.*

The mechanisms that control oestrous expression are not yet completely understood; however, it is widely accepted that oestradiol and progesterone both play a role in oestrous expression in dairy cows (Allrich, 1994). Additionally, stress effects reproduction (Dobson & Smith, 2000; Liptrap, 1993). The chronic pain and stress associated with lameness may impair proper endocrine function having detrimental effects on ovarian cyclicity and oestrous expression (Dobson & Alam, 1987; Li & Wagner, 1983; Dobson et al., 2003). Activation of the hypothalamic-pituitary-adrenal axis by stressors reduces the pulsatility of gonadotrophins ultimately depriving the ovarian follicle of adequate support (Dobson & Smith, 2000) and could thus disrupt the normal hormone milieu required for oestrus expression. *Therefore, a second aim of the present study was to elucidate progesterone, oestradiol and cortisol profiles that accompany unobserved and observed oestrus in the postpartum period in relation to lameness.*

There are many other physiological, psychological and environmental factors that influence the intensity of oestrus expression (Orihuela, 2000). In general, cows in oestrus are restless and spend less time lying down (Singh et al., 1994; Roelofs et al., 2005a). This could affect feeding strategies, nutrition and body condition. Nutrition and reproduction are interrelated and the loss of body fat or negative energy balance results in poor reproductive efficiency (Lucy, 2003; Ferguson, 2005). Negative energy balance affects gonadotrophin pulsatility and/or ovarian steroid synthesis (Butler, 2000), both of which could have a negative impact on oestrous expression (Caraty et al., 2002). Additionally, some studies suggest a relationship between oestrus expression and social dominance in cattle, when higher ranking individuals initiate more mounts and monopolise oestrous cows, preventing other cows from mounting (Hurnik et al., 1975; Wagnon et al., 1966; Kabuga et al., 1992; Thomas & Dobson, 1989). *Therefore, a third aim of the present study was to determine what impact lameness has on social* *behaviours, daily feeding and activity budgets and body condition in relation to oestrous expression.*

Chronic stress and pain is also often associated with disturbances of the hypothalamic-pituitary-adrenal axis, resulting in both functional and structural modifications and long-term alterations in neuroendocrine reactions to subsequent stressors (Ostrander et al., 2006; Ulrich-Lai et al., 2006b). Therefore, it also possible that lame cows do not cope as well as nonlame cows with daily stressors (i.e. re-grouping based on milk yield, social isolation, physical restraint, introduction of new herd-mates or new routines). All of these have the potential to induce fear and activate the hypothalamic-pituitary-adrenal axis in cattle (Boissy, 1995; Hopster & Blokhuis, 1994; Grignard et al., 2001). Understanding how an individual copes with a stressful situation and what factors determine its coping strategies is important as it can lead to improve welfare and reproductive efficiency. *Therefore, the final aim of the present study was to determine if lame cows differed in their behavioural and hormonal coping style to an acute stressor and to investigate whether predictability and control are behavioural character traits that are associated with the adrenal response to an acute stressor in lame cows.*

Chapter 1

Chapter 1

Lameness, oestrus and milk hormone profiles in dairy cattle

Walker SL, Smith RF, Jones DN, Routly, JE and Dobson H

Key words: Lameness, oestrus intensity, behavioural oestrous signs, milk, progesterone, oestradiol, cortisol, postpartum cyclicity, dairy cow

Abstract

Lameness decreases fertility in dairy cows. The objectives of the present study are to determine if lameness affects the expression of oestrus and/or daily profiles of progesterone, oestradiol or cortisol in milk prior to oestrus.

Cows (n=44) more than 20 days postpartum were scored every 2 weeks for lameness [nonlame (LS 1), moderately (LS 2) or severely lame (LS 3)] and observed for oestrus using a weighted summative scoring system (n=36 cows > 100 points = oestrus/Day 0). Clinical treatments for lameness and fertility continued as usual.

Pairwise comparisons revealed that severely lame cows expressed oestrus with lower intensity (284 ± 128 points, n=9) compared to moderately (662 ± 310 points, n=9) or nonlame animals (583 ± 275 points, n=18; p=0.050 and p=0.017, respectively). Severely lame cows tended to exhibit less total mounting activity (mounting the rear and head of other cows plus standing to be mounted; LS 1=15.0 ± 0.7; LS 2=6.9 ± 1.1; LS 3=2.4 ± 0.5; p=0.077) and sniffed the vulva of other cows less than nonlame cows (LS 1=12.9 ± 1.9; LS 2=9.3 ± 1.8; LS 3=6.0 ± 1.0; p=0.031). Results were not affected by clinical fertility treatments.

Increasing lameness score was not associated with differences in profiles of progesterone (Day –18 to 0), oestradiol (Day –6 to 2) or cortisol (Day -18 to 2) around oestrus; maximum oestradiol values (1.8 ± 0.3, 1.8 ± 0.3, 1.9 ± 0.3 pg/ml milk, respectively); or oestradiol concentrations on Day 0 (1.3 ± 0.2, 1.5 ± 0.4, 1.4 ± 0.2 pg/ml milk, respectively; p>0.05). Furthermore, the duration of low progesterone values prior to observed oestrus and prior to maximum oestradiol did not differ with lameness score (p=0.669, p=0.934, respectively). Based on progesterone profiles, the duration of luteal phases, inter-luteal intervals and ovarian cycles were comparable for all lameness scores (p=0.970, p=0.878, p=0.993, respectively). However, post-hoc pairwise comparisons revealed that prior to the first observed oestrus postpartum, severely lame cows had lower

maximum progesterone concentrations compared to nonlame cows (1.3 ± 0.1, 1.2 ± 0.2, 0.7 ± 0.1 ng/ml milk; p=0.042). Results were not affected by clinical fertility treatments.

Irrespective of lameness status, progesterone profiles and cortisol concentrations did not differ between cows seen in oestrus and those not seen in oestrus. Oestradiol concentrations tended to be higher for 4 days following the first detected basal progesterone value for those cows not seen in heat compared to those seen in heat (p<0.10). Resting concentrations of cortisol (Day 20 to 80 postpartum) did not vary between days postpartum or lameness score (nonlame 2.0 ± 0.1, n=22; moderately lame 2.4 ± 0.1, n=11; severely lame 2.3 ± 0.1, n=11). The incidence of oestrus was not affected by lameness as there were 13/24, 10/17 and 7/12 periods of low progesterone that were associated with oestrus expression in nonlame, moderately and severely lame cows, respectively. Additionally, lameness did not affect the number of days postpartum when oestrus was first observed or the number of days postpartum to commencement of luteal activity.

In conclusion, lameness did not affect the incidence of oestrus but reduced the intensity after ovarian cyclicity has began postpartum, the reduced intensity of oestrus in severely lame cows was associated with lower maximum progesterone values prior to oestrus but not abnormal cortisol or oestradiol values.

Introduction

Lameness is associated with poor reproductive performance in dairy cows (Hernandez et al., 2001; Collick et al., 1989; Melendez et al., 2003; Lucey et al., 1986). Furthermore, lameness is a welfare issue for cattle and has important economic implications for the dairy industry, as lame cows take longer to become pregnant (Hernandez et al., 2005; Ward, 2001).

Based on progesterone profiles, lame cows take longer to commence ovarian cyclicity and the first ovulatory oestrus is later in lame cows (Petersson et al., 2006; Garbarino et al., 2004). Lame cows also require more inseminations per pregnancy and have a lower pregnancy rate to first insemination (2.14 *versus* 1.72 and 46% *versus* 56%, respectively (Collick et al., 1989). Many other periparturient diseases are also associated with poor fertility (Borsberry & Dobson, 1989; Fourichon et al., 1999; Huszenicza et al., 2005). Indeed, if cows have mastitis around the time of the first 'silent' oestrus (15-28 days postpartum), both first luteal activity and first oestrus occur later (38.6 *versus* 32 days and 91 *versus* 84 days, respectively; Huszenicza et al., 2005). Cows with mastitis also have smaller follicles than healthy herd-mates (GM Lloyd, *pers. comm.*), which could possibly affect ovarian hormone concentrations. Therefore, it may not just be the physical mechanical limitations of lameness that result in poor fertility but lameness itself may have an impact on ovarian function, compromising the normal hormonal milieu and altering oestrous behaviour.

Oestradiol and progesterone both play a role during oestrous expression in dairy cows (Allrich, 1994). Oestradiol is essential for oestrous expression and frequent oestradiol sampling with intense monitoring of behavioural signs reveals a positive relationship between the intensity of oestrus and maximum oestradiol concentrations suggesting a dose-dependant relationship (Lyimo et al., 2000; Roelofs et al., 2004). Prior to oestrus, progesterone exposure also enhances the activity of oestradiol (Allrich, 1994; Stevenson et al., 1989; Melampy et al., 1957; Carrick & Shelton, 1969; Bell et al., 1983). In the ewe, prior progesterone priming is not only essential for the display of oestrus but increases the intensity of oestrous expression (Karsch et al., 1980; Fabre-Nys & Martin, 1991a; Fabre-Nys & Martin, 1991b). Furthermore, the chronic pain and stress associated with lameness may impair proper endocrine function having detrimental effects on ovarian cyclicity and oestrous behaviour (Dobson & Alam, 1987; Li & Wagner, 1983; Dobson et al., 2003).

The aims of the present study are to investigate the association between severity of lameness and the incidence/intensity of behavioural oestrus in dairy cows, and examine progesterone, oestradiol and cortisol profiles in milk surrounding unobserved and observed oestrus in the post-partum period.

Materials and Methods

Experimental design, animals, feeding and housing

The study was conducted on postpartum Holstein-Friesian cows (n = 44) on a UK commercial dairy farm comprising a total of ~200 year-round calving cows. The average rolling milk yield per cow in the herd was 8300 litres/year. Cows randomly entered into the study at Day 20 postpartum; cows were part of a parallel study (Chapter 4) and at any one time only 12 cows were included in the study. The parity of the study cows was 3.6 ± 0.2 (range 1-7). Clinical treatments for lameness and fertility were recorded and continued as usual. Animals were presented at weekly routine fertility visits if the farmer considered they were due to be inseminated but had not been seen in oestrus. Treatment comprised either of 1) a single 500µg injection of a prostaglandin F2α (PG) analogue (cloprostenol, 2ml, Estrumate® Schering-Plough Animal Health, Uxbridge UK) or 2) EAZI-Breed™ CIDR® device (Animal Reproduction Technologies Ltd., Leominster, UK) inserted intravaginally for 8 days with or without a single 500µg cloprostenol injection 7 days later were administered as deemed appropriate by the attending vet.

Individuals were monitored from Day 20 postpartum until the first observed oestrus postpartum. During the winter months (February to April 2004), animals were housed in a cubicle shed with concrete flooring or were at pasture during the summer (April to November 2004). The temperatures during the study period ranged 0.2 to 27 °C. Milking took place twice a day starting at 6:30am and 4:30pm. All year round animals had access to a total mixed ration (TMR) at a feed-fence after milking. Pastures were of seasonal ryegrass, Italian ryegrass and white clover.

Visual observation of oestrus

Observations for behavioural signs of oestrus were conducted once a day for 30-minutes before morning milking, in the afternoon or following evening milking. Behavioural signs of oestrous were recorded using the weighted scoring methods of (Van Eerdenburg et al., 1996). An individual was considered to be in oestrus when >100 points were recorded in a 30-minute period. The 'intensity' score was then calculated as the total points received and was equivalent to the number of times (termed frequency from here forward) a behavioural oestrous sign was observed in the 30-minute period multiplied by the relevant points (Table 1).

Lameness scoring

Individuals were scored for lameness (score 1-3) every 2 weeks from Day 20 postpartum onwards, based on gait and posture while walking and standing using methods adapted from (Sprecher et al., 1997) and defined in Table 2. Each individual was scored on average 4.5 ± 0.3 times during the study. Ninety-one percent of individuals had the same or one ± 1 lameness score for the duration of the study. Additionally, an individual's average lameness score over the duration of the study and the lameness score taken just prior to oestrus was the same in 97.8% of cases. Therefore, individuals were retrospectively grouped based on their mean lameness score [nonlame LS 1 (1.0-1.5), moderately lame LS 2 (1.6-2.5), severely lame LS 3 (2.6-3.0)].

Hormone assays

Daily milk samples were taken immediately prior to milking to determine progesterone metabolite, oestradiol and cortisol concentrations. Milk progesterone metabolite [hereafter referred to as progesterone as it is the predominant progestagen compound in bovine milk (Purdy et al., 1980)] and cortisol samples were analysed every other day from Day 20 postpartum to ~10 days following observed oestrus. Daily oestradiol samples were analysed around periods of potential oestrus (indicated by low progesterone concentrations).

Enzyme immunoassays

Milk progesterone and cortisol were analysed by enzyme immunoassay (EIA) using the method of (Young et al., 2004) adapted from (Munro & Stabenfeldt, 1984). The progesterone assay employed a progesterone-horseradish peroxidase ligand, monoclonal antiserum (Quidel Clone #425; supplied by CJ Munro, University of California, Davis, CA) and progesterone standards (Sigma-Aldrich, UK). The antiserum cross-reacts 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). The EIA was performed in 96-well microtitre plates (Nunc™-Immuno, Maxisorp Surface; Nunc A/S, Roskilde, Denmark) coated 14-18 hours previously with progesterone antiserum (50μl/well; diluted 1:10,000 in coating buffer; 0.05 M $NaHCO_3$, pH 9.6). Milk samples were assayed at neat concentration. Progesterone standards (50 μl, range 4-200 pg/well, diluted in assay buffer, 0.1M $NaPo_4$, 0.149M NaCL, 0.1% bovine serum albumin, pH 7.0) and samples (50 μl) were combined with progesterone-horseradish peroxidase (50 μl, 1:33,000 dilution in assay buffer). Following incubation at room temperature for 2h, plates were washed 5 times before 100 μl substrate buffer [0.4 mM 2,2'-azino-di-(3-ethlybenzthiazoline sulfonic acid) diammonium salt, 1.6 mM H_2O_2, 0.05 M citrate, pH 4.0] was added to each well. After incubation for 10-15 min, the absorbance was measured at 405nm. The cortisol EIA assay employed a cortisol-horseradish peroxidase ligand [prepared using the methods of (Munro & Stabenfeldt, 1984)], polyclonal antiserum (No. R4866; CJ Munro, University of California, Davis, CA) and cortisol standards (Sigma-Aldrich, UK). The antiserum cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (C.J. Munro, *pers. comm*). The cortisol EIA was performed in the same manner as the progesterone EIA with the exception of a 1h incubation time and working concentrations of cortisol antiserum, cortisol-horseradish peroxidase and standards of 1:8500, 1:40,000 and range 3.9-1000 pg/well, respectively. Parallel displacement curves were obtained for serial diluted pooled milk samples and the progesterone and cortisol standard curves. The intra- and interassay coefficients of variation were <5% and <15% for both assays, respectively.

Milk extraction and oestradiol radioimmunoassay

Prior to oestradiol radioimmunoassay (RIA), milk samples were defatted and extracted using C-18 Sep-Pak® cartridges (Waters, Ireland). Following centrifugation (250 g for 10 min) the resultant fat supernatant was removed and samples were re-spun at 250 g for a further 10 min. C-18 Sep-Pak® cartridges were primed with 2ml methanol (VWR International, UK) followed by 4ml H_2O. Defatted milk samples (2ml) were loaded onto the C-18 Sep-Pak® cartridges followed by 4ml H_2O and 500 μl acetone (VWR International, UK). C-18 Sep-Pak® cartridges were placed over 12x75 mm glass collection tubes and eluted with 2ml acetone. Samples were then evaporated to dryness using a Savant Instrument Speedvac© Concentrator (Holbrook, NY, USA) and stored at –20°C until assayed. The efficiency of steroid extraction was evaluated by adding ^{3}H-oestradiol (16,000 dpm) prior to extraction. The mean recovery ± SD of ^{3}H -oestradiol was 67.4 ± 3.2% (n=26 samples). Oestrogen concentrations were analysed by a previously described RIA (Mann et al., 1995). Briefly, the modified oestrogen RIA based on the oestradiol MAIA Kit (Adaltis Italis S.p.A, Italy) utilized a rabbit anti-oestradiol antibody [50 μl/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 (50 μl/tube; diluted 12,000 dpm in assay buffer), goat anti-rabbit gammaglobulin coupled to magnetic particles (100 μl/tube), oestradiol standards (oestradiol-17β; Sigma-Aldrich, UK; range 0.0625-16 pg/tube made up to 250μl) and samples reconstituted in 250μl assay buffer. The assay cross-reacts with oestradiol-17β 100% and ≤ 2% with all other metabolites tested. The intra- and interassay coefficients of variations were both <20%.

Data analysis

All data are presented as mean ± SEM and were analysed using Minitab 14®. Statistical differences were reported when $p<0.05$; with a tendency considered when $0.06>p<0.10$. Minitab 14® restricts post-hoc comparisons on factors nested within random factors therefore any significant differences detected by a model with factors nested within random factors were analysed using GLM ANOVA comparing within and between nested factors with Tukey's 95% Post-hoc pairwise comparison.

Oestrous behaviours: Oestrus intensity (total points received) was analysed using a General Linear Model (GLM) ANOVA. The model included the fixed factors of lameness (1/2/3), fertility treatment (yes/no), season (summer/winter), where oestrus was observed (inside cubicles/outside on grass) and days postpartum oestrus was observed (20-60 or 60-80+ days postpartum). Pearson correlations were used to assess the relationship between total points and oestradiol concentrations on the day oestrus was observed (Day 0) and maximum oestradiol concentrations. The frequencies of each behavioural sign of oestrus were analysed using GLM ANOVA with fixed factors of lameness and fertility treatment.

Hormones around oestrus: Profiles for oestradiol (Day –6 to 2), progesterone (Day –18 to 0) and cortisol (Day –18 to 2) were aligned to the day oestrus was observed (Day 0 defined as >100 points) and analysed using GLM ANOVA with repeated measures. Steroid concentrations were normalized using logarithmic transformation [log (oestradiol+1), log (progesterone), and log (cortisol)]. The model included the fixed factors of lameness, fertility treatment, days from observed oestrus, the interaction between fertility treatment and lameness, the random factor cow ID, the factors lameness and fertility treatment were also nested within Cow ID (as each cow was only present in one category within each factor).

Other progesterone profile characteristics were calculated as follows: (1) baseline progesterone was defined as the mean plus 2SD progesterone concentration in all cows during potential oestrous periods and was equal to 0.17 ng/ml, (2) the duration of the luteal phase was defined as the time between the first and last progesterone value above baseline plus 2 days, as the analysis of progesterone concentrations every other day introduced an underestimation of (1+1) 2 days, (3) the duration of the inter-luteal interval was the time between the first and last progesterone value below baseline plus 2 days and (4) cycle length was defined as the interval between an increase in progesterone above baseline in one cycle to the increase in progesterone above baseline in the next cycle; this required no adjustment as the sampling bias was cancelled out (1 - 1 = 0 days). The frequencies of the following intervals within lameness groups were analysed using Chi-Square tests; basal progesterone to maximum oestradiol (≤0, 1 or ≥2 days), basal progesterone to oestrus (≤1, 2 or ≥3 days) and maximum oestradiol to oestrus (0, 1 or ≥2 days). Maximum progesterone and oestradiol concentrations prior to oestrus and the durations of luteal phase, inter-luteal interval and cycle length were analysed using a GLM ANOVA, which included the fixed factors of lameness and fertility treatment.

Postpartum cyclicity and fertility: Cortisol (Day 20 to 80 postpartum), defined as resting cortisol concentrations, were analysed using GLM ANOVA with repeated measures. The model included the fixed factors of lameness, days (Day 20 to 80 postpartum), the random factor cow ID and the factor lameness also nested within cow ID. Daily progesterone (Day –18 to 6), oestradiol (Day –6 to 6) and cortisol (Day –18 to 6) profiles in all cows that were seen or not seen in oestrus (considered to be potential periods when oestrus could have been observed and aligned from day of the first detected basal progesterone value) were analysed using a GLM ANOVA model with repeated measures and included the fixed factors: occurrence of oestrus (yes/no), day, the interaction between occurrence of oestrus and day, the random factor cow ID, and the factor occurrence of oestrus was also nested within cow ID. Chi-Square tests were employed to analyse the associations between oestrus, fertility treatment, lameness, potential progesterone cycle where oestrus was observed in (1^{st} ,2^{nd} or $\geq 3^{rd}$ cycle). The day oestrus was observed postpartum and the day ovarian activity started postpartum [defined as the first day postpartum progesterone concentrations were above baseline for two or more days, or Day '20' if progesterone was already above baseline (n=9 cases)] in cows with a naturally occurring oestrus (n=27) were analysed with a GLM ANOVA that included the fixed factor of lameness.

Results

Oestrous behaviours

Out of 44 dairy cows, oestrus was seen in 41 [including: 36 in which oestrus intensity (total points) was recorded, and 5 oestrous periods were seen only by the herdsman] and 3 cows were not observed in oestrus.

Lameness was an explanatory variable in the oestrus intensity model (p=0.019) and pairwise comparisons revealed that severely lame cows scored fewer total points than nonlame or moderately lame cows, and thus had a lower intensity of oestrus (p=0.050 and p=0.017, respectively; Figure 1). Other possible explanatory variables in the model showed that the variation in intensity score was not explained by season (summer n=26, 482.7 ± 53.2; winter n=10, 642 ± 94.8; p=0.321), where oestrus was observed (on grass n=33, 539.8 ± 51.0; in cubicles n=3, 386 ± 67.7; p=0.722), administration of fertility treatment [fertility treatment given n = 10 (of these 2 lame and 1 nonlame had CIDR's), 504.5 ± 93.7; natural oestrus n=26, 535.8 ± 56.0; p=0.742] or days postpartum when oestrus was observed (20-60 days postpartum, n = 18, 497.5 ± 66.6; 60-80+ days postpartum, n = 18, 556.7 ± 68.7; p=0.239).

Lameness was also an explanatory variable in the analysis of individual behavioural signs of oestrus, i.e. for total mounting activity (TMA, included mounting the rear or head side of another cow plus standing to be mounted (STBM); p=0.019) and sniffing the vulva of another cow (p=0.038). Pairwise comparisons revealed that severely lame cows tended to have a lower frequency of total mounting activity (p=0.077) and sniffed less than nonlame cows (p=0.031; Figure 2). Although there was a trend, the frequencies of all other behavioural signs of oestrus were not significantly lower in severely lame compared to nonlame cows (p>0.05; Table 3 and Figure 2). The frequency of behavioural signs of oestrus did not vary between moderately lame and nonlame cows (p>0.05; Table 3).

Variations in behavioural signs of oestrus were not explained by clinical fertility treatment (data not shown; $p>0.05$).

Hormones profiles in mik around oestrus

Daily oestradiol profiles from Day –6 to 2 were similar for nonlame (n=18), moderately lame (n=9) and severely lame (n=9) cows (Figure 3a; p=0.313). Additionally, there was no association between degree of lameness and the interval from maximum oestradiol concentration to observed oestrus (p = 0.726). Maximum oestradiol concentrations and those on Day 0 were the same for the 3 lameness groups (Table 4; p=0.966, p=0.313, respectively) and did not correlate with total points received (p = 0.572, p = 0.644, respectively). The variation in oestradiol profiles (Day –6 to 2) was not explained by fertility treatment (Day –6 to 2; p=0.889) but differences between individual cows did explain a large proportion of the variation (p=0.000; r^2 = 42.4%).

Progesterone profiles over the 18 days prior to oestrus, although lower for both groups of lame cows, did not differ significantly between nonlame (n=18), moderately lame (n=9) and severely lame (n=9) cows (Figure 3b; p=0.497). Other possible explanatory variables in the model showed that the variation in progesterone profiles was not explained by fertility treatment (p=0.358) but again individual cow variation explained a large proportion of the variation (p=0.000; r^2 = 31.7%). Nevertheless, lameness score was an explanatory variable (p=0.050) related to maximum progesterone concentrations prior to oestrus. Post-hoc pairwise comparisons revealed that severely lame cows had lower maximum progesterone concentrations compared to nonlame cows prior to oestrus (Table 4; $p<0.042$). Variations in maximum progesterone values were not explained by clinical fertility treatment (p=0.329). There was no association between lameness and the interval from first detected basal progesterone (prior to oestrus) to maximum oestradiol concentration, or in the interval from basal progesterone to oestrus (p=0.669, p=0.817, respectively). Based on progesterone profiles prior to oestrus, the durations of the luteal phase, inter-luteal interval and cycle length were comparable for all lameness scores (Table 4; p=0.970, p=0.878, p=0.993, respectively).

Cortisol concentrations surrounding oestrus were the same for nonlame, moderate and severely lame cows and variation in the data was not explained by day (–18 to 2) from oestrus (Figure 3c; p=0.922, p=0.557, respectively).

Postpartum cyclicty and fertility

Based on progesterone profiles, irrespective of lameness, the expression of oestrus was associated with 44/67 potential periods (indicated by low progesterone). There were similar proportions of nonlame, moderately and severely lame cows seen in oestrus (seen 12/12/20 and not seen 5/7/11, respectively; p=0.881; Figure 4). The observation of oestrus was associated less with spontaneous potential periods (30/53 cycles) then after fertility treatments (14/14 cycles; p=0.002). Lameness was not associated with a cow receiving a clinical fertility treatment (9/22 nonlame, 2/11 moderately lame and 6/11 severely lame cows; p = 0.206).

Progesterone profiles (Day-18 to 6) that were associated with or without an observed oestrus were similar prior to a potential oestrus (p=0.260; Figure 4b). For the corresponding oestradiol profiles (Day –6 to 6), there tended to be an interaction between occurrence of oestrus and day (p=0.063); post-hoc comparisons also revealed a tendency for oestradiol concentrations from Day 1 to 4 to be higher in cows not seen in oestrus than those seen in oestrus (p<0.10; Figure 4a). Cortisol concentrations (Day –18 to 6) did not differ between cows seen or not seen in oestrus (p=0.250; Figure 4c). Moreover, resting cortisol concentrations (Day 20 to 80 postpartum) did not vary with days postpartum or lameness (nonlame 2.0 ± 0.1, n=22; moderately lame 2.4 ± 0.1, n=11; severely lame, 2.3 ± 0.1 n=11; p=0.602 and p=0.884 respectively; Figure 5).

In all spontaneous oestrous cycles (n=27 cows), lameness was not associated with incidence of oestrus (Table 4; p = 0.948). Lameness did not affect the number of days postpartum oestrus was first observed (p=0.592) or the number of days postpartum when ovarian cyclicity commenced (Table 4; p=0.090). Additionally, the first observed oestrus postpartum in all study cows was equally observed among lameness scores between the 1st, 2nd and ≥ 3rd potential progesterone cycle postpartum (p=0.427).

Discussion

Oestrous behaviour

Severely lame cows had a less intense oestrus than nonlame cows. The reduction in intensity was related to a decrease in general mounting activity. Although not statistically significant, severely lame cows had fewer standing events compared to nonlame cows, and attempted to mount the rear of another cow on fewer occasions. Mounting the head of another cow was a very rare event in the present study and was only observed on 3 occasions in 3 different cows. Moderately lame cows expressed oestrus with similar intensity to nonlame cows. Using the same weighted scoring system to quantify oestrus intensity over the total duration of oestrus, (Gomez et al., 2003) also found that moderate lameness was not associated with low oestrus expression. Similarly, (Sood & Nanda, 2006) reported that moderate lameness in crossbred cows in tropical India did not impact oestrus intensity over the total duration of oestrus. In the present study, the frequency of standing events in moderately lame cows and nonlame cows were comparable, and although (Sood & Nanda, 2006) reported a 'normal' oestrus in moderately lame cows, cows had fewer total standing events over the duration of oestrus compared with nonlame cows (2.4 ± 0.4 *versus* 8.0 ± 2.5, respectively).

In the present study, the reduction in oestrus intensity in severely lame cows was also attributable to less 'sniffing'. Severely lame cows had a propensity to exhibit less chin resting and flehmen but this was not significantly different from nonlame cows. Cows in oestrus are restless and more active (Roelofs et al., 2005a). Cows in oestrus also tend to form very mobile sexually active groups (*personal observation*). Sniffing requires cows to be in close contact with female herd-mates. It is possible that lame cows are not motivated or rewarded sufficiently to remain with the sexually active group, therefore, exhibit fewer behavioural signs of oestrus. In another study, we have shown that lame cows spend more time lying down and less time standing up, walking and expressing an oestrous behaviour compared to nonlame cows (Chapter 3). As the number of cows participating in the sexually active group increases, so does the chance to display a behavioural sign of oestrus resulting in an increase in oestrus intensity (Roelofs et al., 2005b). However, (Sood & Nanda, 2006) observed that oestrus intensity in moderately lame cows does not increase with an increasing number of cows in oestrus as it does with nonlame cows. In the present study, comparisons between oestrus intensity and an increasing numbers of cows in the sexually active group were not made. Nevertheless, it is possible that the pain of lameness reduces oestrus intensity by hampering the number of interactions between cows in oestrus.

The purpose of chin resting, flehmen and sniffing may be to permit the transmission of chemical signals or pheromones between oestrous herd-mates. Pheromones are multifunctional, serving not only to attract but to induce sexual behaviours in others (Izard, 1983). Pheromones from females cows are involved in inducing and synchronizing oestrus in herd-mates. The application of cervical mucus from cows in oestrus to cows following prostaglandin treatment results in a more uniform synchronization of oestrus (Izard & Vandenbergh, 1982b). Additionally, when samples of vaginal mucus from a cow in oestrus are stored and later re-applied to the perineal region of the same cow in the mid-luteal phase, the recipient cow is sniffed more than if the cows mucus is applied to an 'alien' cow. This suggests that vaginal mucus contains not only oestrus-related odours, but also individual distinctive odours (Nishimura et al., 1991). Although a lame cow is less likely to stand when mounted as a result of pain, it may also be possible that lameness may diminish how 'pheromonally attractive' a cow is or the cow may be emitting 'stress-related' pheromones as there is evidence to indicate that heifers can perceive an increased state of stress in herd-mates by olfactory cues (Boissy et al., 1998). Therefore, a diseased, stressed or lame cow may be less likely to be mounted by fellow herd-mates. In the present study, severely lame cows were mounted less than fellow herd-mates when they were first detected in oestrus, but the difference was statistically not significant. Therefore, intensive observations over a complete oestrus, to determine the duration and total mounts, may be a more appropriate measure to detect a relationship between lameness and 'attractivity'. Pheromones may also be important to drive oestrous behaviour in a positive feedback fashion. It has been hypothesised in sheep, that increased "dopaminergic rewards" are stimulated by pheromones during initial sexual interactions, reinforcing the expression of further behaviour (Fabre-Nys et al., 2003). However, the exact control of the "reward" mechanism is still not clear at present. Nevertheless, due to a reduced sniffing activity, severely lame cows may not be receiving the same level of positive feedback and, therefore, express a less intense oestrus.

Milk hormones profiles around oestrus

The unaltered oestradiol profiles in lame cows suggest that the reduced oestrus intensity in severely lame cows is not due to low oestradiol concentrations. (Lyimo et al., 2000) and (Roelofs et al., 2004) using the same oestrous scoring system found a positive relationship between the oestrus intensity and maximum serum oestradiol concentration. However, the latter study suggested that this relationship exists mainly when oestradiol concentrations are low. The lack of correlation between oestrus intensity and oestradiol concentrations observed in the present study may be a result of once daily oestrus observation and once a day milk sampling. However, several other studies find no relationship between oestradiol and behavioural signs of oestrus (e.g. incidence of standing or mounting activities, chin resting or sniffing) and this suggests that for individual cows threshold values may be involved to induce oestrous behaviours (Glencross et al., 1981; Cook et al., 1987; Coe & Allrich, 1989; Walton et al., 1987). Indeed, in the present study, the majority of variation in oestradiol profiles surrounding oestrus was explained by individual animal variation. Additionally, although there was no difference in oestradiol concentrations between lame and nonlame cows, (Lopez et al., 2002) and (Roelofs et al., 2004) found no correlation between oestradiol concentrations and pre-ovulatory follicle size. Therefore, there maybe an impact of lameness on follicular growth and non-oestrogenic function of the follicle.

In the present study, progesterone profiles are related to the first observed oestrus (>20 days postpartum) after the commencement of ovarian cyclicity. Progesterone concentrations over the whole 18 days prior to observed oestrus, although lower, did not differ between lame and nonlame cows. However, severely lame cows did have lower maximum progesterone concentrations prior to oestrus compared to nonlame cows. The lower oestrus intensity observed in severely lame cows may be related to insufficient progesterone exposure prior to oestrus. Prevailing progesterone concentrations are crucial for the expression of oestrus. In the ewe, prior progesterone priming is not only essential for the display of oestrus but increased concentrations increase the intensity of oestrus expression (Fabre-Nys & Martin, 1991a). Progesterone increases the number of oestradiol receptors in the mediobasal hypothalamus and increases sensitivity to oestradiol (Blache et al., 1994; Blache et al., 1991). Similar to sheep, priming with progesterone in cattle regulates the occurrence of oestrous behaviours (Carrick & Shelton, 1969; Melampy et al., 1957). Cows that exhibit oestrus at the first postpartum ovulation often have transient increases in progesterone prior to ovulation, whereas an increase in progesterone is not seen in cows that fail to exhibit oestrus (Bell et al., 1983). The intensity of oestrus may be affected, as prior treatment with progesterone for 5 days in oestradiol treated ovariectomized cows resulted in more mounts, chin resting and sniffing but the difference was not statistically significant (Vailes et al., 1992).

Resting cortisol concentrations surrounding oestrus did not differ between lame and nonlame cows and significant elevations in cortisol concentrations were not observed around oestrus. (Dieleman et al., 1986) did not observe a peak in cortisol in the peri-oestrus period. However, (Lyimo et al., 2000) demonstrated an increase in serum cortisol and a disruption in diurnal rhythm at the time of maximum oestrus intensity. Cortisol milk samples in the current study were analysed every other day, which may be insufficient to detect differences associated with oestrus. A small pilot study demonstrated a diurnal rhythm in milk cortisol concentration was not present in the present study (data not shown – Appendix I).

Postpartum cyclicity and fertility

Lameness had no effect on the incidence of spontaneous oestrus; therefore following progesterone exposure, lame cows were just as likely to be observed in oestrus as nonlame cows. In this study, lameness did not delay the onset of oestrus or the day postpartum luteal activity started. (Garbarino et al., 2004) and (Petersson et al., 2006) reported that lameness delayed the commencement ovarian cyclicity by 3-16 days compared to nonlame cows. (Petersson et al., 2006) reported a delay in the onset to oestrus in lame cows compared to nonlame cows (88.4 ± 36.1 *versus* 60.4 ± 30.3 days postpartum). These other studies were based on 200-500 cows, compared with the 27 cows with a spontaneous oestrus in the present study. All cows that received a clinical fertility treatment in the present study were observed in oestrus. Lame cows were no more likely to receive a clinical fertility treatment compared to nonlame cows and it did not take longer (ie second or third progesterone cycle observed postpartum) for lame cows to been seen in oestrus. Therefore in the present study, lame cows were just as likely to be observed in oestrus following the first progesterone cycle postpartum as nonlame cows.

Examination of progesterone profiles postpartum associated with or without oestrus (regardless of lameness score) revealed similar concentrations of progesterone and cortisol. However, cows not seen in oestrus had continued elevated oestradiol concentrations for 4 days beyond the decrease to basal progesterone concentrations; presumably representing continued follicle growth and a delay or impairment of the LH surge and/or ovulation. Therefore, a 'silent' oestrus in the present study was not associated with low prior progesterone exposure, elevated progesterone values during the period of expected oestrus or altered cortisol concentrations but with continued elevated oestradiol around the expected time of ovulation. (Melendez et al., 2003) suggests cows that become lame within the first 30 days postpartum have a higher incidence of ovarian cysts. Ovarian cysts are associated with altered follicular growth and the delay or inhibition of LH surge (Dobson et al., 2000; Nanda et al., 1990; Hamilton et al., 1995). However, in the present study, there were similar numbers of lame and nonlame cows with continued elevated oestradiol concentrations around the expected time of oestrus.

Lameness is a painful and stressful process characterized by hyperalgesia (Whay et al., 1997). The activation of the hypothalamic-pituitary-adrenal axis by stressors reduces the pulsitility of GnRH by actions at both the hypothalamus and pituitary gland that ultimately deprives the ovarian follicle of adequate support (Dobson & Smith, 2000). Chronic stress, such as lameness, may affect the amplitude and/or frequency of GnRH pulsatility. The chronic administration of ACTH (100 IU every 12hrs for 7 days) disrupts pulsatile patterns of LH in cows and is associated with lower oestradiol concentrations (Dobson et al., 2000). Similarly, a key event during the return of ovarian cyclicity is the re-establishment of suffient frequency of pulsatile LH secretion (Butler, 2000). Cows with chronic stressors such milk fever, retained fetal membranes, endometritis and mastitis also have a delay in the commencement of luteal activity postpartum inferring a delay in re-establishment of sufficient frequency of pulsatile LH secretion (Morrow et al., 1966; Huszenicza et al., 2005). In the current study, cortisol concentrations did not vary with days postpartum (20-80) and there was no difference between resting cortisol concentrations in severely lame, moderately lame or nonlame cows. Similar to the present study, (Ley et al., 1996), who from a single blood sample, found no difference in cortisol concentrations between lame and nonlame cows. Stress and acute pain in cattle are associated with elevated concentrations of stress-induced and glucocorticoids (Wohlt et al., 1994; Cohen et al., 1990; Mitchell et al., 1988). However, the chronic pain associated with lameness does not appear to increase cortisol concentrations. In studies with rodents, the adrenal response to a repeated stress is adaptation or habituation so that the same stressor evokes less of an adrenal response to each successive stressor (Kant et al., 1983; Tache et al., 1976). In continually stressed (round the clock unpredictable footshock for 14 days) rats, initial corticoid concentrations are greater, however, over time concentrations do not differ between stressed and nonstressed animals (Kant et al., 1987). The similar resting cortisol concentrations observed in the present study between lame and nonlame cows may be the result of adaptation to the stress and pain of lameness. Chronic stress and pain is also often associated with disturbances of the hypothalamic-pituitary-adrenal axis, resulting in both functional and structural modifications and long-term alterations in neuroendocrine reactivity to subsequent stressors (Ostrander et al., 2006; Ulrich-Lai et al., 2006b). Therefore, although cortisol concentrations did not differ with lameness, dysfunction in the hypothalamic-pituitary-adrenal cannot be excluded and should be investigated futher.

In conclusion, following the commencement of luteal activity lameness does not affect the incidence of oestrus. Severely lame cows express oestrus with less intensity compared to nonlame cows and the diminished oestrus intensity is associated with lower maximum progesterone concentrations prior to oestrus but not with abnormal oestradiol or cortisol profiles.

Acknowledgements

The authors would like to thank farm staff for their cooperation with the study. Thank you to Hilary Pursell for technical assistance and Coralie Munro for supplying the EIA reagents. A very special thanks to Donald Tyson and Paul Fox for their assistance with the project. Scholarships (to SLW) were provided by Natural Sciences and Engineering Research Council of Canada (NSERC), Universities UK and University of Liverpool. This work was presented in part at the annual meetings of the European Society of Domestic Animal Reproduction in 2005 and 2006.

Table 1. Point scale for observed behavioural signs of oestrus [1]

Oestrus Signs	Points
Flehmen	3
Restlessness [2]	5
Sniffing the vulva of another cow	10
Mounting received but did not stand	10
Resting chin on the back of another cow	15
Mounting the rear of other cows	35
Mounting the head side of another cow	45
Stood to be mounted (STBM)	100

Each time an oestrus sign was observed, the assigned number of points were recorded

[1] modified after (Van Eerdenburg et al., 1996) by removing cajoling and mucous vaginal discharge and adding flehmen response

[2] can only be recorded once during a single 30-minute observation period

Table 2. Lameness scoring scale[1]

Lameness Score	Description	Assessment Criteria		
		Stands	**Walking**	**Gait**
1	**Nonlame**	Level back posture	Level back posture	Normal
2	**Moderately Lame**	Level back posture	Arched back	Normal to short-striding
3	**Severely Lame**	Arched posture	Arched back	Takes one step at a time/reluctance to bear weight on one or more limbs/feet

[1] modified after (Sprecher et al., 1997) 5 point scale, in which scores 1 and 2 are comparable and 3-5 are grouped and equivalent to score 3 as defined above

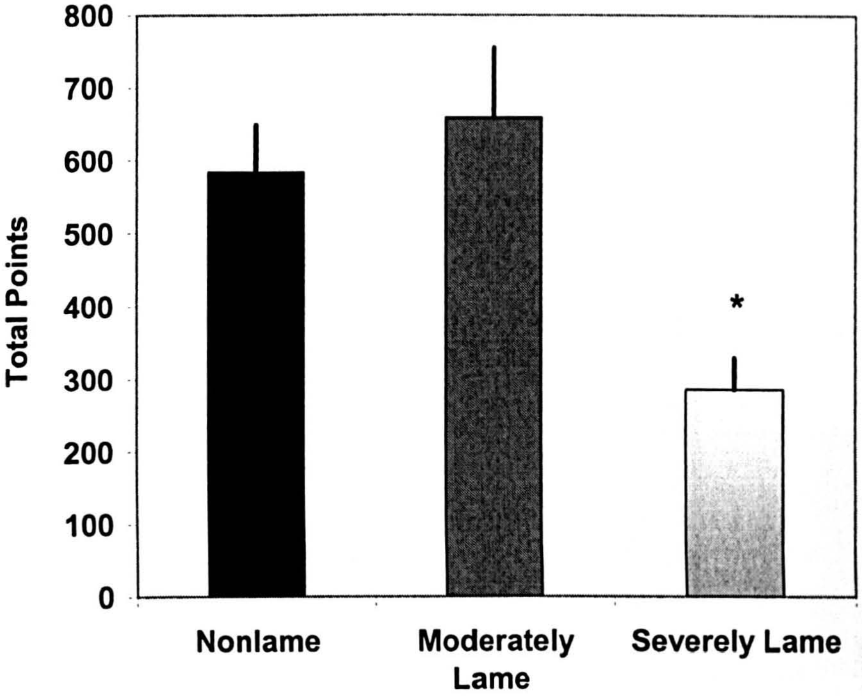

Figure 1. Oestrus intensity as the total points received (± SEM) in normal (n = 18), moderately (n = 9) and severely lame (n = 9) cows (*p=0.019) within one 30-minute period per day (*p<0.05). The point scoring system is shown in Table 1.

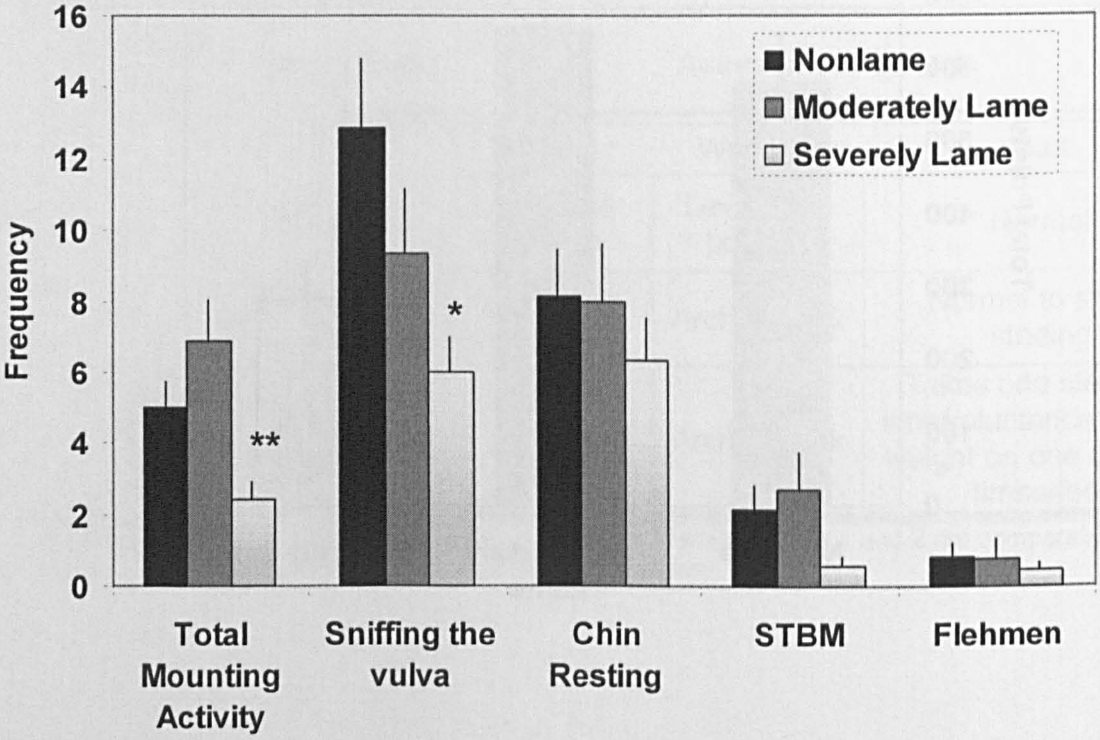

Figure 2. Frequencies of different behavioural signs of oestrus exhibited by normal (n =18), moderately lame (n = 9) and severely lame (n = 9) cows within one 30-minute period per day (*p<0.05; **p<0.10). The point scoring system is shown in Table 1.

Table 3. Frequency of behavioural signs of oestrus in nonlame, moderately lame and severely lame cows[1]

Oestrous Signs	Nonlame	Moderately Lame	Severely Lame	P value
Total mounting activity (TMA)	5.0 ± 0.7 ^{a}	6.9 ± 1.1 ^{a}	2.4 ± 0.5 ^{b*}	-
Sniffing the vulva of another cow	12.9 ± 1.9 ^{a}	9.3 ± 1.8 ^{ab}	6.0 ± 1.0 ^{b}	-
Chin resting	8.1 ± 1.3	8.0 ± 1.6	6.3 ± 1.2	p=0.467
Stood to be mounted (STBM)	2.2 ± 0.7	2.7 ± 1.1	0.6 ± 0.2	p=0.243
Flehmen	0.8 ± 0.4	0.8 ± 0.5	0.4 ± 0.2	p=0.817
Mounting rear of other cows	2.7± 0.4	4.2± 1.2	1.9± 0.4	p=0.238
Mounting head side of other cows	0.1± 0.1	0.0± 0.0	0.0± 0.0	p=0.367
Mounted but did not STBM	0.7± 0.2	1.1 ± 0.6	0.1± 0.1	p=0.240

[1] Frequency ± SEM within one 30-minute period per day. The point scoring system is shown in Table 1. Significant differences $p<0.05^{a,b}$ within a row and * $p<0.10$ compared to nonlame cows

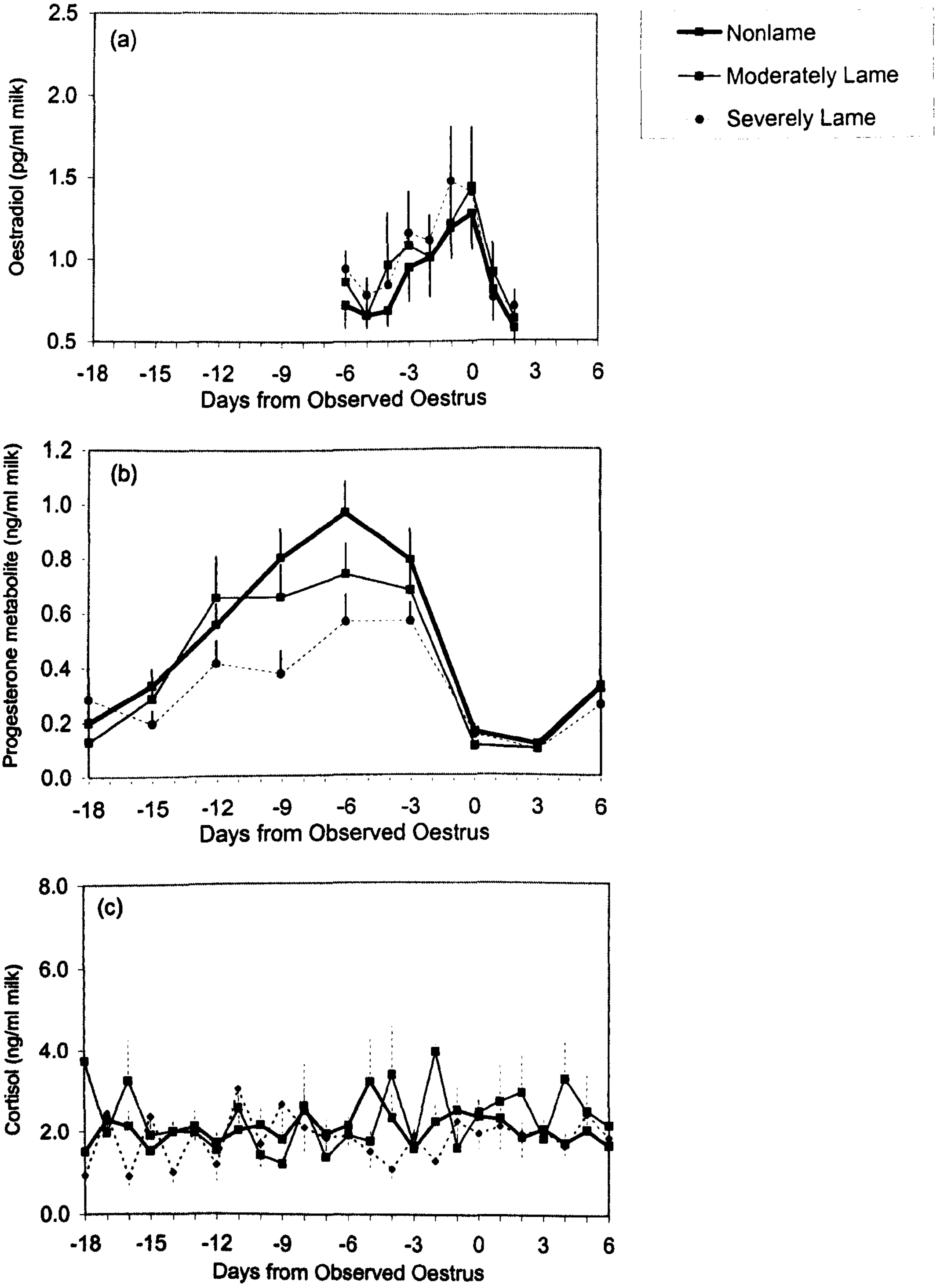

Figure 3. Mean ± SEM milk (a) progesterone (ng/ml) (b) oestradiol (pg/ml) and (c) cortisol (ng/ml) concentrations in nonlame (n=18), moderately (n=9) and severely lame (n=9) cows aligned to the day of observed oestrus (Day 0 = oestrus intensity >100 points).

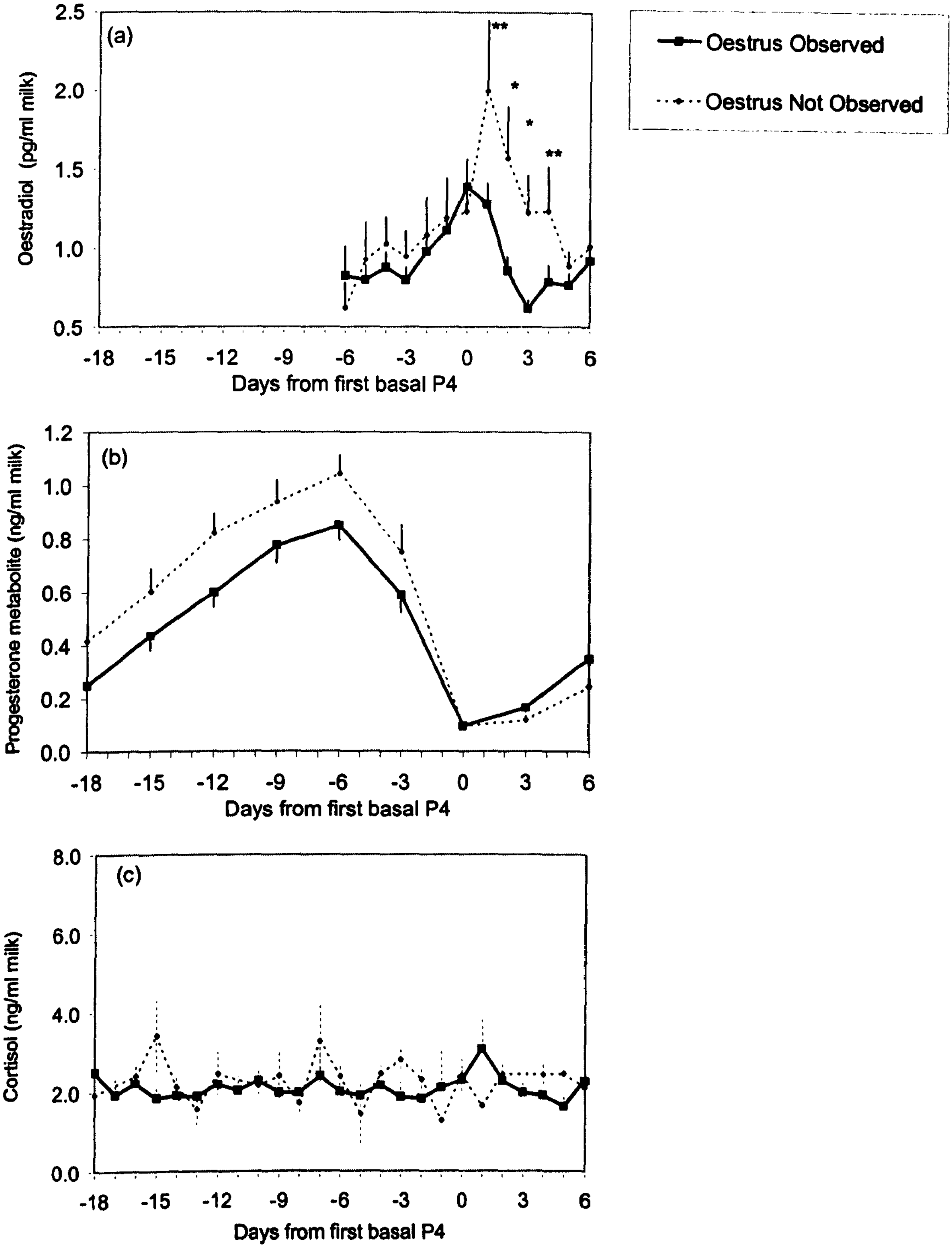

Figure 4. Mean (± SEM) milk (a) progesterone (ng/ml) (b) oestradiol (pg/ml) and (c) cortisol (ng/ml) concentrations in cycles when oestrus was observed (n=44) or not observed (n=23). Data are aligned to the day of first basal progesterone concentration (*p<0.05,**p<0.10). There are equal propotions of nonlame, moderately lame and severly lame cows observed and not observed in oestrus (p=0.881).

Table 4. Parameters of cycle characteristics in nonlame, moderately and severely lame cows

	Nonlame (n=18)	**Moderately Lame** (n=9)	**Severely Lame** (n=9)
Parameters surrounding first observed oestrus postpartum*			
Maximum oestradiol (pg/ml)	1.8 ± 0.3	1.8 ± 0.3	1.9 ± 0.3
Oestradiol on Day 0 (pg/ml)	1.3 ± 0.2	1.5 ± 0.4	1.4 ± 0.2
Maximum progesterone (ng/ml)	1.3 ± 0.1^{a}	1.2 ± 0.2^{ab}	0.7 ± 0.1^{b}
Luteal phase duration (days)	12.8 ± 1.3 (2;20)	12.4 ± 1.2 (6;16)	13.1 ± 2.2 (4;26)
Inter-luteal Interval (days)	6.0 ± 0.5 (2;10)	6.0 ± 0.6 (2;8)	5.8 ± 0.7 (4;10)
Cycle length (days)	18.7 ± 1.5 (4;28)	18.9 ±1.4 (12;22)	18.9 ± 2.9 (8;36)
	Nonlame (n=13)	**Moderately Lame** (n=9)	**Severely Lame** (n=5)
Postpartum parameters**			
Days postpartum oestrus observed	54.6 ± 3.9 (38;82)	49.2 ± 5.1 (31;81)	56.2 ± 5.9 (39;73)
Days postpartum ovarian cyclicity commenced	33.5 ± 3.4('20';54)	22.4 ± 1.6 ('20';32)	33.6 ± 4.5 ('20';48)
Incidence of oestrus (observed/potential periods)	13/24	10/17	7/12

Significant differences ^{a,b} within a row $p<0.05$

Data are presented as mean ± SEM (range)

* data collected from 36 oestrus periods scored for intensity using a point scoring system (Table 1)

** data collected from 27 spontaneous oestrus periods

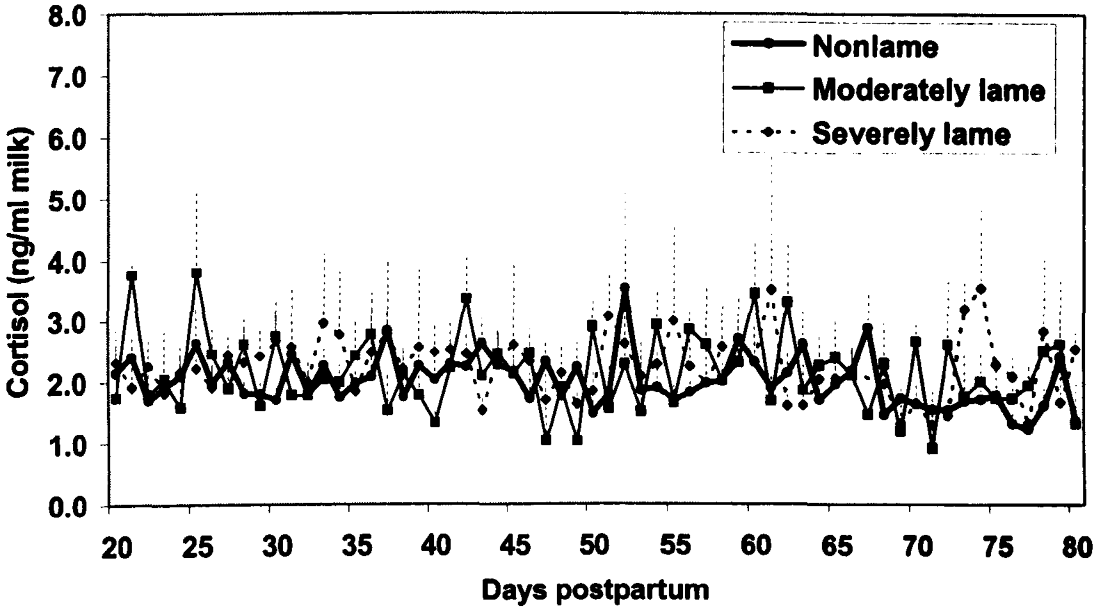

Figure 5. Mean ± SEM cortisol concentration (ng/ml milk) in nonlame (n=22), moderately (n=11) and severely lame (n=11) cows between Days 20 to 80 postpartum.

Chapter 2

Chapter 2

Lameness, milk hormone profiles, intensity and duration of oestrus in dairy cattle

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Key words: lameness, behavioural signs of oestrous, oestrus intensity, oestrus duration, body condition score, milk, progesterone, oestradiol, cortisol, dairy cow

Abstract

Lameness reduces fertility in dairy cows. The primary objectives of the present study are to determine if lameness affects the duration and frequency of several primary and secondary behavioural signs of oestrus and/or associated progesterone, oestradiol and cortisol hormone profiles in milk. A further objective was to determine if lameness was associated with body condition score (BCS) and/or the incidence of oestrus.

Oestrus was synchronized in 59 cows (range 30-75 days postpartum) using GnRH followed by prostaglandin F2α (PG) 7 days later. Cows were scored weekly for lameness (lame, n=39; nonlame, n=20) and body condition score (low BCS, n=10; moderate BCS, n=49) and observed for behavioural signs of oestrus for 30 minutes every 3 h for 7 days after PG.

Following PG, cows responded in 3 ways: expressed oestrus after prior high values of progesterone (n=33; 18 lame and 15 nonlame); no oestrus observed but had prior high progesterone (n=7; 6 lame and 1 nonlame); no oestrus and had low prior progesterone (n=12; 10 lame and 2 nonlame). Seven cows had variable progesterone profiles and data were discarded.

In cows with prior progesterone, the duration of oestrus was the same for nonlame (15.2 ± 1.3; range 3-24 h) and lame cows (12.3 ± 1.3; range 3-21 h). Lame cows had a less intense oestrus (nonlame, 2260 ± 307 points; lame, 1417 ± 206 points; p=0.029) as result of: 1) decreased total frequency of mounting and chin resting 2) shorter duration of being mounted by herd-mates 3) shorter duration of high frequency sniffing, chin resting, restlessness, mounting and being mounted by herd-mates 4) lower average frequency of chin resting, mounting and being mounted by herd-mates over the duration of oestrus, and 5) surrounding maximum oestrus intensity, lameness diminished the proportion of cows displaying chin resting, mounting and standing to be mounted.

Lame cows had lower maximum progesterone concentrations (0.9 ± 0.1 *versus* 1.2 ± 0.2 ng/ml milk; p=0.023) and lower progesterone concentrations for a 6-day period prior to observed oestrus. Oestradiol profiles prior to observed oestrus and maximum oestradiol (nonlame, 1.2 ± 0.2; lame, 1.5 ± 0.2 pg/ml milk) did not differ between lame or nonlame cows. Additionally, maximum oestradiol and total or maximum oestrus points were not correlated. Resting cortisol concentrations did not differ between lame or nonlame cows. Fewer lame cows were observed in oestrus following PG (nonlame 15/18, lame 18/34; p=0.030). When cows with low prior progesterone were removed (n=2 nonlame and n=10 lame), lameness was no longer associated with oestrus (nonlame 15/16, lame 18/24). Progesterone profiles in cows with prior progesterone seen or not seen in oestrus were similar except for Day 1 following PG injection when cows seen in oestrus tended to have higher progesterone concentrations (p=0.087). Oestradiol profiles did not differ between cows seen or not seen in oestrus except on Day 5 after PG, when oestradiol concentrations were higher in cows that were not seen (p=0.025).

Lame cows were more likely to have a low BCS (0/18 nonlame and 10/34 lame had a low BCS; p=0.010) and fewer cows with low BCS were seen in oestrus (3/33 low BCS and 7/19 moderate BCS; p=0.014).

In conclusion, lame cows express oestrus of a similar duration but with lower intensity and fewer primary and secondary signs. The reduced intensity of oestrus is associated with a low progesterone profile prior to the observed oestrus but not with abnormal oestradiol or cortisol profiles. Lame cows are more likely to have a low BCS and are less likely to be seen in oestrus following oestrus synchronization.

Introduction

Lameness is associated with pain and poor fertility in dairy cows. With a high incidence of lameness this is an important welfare and economic issue for the UK dairy industry (Collick et al., 1989; Greenough et al., 1997; Whay et al., 1997; Ward, 2001).

In general to maximize milk production, a dairy cow should produce one calf per year, but lameness increases this interval resulting in poor productivity (Collick et al., 1989; Lucey et al., 1986; Hernandez et al., 2001; Hernandez et al., 2005). The mechanisms by which lameness has an impact on reproductive function are not completely understood. Lameness appears to reduce reproductive efficiency by delaying postpartum reproductive events and/or increases the chance of a missed or inappropriately-timed insemination. Based on progesterone profiles, lameness delays the commencement of ovarian activity following parturition and delays the onset to the first postpartum oestrus (Petersson et al., 2006; Garbarino et al., 2004). Lameness also increases the number of inseminations required per pregnancy and lowers the pregnancy rate to first insemination, inferring poor oocyte quality and/or an inappropriately-timed insemination (Collick et al., 1989; Hernandez et al., 2001; Melendez et al., 2003). We have also shown in (Chapter 1, Walker et al., 2005), that lameness diminishes the expression of oestrus resulting in a less intense oestrus. This could decrease the chances of detection, result in a missed or poorly timed insemination and decreased reproductive efficiency.

Oestradiol and progesterone both play a role in oestrous expression in dairy cows (Allrich, 1994). Prior to oestrus, progesterone exposure facilitates the actions of oestradiol (Allrich, 1994; Stevenson et al., 1989; Melampy et al., 1957; Carrick & Shelton, 1969; Bell et al., 1983). A chronic stressor such as lameness can have detrimental effects on ovarian cyclicity and delay re-establishment of sufficient frequency of pulsatile LH secretion, ultimately depriving the ovarian follicle of adequate LH support and decreasing steroidogenesis (Dobson & Alam, 1987; Li & Wagner, 1983; Dobson et al., 2003; Dobson & Smith, 2000). In lame cows, less intense oestrus is associated with lower maximum progesterone concentrations prior the first observed oestrus postpartum (Chapter 1, Walker et al., 2006). Therefore, it may not just be the physical mechanical limitations of lameness that result in poor fertility but lameness may have an impact on ovarian function, compromising the normal hormonal milieu and altering oestrous behaviour. A primary aim of the present study was to establish the association between lameness, oestrous intensity and low progesterone exposure prior to oestrus and to confirm that similar oestradiol and cortisol concentrations occur around oestrus in both lame and nonlame cows as observed in (Chapter 1, Walker et al., 2006). Additionally, in order to meet a calving interval of 365 days, cows should by pregnant by no later than 80 days postpartum; therefore, in the present study oestrus was synchronized in cows 30-75 days postpartum using GnRH and PG analogues. Exogenous progesterone was not used for oestrus synchronization as this could mask any defects in 'natural' progesterone production in lame cows.

The less intense oestrus in lame cows is associated with a decrease in the amount of mounting activity and sniffing when the cows are first detected in oestrus (Chapter 1, Walker et al., 2005). The decreased intensity of oestrus also appeared to be associated with less mounting by fellow female herd-mates and fewer chin rests and flehmen responses. These behaviours could be related to the production and/or detection of oestrus-related pheromones, as pheromones serve not only to attract but also to induce sexual behaviours in others (Izard, 1983). A further aim of the present study is to monitor the total duration and frequency of primary and secondary behavioural signs of oestrus during the entire oestrous period, and not just when cows are first detected in oestrus, to determine if lameness alters the duration and/or total frequency and possibly how 'attractive' lame cows are in terms of being mounted by herd-mates.

Lastly, nutrition has an impact on reproduction and may influence oestrous expression in cows (Orihuela, 2000). Nutrition and reproduction are interrelated and the loss of body fat (negative energy balance) results in poor reproductive efficiency (Lucy, 2003; Ferguson, 2005). Increasing levels of nutrient intake and/or cows that maintain body condition are associated with shorter intervals to the first postpartum oestrus (Rutter & Randel, 1984; Hurnik, 1987). A negative energy balance affects LH pulsatility and/or ovarian steroid synthesis (Butler, 2000), both of which could have a negative impact on oestrous expression (Caraty et al., 2002). Therefore, an additional aim of the present study was to investigate the association between body condition score, the incidence of lameness and oestrus expression.

Thus, the aims of the present study are to monitor the frequency and duration of eight different behavioural signs of oestrus over the duration of oestrus in synchronized lame and nonlame postpartum dairy cows, in relation to body condition score and the accompanying progesterone, oestradiol and cortisol profiles.

Materials and Methods

Experimental design, animals, feeding and housing

The study was conducted on postpartum Holstein-Friesian cows (n = 59) on a UK commercial dairy farm comprising a total of ~200 year-round calving cows. The parity and days postpartum of the study cows was 3.9 ± 0.2 (range 2-10) and 51.8 ± 1.4 (range 30 – 75), respectively. Cows were selected based on lameness score (see below) and days postpartum and oestrus was synchronized in 5 groups of ~12 animals (including at least 4 nonlame or control cows) between May and September 2005 (temperatures ranged 5 to 28 °C) using a 100 μg im injection of a gonadotrophin releasing hormone (GnRH) analogue (Buserelin, 2.5ml Receptal®, Intervet Ltd. Bucks, UK) followed by a single 500 μg im injection of a prostaglandin F2α (PG) analogue (cloprostenol, 2ml, Estrumate® Schering-Plough Animal Health, Uxbridge, UK) 7 days later. Animals were at pasture (seasonal ryegrass, Italian ryegrass and white clover) for the duration of the study with additional access to total mixed rations (TMR) inside at a feed-fence after milking twice a day. The average rolling milk yield per cow in the herd was 8500 litres/year.

Visual observations of oestrus

To establish the frequency, duration and intensity of primary and secondary behavioural signs of oestrus, observations were carried out for 30 min every 3 h for 7 days following PG injection. Behavioural signs of oestrus were recorded using the weighted scoring method of (Van Eerdenburg et al., 1996). Animals received points based on the number of times a behavioural sign of oestrus was observed in a 30-min observation period (hereafter termed 'frequency'; Table 1). When the sum of points in a consecutive 30-min observation period was >100 points, an animal was considered to be in oestrus. An animal was no longer in oestrus when 2 or more consecutive periods achieved <100 points. The beginning or end of oestrus were defined as the first (minus 1.5 h) and last (plus 1.5 h) 30-minute observation period the animal exhibited a behavioural sign, respectively. The duration of oestrus was the sum of 3-h intervals from the beginning to the end of oestrus. The total points received over the duration of oestrus were considered to be a measure of 'oestrus intensity'.

Lameness and body condition scoring

Individuals were scored for lameness (score 1-3) for 4 weeks prior to the commencement of the study, based on gait and posture while walking and standing, using methods adapted from (Sprecher et al., 1997) and summarized in Table 2. Clinical treatment of lameness continued as usual on the farm. Retrospectively, ninety-five percent of individuals had the same or one ± 1 lameness score for the duration of the study and were therefore grouped based on an average lameness score. Any cow with average score of ≥ 2 was considered to be lame and animals were grouped as either nonlame (score of 1; n = 20) or lame (score of 2 or 3; n = 39). Concurrently, animals were scored for body condition (BCS) on a scale of 1-5 using the methods described in (Chamberlain & Wilkinson, 1996). In summary, only 3 cows had an average BCS of 3 or 4 therefore animals were grouped as low BCS (score 1; n=10) or moderate BCS (score 2-4; n = 49).

Hormone assays

Milk samples were collected daily (week prior to PG injection) and twice daily (week following PG injection), immediately prior to milking to determine progesterone metabolite [hereafter referred to as progesterone as it is the predominant progestagen compound in bovine milk (Purdy et al., 1980)], oestradiol and cortisol concentrations.

Enzyme immunoassays

Milk progesterone and cortisol were analysed by previously described (Chapter 1, Young et al., 2004) enzyme immunoassay's (EIA) using methods adapted from (Munro & Stabenfeldt, 1984). Briefly, the EIA utilized an antibody (monoclonal antiserum progesterone metabolite Quidel Clone #425, polyclonal cortisol antiserum R4866; supplied by CJ Munro, University of California, Davis, CA), horseradish peroxidase conjugated label (progesterone and cortisol) and standard (progesterone, hydrocortisone; Sigma-Aldrich, UK). In general, assay procedures were as follows: 1) antiserum was diluted at 1:10,000 for progesterone, and 1:8500 for cortisol 2) standards (progesterone, 4-200 pg/well and cortisol, 3.9-1000 pg/well) and samples (ran undiluted for progesterone and cortisol) were loaded (50 µl/well for progesterone and cortisol) onto the plate; and 3) the horseradish peroxidase conjugate was used at a dilution of 1:33,000 for progesterone and 1:40,000 for cortisol. The progesterone antiserum cross-reacts 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). The cortisol antiserum cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (C.J. Munro, pers. comm.). Parallel displacement curves were obtained for serial diluted pooled milk samples and the progesterone and cortisol standard curves. The intra- and interassay coefficients of variation were <5% and <15% in both assays, respectively.

Milk extraction and oestradiol radioimmunoassay

Milk oestradiol was extracted and assayed by previous described methods (Chapter 1). Briefly, following centrifugation defatted milk samples (2ml) were extracted with acetone using methanol and water primed C-18 Sep-Pak® cartridges (Waters, Ireland, UK). Samples were evaporated to dryness using a Savant Instrument Speedvac© Concentrator (Holbrook, NY, USA) and stored at –20°C until assayed. The efficiency of steroid extraction was 67.4 ± 3.2% (n=26 samples). Oestrogen concentrations were analysed by a previously described radioimmunoassay (RIA, Chapter 1, Mann et al., 1995). Briefly, the modified oestrogen RIA based on the oestradiol MAIA Kit (Adaltis Italis S.p.A, Italy) utilized a rabbit anti-oestradiol antibody [50 µl/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 (50 µl/tube; diluted 12,000 dpm in assay buffer), goat anti-rabbit gammaglobulin coupled to magnetic particles (100 µl/tube), oestradiol standards (oestradiol-17β; Sigma-Aldrich, UK; range 0.0625-16 pg/tube made up to 250µl) and samples reconstituted in 250µl assay buffer. The assay cross-reacts with oestradiol-17β 100% and ≤ 2% with all other metabolites tested. The intra- and interassay coefficients of variations were both <20%.

Data analysis

All data are presented as mean ± SEM and were analysed using Minitab 14®. Statistical differences were reported when $p<0.05$; with a tendency considered as a difference when $0.06>p<0.10$. Minitab 14® restricts Post-hoc comparisons on factors nested within random factors; therefore, any significant differences detected by a model with factors nested within random factors were analysed using GLM ANOVA comparing within and between nested factors and Tukey's 95% Post-hoc pairwise comparison.

Oestrous behaviours

Behavioural signs of oestrus were aligned (in 3-h intervals) relative to the first 3-interval a cow stood to be mounted (STBM). A GLM ANOVA with repeated measures was used to analyse data concerning the average frequency of behavioural signs and average total points received over time from first STBM. The model included the fixed factors of lameness (nonlame/lame) and time (3-h intervals), the interaction of lameness and time, the random factor cow ID, and the factor lameness also nested within cow ID (as each cow was only present in one category within the factor lameness). The duration of 'elevated frequency' for each behavioural sign and total points received is defined as the total number of 3-h intervals that had the same frequency as the 3-h interval of the first observed STBM.

A GLM ANOVA with the fixed factor of lameness was used to analyse total points received, and the following for each behavioural sign: maximum frequency, time of maximum frequency from first STBM, total frequency, total duration. Chi-Square tests were used to compare the proportion of nonlame and lame cows expressing one or more behavioural sign at each 3-h interval. Pearson's correlation was used to compare total or maximum points received with maximum oestradiol concentrations. The associations between lameness, BCS (low/moderate) and the observation of oestrus (yes/no) were analysed using Chi-square tests. The time to the start of oestrus from PG injection was analysed using a Kaplan-Meier Survival Method with a Log-Rank test.

Hormones

Hormone profiles were aligned to the time of first STBM by grouping four 3-h intervals in 12-h (0.5-day) intervals. Profiles of progesterone and oestradiol over time (time from first STBM) were analysed using a GLM ANOVA with repeated measures. Steroid concentrations were normalized using logarithmic transformations [log (oestradiol+1) and log (progesterone)]. The model included the fixed factors of lameness (lame/nonlame) and time (from first STBM for progesterone and oestradiol), the interaction between lameness and time, the random factor of cow ID, and the factor of lameness was also nested within cow ID. Maximum progesterone and oestradiol concentrations and the time first STBM was observed were analysed with a GLM ANOVA with the fixed factor of lameness. The association between lameness and basal progesterone concentrations following GnRH was analysed using a Chi-square test.

Profiles of cortisol over time (day from PG) were analysed using a GLM ANOVA with repeated measures. Cortisol concentrations were normalized using logarithmic transformations. The model included the fixed factors of lameness and time, the interaction between lameness and time, the random factor of cow ID, and the factor of lameness was also nested in cow ID.

The hormone profiles of cows seen or not seen in oestrus [progesterone (Day –6 to 5), and oestradiol (Day –1 to 6)] were aligned from the time of PG injection and were analysed with a GLM ANOVA with repeated measures with the fixed factors of oestrus observed (yes/no), time, the interaction between oestrus and time, the random factor of cow ID, and the factor of oestrus was also nested in cow ID.

Results

Of the 59 dairy cows, 52 were considered to have responded in one of 3 ways: a) expressed oestrus with prior high progesterone values (defined as a concentration of progesterone >0.2 ng/ml for >3 days prior to PG followed by a decrease in progesterone to concentrations <0.2 ng/ml within 2 days of PG; total n=33; 18 lame and 15 nonlame); b) not seen in oestrus and had 'prior progesterone' exposure (total n=7; 6 lame and 1 nonlame); or c) were not seen in oestrus and progesterone remained at <0.2 ng/ml ('low prior progesterone'; total n=12; 10 lame and 2 nonlame). Seven cows had variable progesterone profiles [4 of which were seen in heat (all lame) and 3 that were not (1 lame and 2 nonlame)] and were removed from subsequent analysis.

Associations between lameness, BCS and oestrus

Fewer lame cows were observed in oestrus (nonlame 15/18, lame 18/34; p=0.030); however, when cows with low prior progesterone exposure were excluded (n=2 nonlame and n=10 lame), lameness was no longer associated with the frequency of oestrus (nonlame 15/16, lame 18/24; p=0.126; Figure 1). The survival time from PG injection to the onset of oestrus was not different (p=0.165), but lame cows that came into oestrus do so by 90 h following PG, whereas nonlame cows continued to come into oestrus until 123h following PG (Figure 2). Lameness was also associated with BCS (0/18 nonlame and 10/34 lame cows had a low BCS; p=0.010). Additionally, fewer cows with low BCS were seen in oestrus (3/33 low BCS and 7/19 moderate BCS; p=0.014). Removing cows with low prior progesterone exposure, BCS was still associated with oestrous expression (3/30 low BCS and 7/12 moderate BCS were seen in oestrus; p=0.001).

Hormone profiles in milk from cows observed or not observed in oestrus

Milk progesterone profiles from cows with prior progesterone seen or not seen in oestrus (regardless of lameness score) did not differ ($p \geq 0.05$), with the exception of Day 1 following PG injection when cows seen in oestrus tended to have higher progesterone concentrations (p=0.087; Figure 3a). Post-hoc comparisons also revealed that progesterone profiles from cows with low prior progesterone were significantly lower from Day –6 to 0 from PG ($p \leq 0.05$; Figure 3a).

Oestradiol profiles (Day –1 to 6) did not differ between all three groups (p=0.789; Figure 3b). However, in cows that had prior progesterone exposure, post-hoc comparisons revealed that milk oestradiol concentrations on Day 5 after PG were higher in cows not seen in oestrus (p=0.025; Figure 3b).

Hormone profiles in milk from lame and nonlame cows observed in oestrus

Progesterone profiles only from those cows observed in oestrus, aligned from Day -9 to 5 from first STBM, were lower in lame (n=18) compared nonlame cows (n=15; p=0.048; Figure 4a). Post-hoc comparisons revealed that lame cows had lower progesterone concentrations prior to observed oestrus from Day –9 to –4 from first STBM (p<0.05). Additionally, maximum progesterone concentrations before oestrus were lower (p=0.023) and occurred closer to oestrus (p=0.014) in lame (0.87 ± 0.11 ng/ml milk; –3.94 ± 0.42 days from first STBM, respectively) compared to nonlame cows (1.17 ± 0.16 ng/ml milk; –4.53 ± 0.35 days from first STBM, respectively). The same progesterone data aligned by Day of PG injection similarly revealed that progesterone concentrations in lame cows were lower ($p=0.035$) from the day following GnRH injection (Day –6) to the day of PG injection (Day 0) compared to nonlame cows ($p<0.05$; Figure 5). Additionally, more lame cows had basal progesterone concentrations (11/18 lame and 3/15 nonlame cows; $p=0.017$) on the day following the GnRH injection (Figure 5).

Oestradiol profiles (Day –5 to 2 from first STBM; $p=0.250$; Figure 4b), maximum oestradiol concentration (nonlame, 1.17 ± 0.152 pg/ml milk; lame, 1.544 ± 0.21; $p=0.120$) or day of maximum oestradiol from first STBM (nonlame, 1.27 ± 0.28 pg/ml milk; lame, 0.972 ± 0.15; $p=0.211$) did not differ between nonlame and lame cows.

Maximum oestradiol concentrations and total or maximum oestrous points were not correlated ($p=0.724$, $r^2=-0.064$; $p=0.958$, $r^2=-0.095$, respectively; Figure 6). In a parallel study, when corresponding daily serum samples were compared, mean milk and serum concentrations were correlated ($r^2=0.91$); however, milk oestradiol was 6-fold lower in concentration (Appendix II).

Cortisol concentrations (Day –6 to 6 from PG injection) during the study were similar for nonlame and lame cows ($p=0.331$; Figure 7).

Oestrous behaviours

Lame cows had a less intense oestrus as revealed by a lower number of total points during oestrus ($p=0.029$; Figure 8). Analysis of the total frequency of each behavioural sign of oestrus revealed that lame cows had less total mounting activity (TMA included: mounting rear and head of another cow and standing to be mounted; $p=0.012$), mounted the rear of other cows less ($p=0.020$), and tended to chin rest less than nonlame cows ($p=0.075$; Table 3). The total frequency of all other behavioural signs of oestrus did not differ between lame and nonlame cows (Table 3; $p\geq0.05$).

The maximum frequency that cows mounted the rear of another cow ($p=0.016$) and the TMA ($p=0.012$) in a 30-minute observation period were both lower in lame cows compared to nonlame cows (Table 3). Maximum values for all other behavioural signs of oestrus were similar between lame and nonlame cows ($p>0.05$; Table 3). The time from first STBM that the maximum frequency of each behavioural sign of oestrus was observed was the same for nonlame and lame cows ($p>0.05$; Table 3).

The overall duration of oestrous activity was the same for nonlame (15.20 ± 1.32; range 3-24 h) and lame cows (12.33 ± 1.26; range 3-21 h; $p=0.163$). Similarly, the durations of TMA ($p=0.386$), mounting the rear of another cow ($p=0.486$) or STBM ($p=0.122$) were shorter for lame cows but not significantly different compared to nonlame cows (Table

3). The durations of mounting the head of another cow (p=0.577), sniffing (p=0.617), chin resting (p=0.252), flehmen (p=0.712) and restlessness (p=0.821) were similar between nonlame and lame cows (Table 3). In contrast, lame cows had a shorter duration of being mounted but not STBM (p=0.042) and TMR (included: being mounted rear and STBM; p=0.054;Table 3).

Analysis of the frequency of behavioural signs of oestrus expressed over time, aligned from first STBM, revealed that lame and nonlame cows expressed most oestrous signs in a similar pattern (p<0.05; Figure 9). Surrounding the first observed STBM, with the exception of flehmen and mounting the head of another cow, lame and nonlame cows demonstrated a period of 'elevated frequency' in the expression of a behavioural sign of oestrus (p>0.05; Figure 9). Lame cows had a shorter (in 3-h intervals) elevated frequency of sniffing (6 ± 3 h shorter; p<0.05), chin resting (6 ± 3 h shorter; p<0.05), restlessness (3 ± 3 h shorter; p<0.05), mounting the rear of another cow (15 ± 3 h shorter; p<0.05), mounted but not STBM (12 ± 3 h shorter; p<0.05), TMA (3 ± 3 h shorter; p<0.05) and TMR (3 ± 3 h shorter; p<0.05; Figure 9c,e,b,f,l,h,k, respectively). Lame and nonlame cows had the same duration of elevated frequency of STBM (12 ± 3 h; p<0.05) and total points acquired (9 ± 3 h, p<0.05; Figure 9l,a respectively). Mounting the head of another cow (p=0.140) or flehmen (p=0.160) did not exhibit periods of elevated frequency and therefore could not compared between lame and nonlame cows; Figure 9j,d, respectively). Similar to the total frequencies, lame cows had a lower average frequency over time of mounting the rear of another cow (p=0.022; Figure 9f), TMA (p=0.012; Figure 9h), and lame cows received lower average total points (p=0.029; Figure 9a). The average frequency of TMR and chin resting over time by lame cows also tended to be lower than for nonlame cows (p=0.095, p=0.075; Figure 9k,e, respectively). The average frequency of all other behavioural signs of oestrus over time from first STBM did not differ between lame and nonlame cows (p>0.05; Figure 9).

The proportion of cows that expressed a particular behavioural sign of oestrus at least once in a 3-h interval from first STBM is shown in Figure 10. A smaller proportion of lame cows received points 3 h prior to the first STBM (p=0.022). Similarly, there was a smaller proportion of lame cows that displayed chin resting 3 h prior to the first observed STBM (p=0.037). Following the first observed STBM, the proportion of lame cows mounting the rear of another cow was lower within 3 h compared to nonlame cows (p=0.048) and at 3 and 6 h following the first STBM, the proportion of lame cows exhibiting STBM tended to be lower than nonlame cows (p=0.095; p=0.056; respectively). The proportion of lame cows that were mounted (TMR) also tended to be lower 3 h following the first observed STBM (p=0.095). For all other behavioural signs of oestrus, there was no difference between lame and nonlame cows (p>0.05; Figure 10).

Discussion

In general, all behavioural signs of oestrus gradually increased and decreased around a period of elevated activity concurrent with the first recorded STBM. The total duration of oestrus was not significantly different between lame and nonlame cows. Similarly, in a study that used the same scoring method for oestrus detection in crossbred cows in tropical India, mild lameness did not affect the overall duration of oestrus (18.7 ± 1.2 *versus* 17.2 ± 0.9 h; (Sood & Nanda, 2006). The range and total duration of oestrus reported in the current study was comparable to other studies in nonlame cows that used the same scoring method for oestrus detection (Lyimo et al., 2000; Roelofs et al., 2004; Van Vliet & Van Eerdenburg, 1996). Therefore, lameness does not affect the time period in which lame cows could be detected in oestrus.

Although lameness does not affect the duration of oestrus, lame cows expressed oestrus with less intensity compared to nonlame cows. Moreover, the different behavioural signs of oestrus, primary or secondary, were affected to varying degrees by lameness. In brief, lameness had an impact on general mounting activities (mounting and being mounted), specifically mounting the rear of another cow, standing to be mounted (STMB), being mounted but not STBM and several secondary behavioural signs of oestrus including, chin resting, sniffing and restlessness. The only two behavioural signs of oestrus that appeared to be unaffected by lameness were mounting the head of other cows and the flehmen response. In contrary to the present study, (Sood & Nanda, 2006) stated that mild lameness did not have an impact on oestrus intensity (1100 ± 179 *versus* 1610 ± 357 total points). However, the authors did state that lameness alters certain characteristics of oestrus, so that oestrus of a shorter duration was observed more often in mildly lame cows and mildly lame cows exhibited fewer standing events. The diminished oestrus intensity in lame cows in the present study was reflected in a decrease in the total amount of certain behavioural signs of oestrus, the total period they were displayed for and/or the time they were displayed at an elevated frequency. Lame cows were involved in fewer mounting activities over the course of oestrus and the total amount of time lame cows maintained a high frequency of mounting activities was shorter compared to nonlame cows. However, the total duration of mounting activities did not differ between lame and nonlame cows; therefore, similar to the total duration of oestrus, lameness had no affect on the duration of mounting activities but did affect their frequency. This suggests that lame cows do mount other cows but are unable or not motivated to maintain high frequencies over long periods as observed in nonlame cows. This reduction probably leads to lame cows not being detected in oestrus. The reason for the reduction in mounting activity may simply be because it is too painful to mount another cow. Additionally, lame cows may have a less chance of expressing mounting behaviours. Cows in oestrus are restless and have increased activity levels (Roelofs et al., 2005a). Cows in oestrus tend to form very mobile sexually active groups (*personal observation*). As the number of cows participating in the sexually active group increases, so does the chance to display signs of oestrus resulting in an increase in oestrus intensity (Roelofs et al., 2005b). In the present study, lame cows were restless for the same duration as nonlame cows; however, the duration of maximum restless was longer in nonlame cows. Therefore, lame cows may not have been moving with the sexually active group and thus had no partners with which to engage in mounting behaviour. In a parallel study in this same group of cows, we have shown that lame cows spend a greater proportion of their time time lying down and a smaller proportion of time walking and expressing an oestrous behaviour and compared to nonlame cows (Chapter 3). Furthermore, lame cows may not be as sexually motivated or rewarded by pheromones to express mounting behaviour.

Lameness also affected the number of mounts received. Lameness shortened the total period when cows were mounted, shortened the period of being frequently mounted and tended to decrease the proportion of lame cows that mounted in the 3 hours after the first STBM. The total number of times cows STBM was not significantly different between lame and nonlame cows, contrary to the findings of (Sood & Nanda, 2006). In the present study, lameness did not affect the total period of time lame cows were seen STBM, or the duration of elevated frequency of STBM. However, lameness did tend to affect the proportion of lame cows that STBM following the first observed STBM, i.e., fewer lame cows STBM at 3 and 6 hours following the first STBM. This implies that the only effect lameness had on STBM was that a lower proportion of lame cows STBM for a shorter period. Indeed, lameness had a negative impact on being mounted. Possibly because it may be painful and/or lame cows are too distant from the sexually active group. However, lame cows STBM as frequently as nonlame cows, implying that they are willing to be mounted but overall they receive fewer attempts at being mounted. Perhaps lame cows do not solicit mounting in the same way as nonlame cows and possibly lame cows produce less pheromones so they are not as 'attractive' as nonlame cows, or the lame cows may be emitting 'stress-related' pheromones. Indeed, there is evidence to indicate that heifers perceive an increased state of stress in herd-mates by olfactory cues (Boissy et al., 1998).

Previously, we have shown that the frequency of sniffing when cows were first detected in oestrus, was lower in severely lame cows (Chapter 1, Walker et al., 2005). However in the present study, the total amount of sniffing the vulva of another cows throughout oestrus was similar for lame and nonlame cows but lameness did decrease the duration of elevated frequency of sniffing around the first STBM. (Sood & Nanda, 2006) also recorded fewer 'sniffs' by lame cows (lame 13.8 ± 1.7 *versus* nonlame 16.2 ± 2.6), but as in the present study the difference was not statistically significant. However, lame cows chin rested with less overall frequency and for a shorter period of elevated frequency compared to nonlame cows. Lameness also decreased the proportion of cows that chin rested 3 hours prior to the first observed STBM. Therefore, lameness does affect secondary signs of oestrus and in particular chin resting. Similar to mounting and being mounted this may be because lame cows are distant from the sexually active group. The purpose of chin resting and sniffing may be to permit the transmission of chemical signals or pheromones between oestrous herd-mates. The receipt of pheromones is important to drive oestrous behaviour in a positive feedback fashion. It has been hypothesised in sheep, that an increased "dopaminergic reward" is stimulated by pheromones during initial sexual interactions, reinforcing the expression of further oestrous behaviour (Fabre-Nys et al., 2003). However, the exact control of the "reward" mechanism is not clear at present. Lame cows may be unable to receive the same level of positive feedback and, therefore, express less intense oestrus. Overall from a behavioural point of view, lameness may have an impact on reproductive efficiency by decreasing oestrous intensity and thus increasing the chance of a missed or inappropriately timed insemination.

In general, oestrous behaviour is the result of the final ovarian follicle maturation, increasing concentrations of oestradiol and, in the presence of very low concentrations of progesterone, oestradiol acts on the hypothalamus to induce oestrus (Allrich, 1994). Lameness, however, was not associated with abnormal maximum oestradiol concentrations or abnormal oestradiol profiles prior to oestrus. Furthermore, maximum oestradiol concentrations were not related to total or maximum oestrous intensity (points). These results are in agreement with previous studies (Chapter 1, Walker et al., 2006). This would suggest the lower oestrous intensity observed in severely lame cows was not due to low oestradiol concentrations.

In addition to oestradiol, progesterone exposure prior to oestrus is important. Prior progesterone facilitates the actions of oestradiol (Allrich, 1994; Stevenson et al., 1989; Melampy et al., 1957; Carrick & Shelton, 1969; Bell et al., 1983). The results from the present study highlight the importance of progesterone exposure prior to oestrus. The lower oestrus intensity observed in lame cows may be related to abnormal prior progesterone exposure. In the ewe, prior progesterone priming is not only essential for the display of oestrus (Karsch et al., 1980; Fabre-Nys & Martin, 1991a) but increases the intensity of oestrus expression (Fabre-Nys & Martin, 1991b). Progesterone increases the number of oestradiol receptors in the mediobasal hypothalamus and increases sensitivity to oestradiol (Blache et al., 1994; Blache et al., 1991). Similarly, in cattle, progesterone regulates the occurrence of oestrous behaviour (Carrick & Shelton, 1969; Melampy et al., 1957; Bell et al., 1983). In the present study, lame cows had lower maximum progesterone concentrations and lower progesterone concentrations for a 6-day period prior to oestrus confirming earlier work (Chapter 1, Walker et al., 2006). Therefore similar to sheep, the intensity of oestrus expression in cattle is probably regulated by prior progesterone exposure.

In a simultaneous study on this same group of cows, ovulation was confirmed by ultrasound in all cows seen in oestrus, and all cows that were not seen in oestrus did not ovulate (Morris et al., 2006). This was reflected in prolonged high oestradiol concentrations, as cows that were not seen in oestrus and had prior progesterone exposure, had higher oestradiol concentrations on Day 5 following PG injection, presumably this suggests that they had an absence or insufficient LH surge and therefore oestradiol concentrations remained elevated. In this study lame cows were just as likely to be observed in oestrus following progesterone exposure compared to nonlame cows, therefore following the commencement of luteal activity (i.e. progesterone exposure) lameness has no affect on the incidence of oestrus.

When profiles were aligned from the PG injection there were more lame cows with basal progesterone concentrations following the GnRH injection compared to nonlame cows, therefore at that at the time of oestrous synchronization (~30 to 75 days postpartum) more nonlame cows possessed luteal structures, indicative of ovarian cyclity.

In this study, following oestrous synchronization in postpartum cows fewer lame cows were observed in oestrus compared to nonlame cows. This contradicts earlier studies in which lameness was not associated with the incidence of oestrus (Chapter 1, Walker et al., 2005). In the present study, some cows had low continuous progesterone concentrations following the GnRH injection, but these cows would not have been included in earlier studies (Chapter 1), because only cows that were seen in oestrus with prior progesterone were monitored. When cows with low prior progesterone were removed from the present study, lameness was no longer associated with the incidence of oestrus as observed in Chapter 1.

Resting cortisol concentration around oestrus did not differ between lame and nonlame cows in the present study in agreement with earlier studies (Chapter 1, Walker et al., 2006). Lameness is a painful and stressful process characterized by hyperalgesia (Whay et al., 1997). Stress can disrupt normal reproductive function by reducing the pulsatility of GnRH/LH by actions at both the hypothalamus and pituitary gland ultimately depriving the ovarian follicle of adequate support (Dobson & Smith, 2000; Caraty et al., 2002). Chronic stress and pain is also often associated with disturbances of the hypothalamic-pituitary-adrenal axis, resulting in both functional and structural modifications and long-term alterations in neuroendocrine reactivity to subsequent stressors (Ostrander et al., 2006; Ulrich-Lai et al., 2006b). Lame cows may have adapted to the stress and pain of their condition (Kant et al., 1987). Therefore, although cortisol concentrations did not differ with lameness, the role of dysfunction of the hypothalamic-pituitary-adrenal axis cannot be excluded.

Lame cows in the present study were also more likely to have a low BCS and fewer cows with low BCS were seen in oestrus. Cows that can maintain body condition postpartum are associated with shorter intervals to the first postpartum oestrus (Rutter & Randel, 1984; Hurnik, 1987) and severe negative energy balance affects LH pulsatility and/or ovarian steroid synthesis (Butler, 2000), both of which have a negative impact on oestrOus expression (Caraty et al., 2002). All cows strive to deal with a negative energy balance after calving, and as suggested several years ago, any stressor (such as lameness) that disrupts this knife-edge balance, will ultimately lead to impaired reproductive function including poor expression of oestrus (Dobson & Smith, 2000; Dobson & Alam, 1987).

In conclusion, lameness does not shorten the duration of oestrus or the incidence of oestrus following progesterone exposure but lameness does diminish oestrous intensity through a reduction in the expression of several primary and secondary behavioural signs of oestrus. The decreased oestrus intensity in lame cows is associated with poor body condition score and abnormally low progesterone profiles prior to oestrus but not with abnormal oestradiol or cortisol profiles.

Acknowledgements

The authors wish to thank farm staff for their cooperation and support with the study. Also, thank you to Hilary Pursell for technical assistance and Coralie Munro for supplying the EIA reagents. A special thanks to Donald Tyson and Paul Fox for their assistance with the project. Scholarships to SLW were provided by Natural Sciences and Engineering Research Council of Canada (NSERC), Universities UK and University of Liverpool and MJM is Wellcome Trust Clinical Fellow.

Table 1. Point scale for observed behavioural signs of oestrus [1]

Oestrus Signs	Points
Flehmen	3
Restlessness [2]	5
Sniffing the vulva of another cow	10
Mounted but did not stand	10
Resting chin on the back of another cow	15
Mounting the rear of other cows	35
Mounting the head side of another cow	45
Stood to be mounted (STBM)	100

Each time an oestrus sign was observed, the assigned number of points were recorded

[1] modified after (Van Eerdenburg et al., 1996) by removing cajoling and mucous vaginal discharge and adding flehmen response

[2] can only be recorded once during a single 30-minute observation period

Table 2. Lameness scoring scale[1]

Lameness Score	Description	Assessment Criteria		
		Stands	**Walking**	**Gait**
1	Normal	Level back posture	Level back posture	Normal
2	**Moderately Lame**	Level back posture	Arched back	Normal to short-striding
3	**Severely lame**	Arched posture	Arched back	Takes one step at a time/reluctance to bear weight on one or more limbs/feet

[1] modified after (Sprecher et al., 1997) 5 point scale, in which scores 1 and 2 are comparable and 3-5 are grouped and equivalent to score 3 as defined above

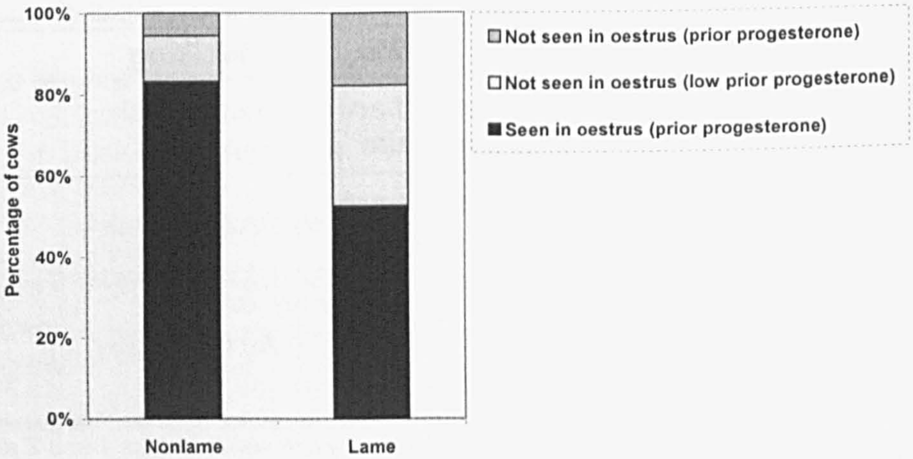


Figure 1. The percentage of nonlame (n=18) and lame cows (n=34) seen in oestrus (with prior progesterone exposure) and not seen in oestrus (with prior progesterone or with low prior progesterone exposure).

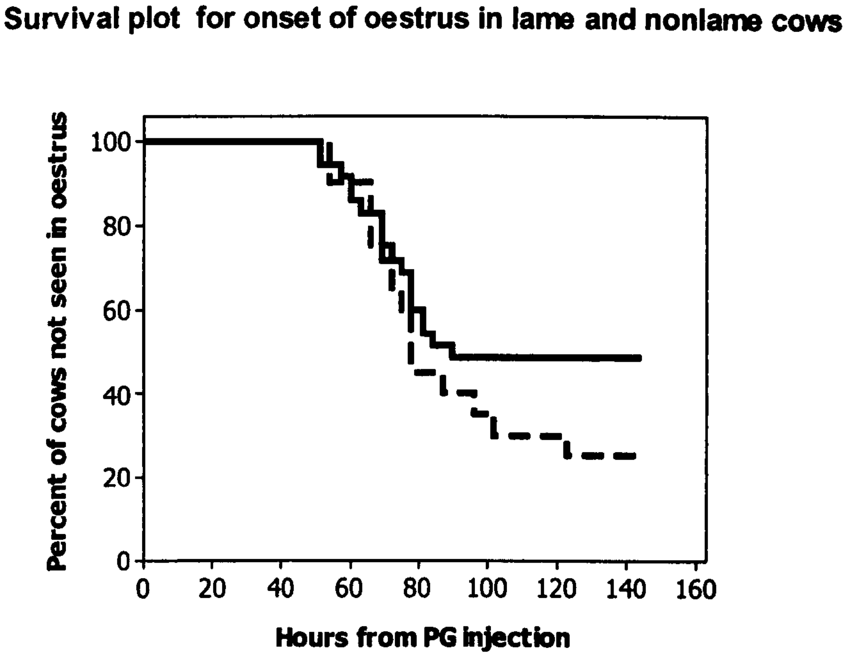


Figure 2. The Kaplan-Meier survival plot of the onset of oestrus after PG in lame (n=18; solid line) and nonlame cows (n=15; broken line).

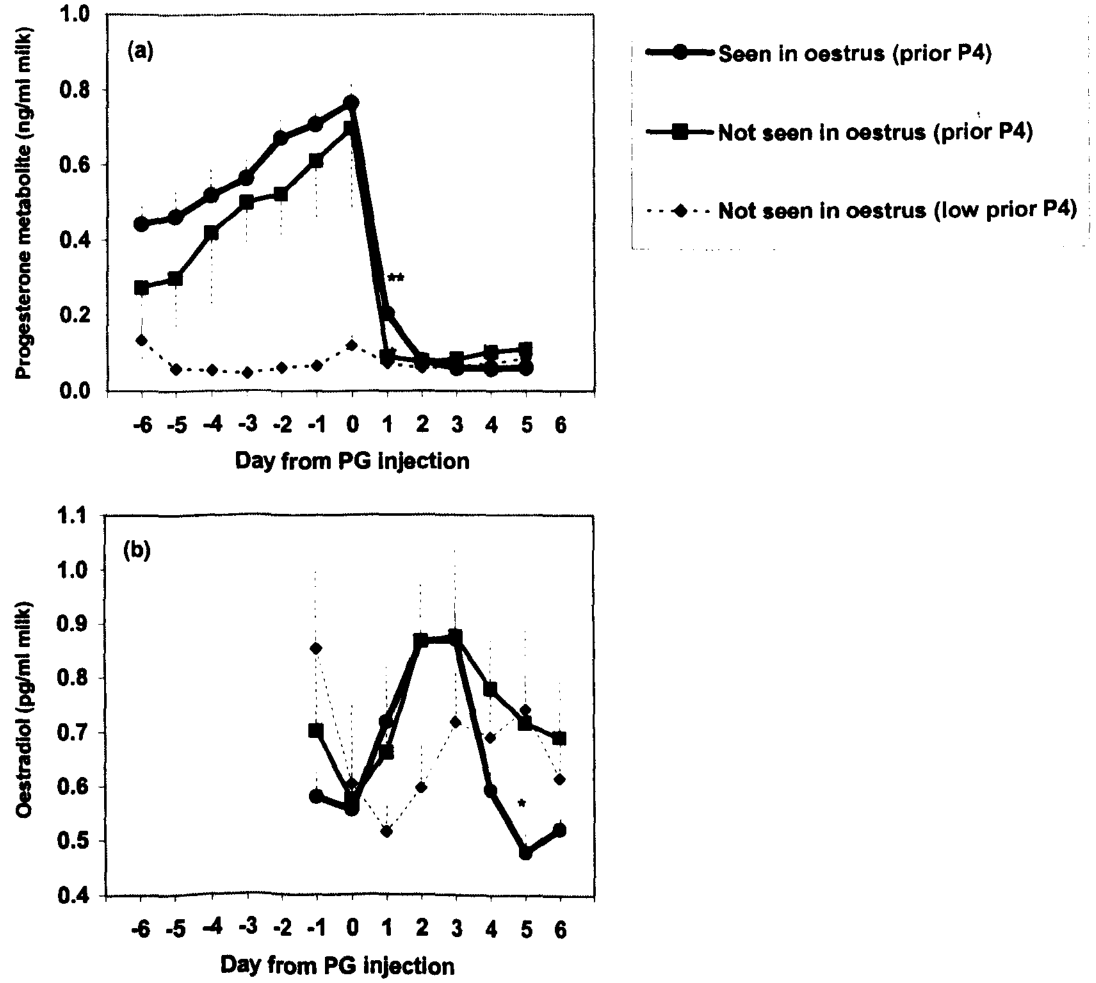


Figure 3. Progesterone metabolite ng/ml milk (a) and oestradiol pg/ml milk (b) profiles for all cows seen in oestrus (n=33 with prior progesterone exposure) and those not seen in oestrus (n=7 with prior progesterone exposure and n=12 with low prior progesterone) aligned from Day to PG injection (*p<0.05; ** p<0.10).

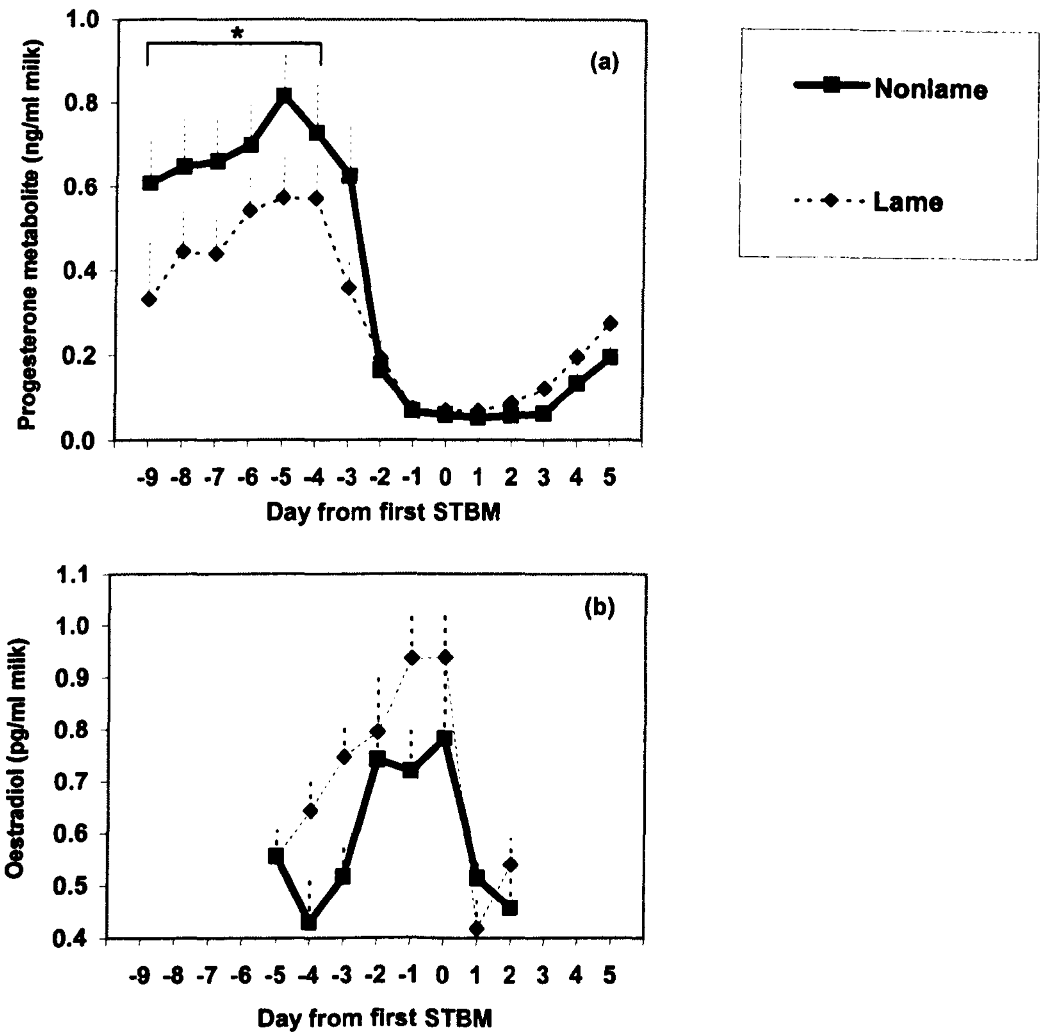


Figure 4. Progesterone (a) metabolite ng/ml milk and oestradiol (b) pg/ml milk profiles from nonlame (n=15) and lame (n=18) cow seen in oestrus aligned to first STBM (*$p<0.05$)

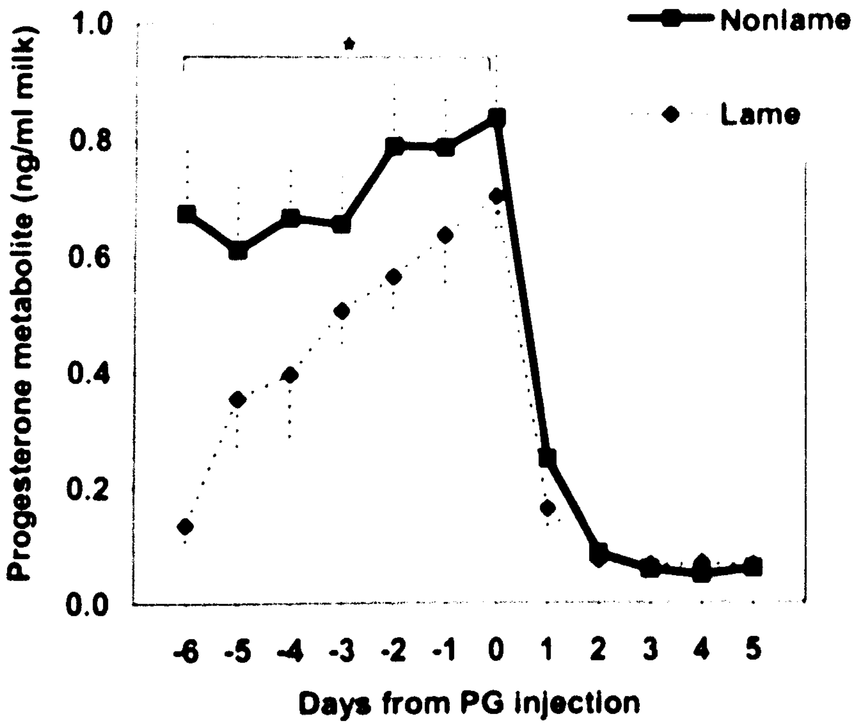


Figure 5. Progesterone metabolite concentrations (ng/ml milk) from nonlame (n=15) and lame (n=18) cows seen in oestrus aligned to day of PG injection (*$p<0.05$).

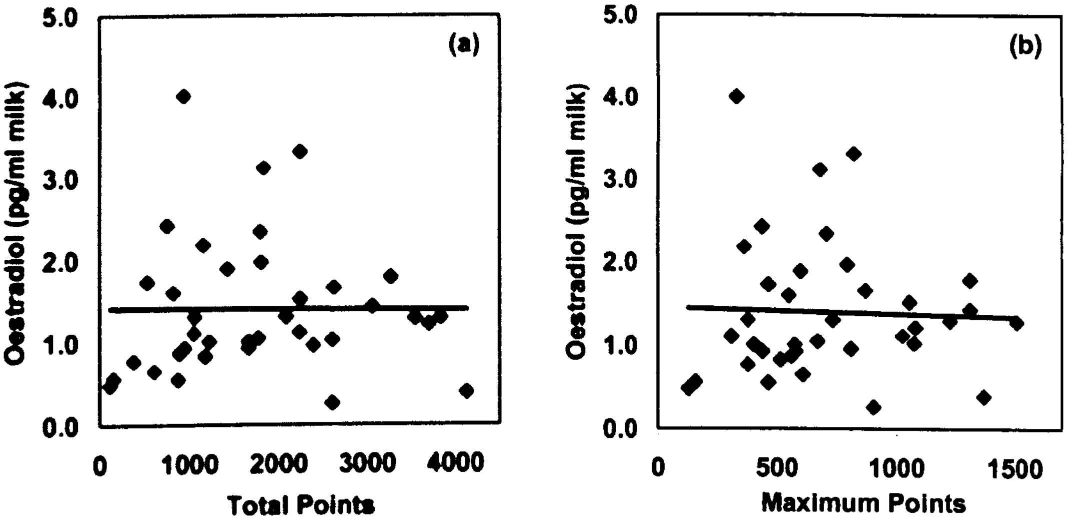


Figure 6. Correlations between maximum oestradiol (pg/ml milk) and (a) total points received during oestrus (p=0.724, r^2=-0.064) or (b) maximum oestrus points (p=0.958, r^2=-0.095) in one 30-minute observation (n=33)

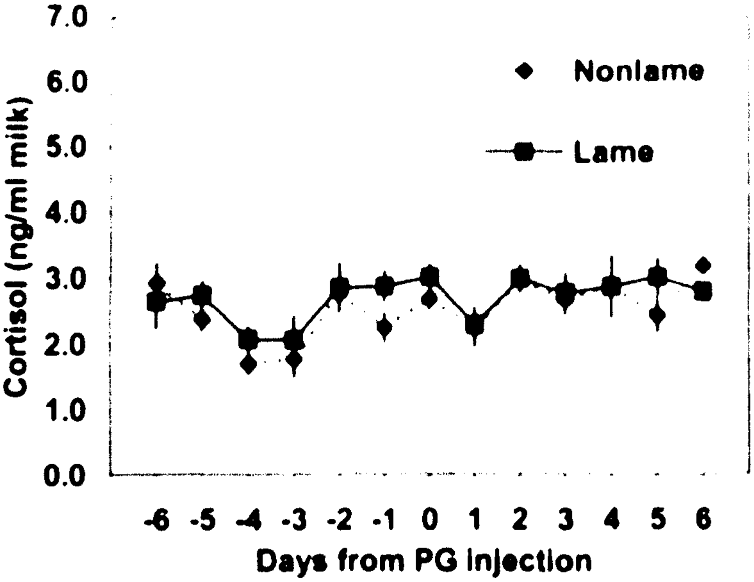


Figure 7. Cortisol concentrations (ng/ml milk) from nonlame (n=15) and lame (n=18) cows aligned to day of PG injection

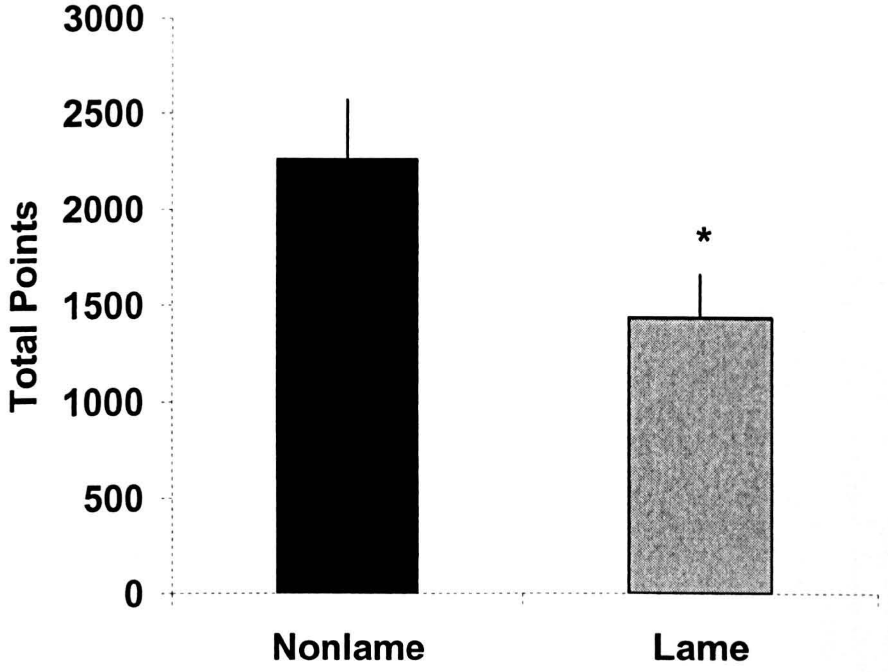


Figure 8. Total oestrus points received (± SEM) in nonlame (n=15) and lame (n=18) (*p=0.029)

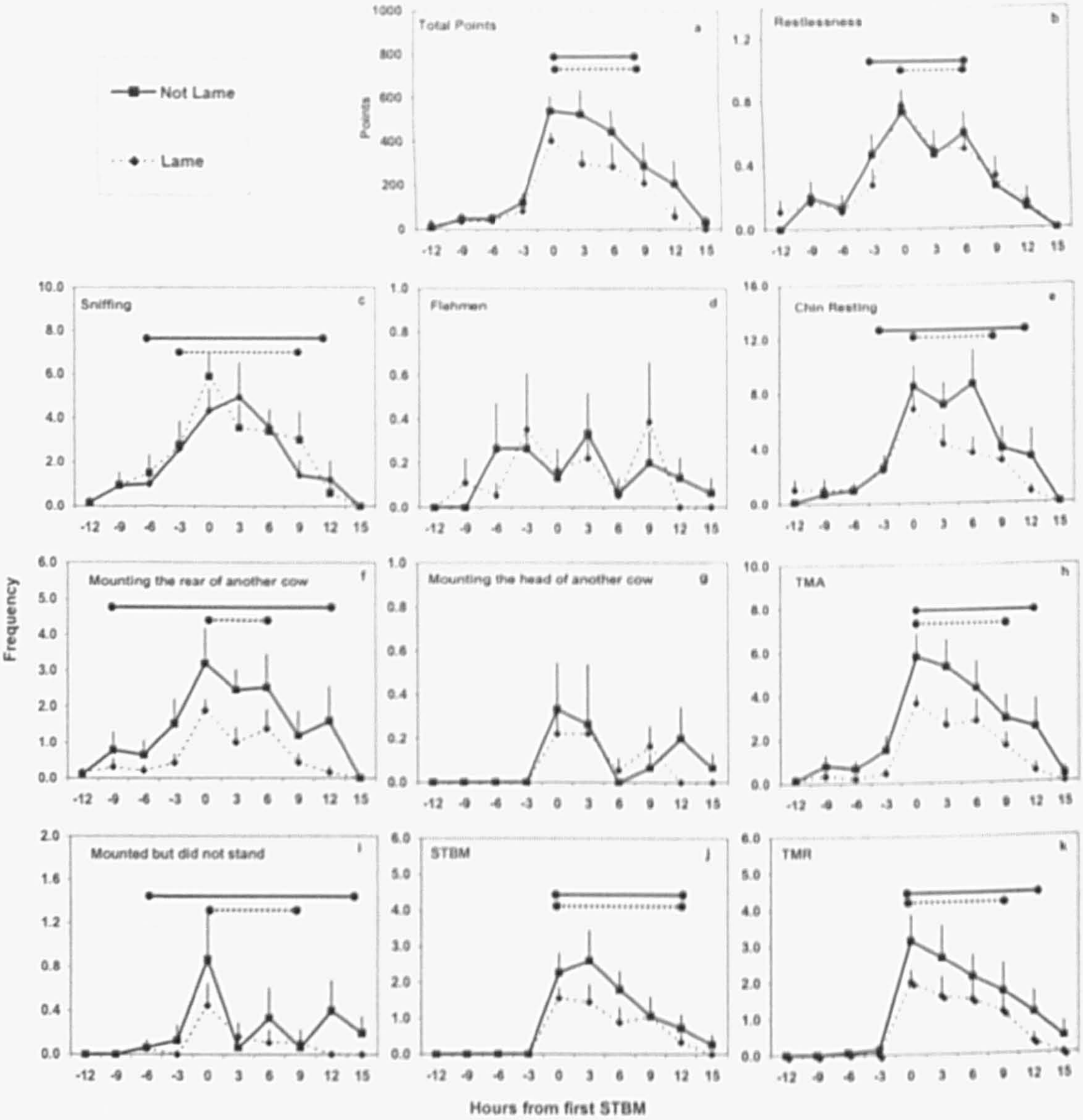


Figure 9. The average frequency (per 3-h interval) in lame (n=18) and nonlame (n=15) cows of total oestrus points (a) and the following behavioural signs of oestrous: restlessness (b), sniffing (c), Flehmen (d), chin resting (e), mounting the rear (f) and head of another cow (g), TMA (h), mounted but did stand (i), STBM (j) and TMR (k) over time aligned to first STBM. Horizontal bars represent the duration of 'high frequency' for each behaviour in lame and nonlame cows.

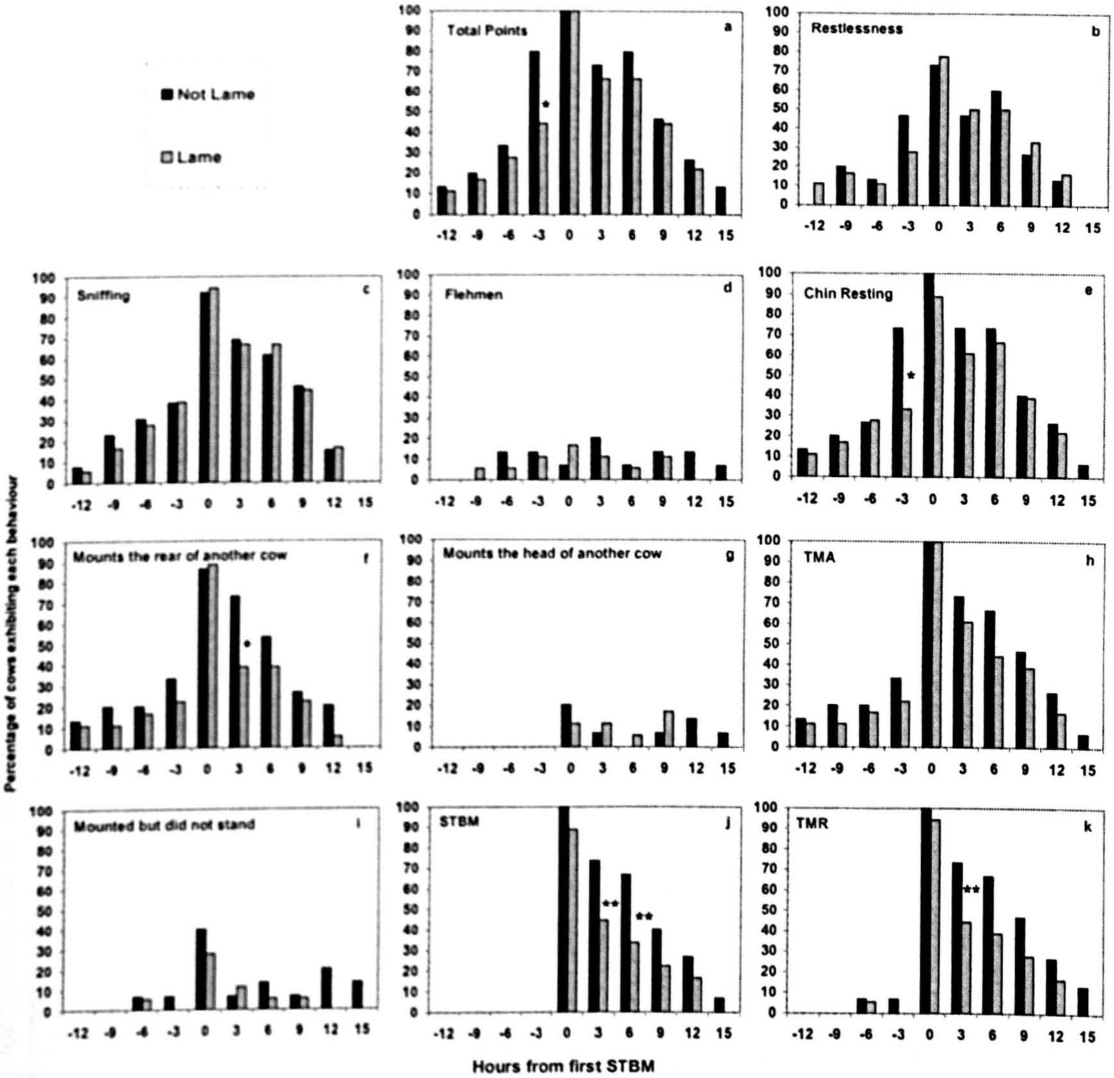


Figure 10. The proportion (%) of lame (n=18) and nonlame (n=15) cows that acquired >100 points in total (a) or expressed a behavioural sign of oestrus [including: restlessness (b), sniffing (c), Flehmen (d), chin resting (e), mounting the rear (f) and head of another cow (g), TMA (h), mounted but did not stand (i), STBM (j) and TMR (k)] over time (3 hour intervals from) aligned from the first observed STBM (*p<0.05, **p<0.10).

Table 3. Mean ± SEM (range) of the total frequency, maximum frequency, time of maximum count and duration of behavioural signs of oestrus in nonlame and lame cows

Oestrous Signs		Total Frequency		Max Frequency [a]		Time [b] of Max Frequency		Duration (hours)	
Total mounting activity (TMA)	Nonlame	**24.40 ± 4.13**	(4-52)	**9.47 ± 1.33**	(4-18)	3.20 ± 1.19	(-3-12)	13.40 ± 1.40	(3-24)
	Lame	**12.50 ± 2.14***	(1-37)	**5.61 ± 0.82***	(1-15)	1.33 ± 1.36	(-12-9)	11.17 ±1.18	(3-18)
Mounting rear of another cow	Nonlame	**14.13 ± 3.41**	(1-42)	**5.93 ± 1.10**	(1-15)	1.80 ± 1.40	(-9-12)	11.40 ± 1.64	(3-24)
	Lame	**6.06 ± 1.12***	(0-19)	**3.06 ± 0.383***	(0-6)	-0.88 ± 1.25	(-12-6)	9.33 ±1.26	(0-18)
Mounting head side of another cow	Nonlame	0.93 ± 0.49	(0-7)	0.28 ± 0.15	(0-2)	7.00 ± 3.61	(0-12)	1.00 ± 0.56	(0-6)
	Lame	0.68 ± 0.40	(0-7)	0.39 ± 0.18	(0-3)	3.60 ± 1.75	(0-9)	1.67 ± 0.81	(0-12)
Mounted but did not stand	Nonlame	2.13 ± 0.74	(0-10)	1.27 ± 0.43	(0-6)	0.90 ± 1.55	(-6-12)	**5.20 ± 1.53**	(0-15)
	Lame	0.94 ± 0.41	(0-7)	0.68 ± 0.21	(0-3)	-1.50 ± 0.80	(-6-0)	**1.83 ± 0.69***	(0-12)
STBM	Nonlame	9.33 ± 2.01	(2-26)	4.47 ± 0.84	(1-12)	3.40 ± 1.01	(0-12)	10.00 ± 1.16	(3-18)
	Lame	5.78 ± 1.41	(0-18)	3.22 ± 0.68	(0-10)	2.06 ± 0.85	(0-9)	6.83 ±1.26	(0-15)
Total mounting activity received (TMR)	Nonlame	11.47 ± 2.56	(2-36)	5.28 ± 0.87	(2-13)	2.40 ± 0.89	(-3-9)	**11.00 ± 1 .23**	(3-18)
	Lame	6.72 ± 1.43	(0-21)	4.05 ± 0.81	(0-13)	1.59 ± 0.93	(-6-9)	**7.50 ± 1.22***	(0-15)
Sniffing the vulva of another cow	Nonlame	20.20 ± 3.10	(0-41)	8.47 ± 1.39	(0-21)	0.21 ± 1.52	(-12-12)	13.20 ± 1.20	(0-18)
	Lame	21.89 ± 3.11	(1-49)	9.78 ± 1.13	(1-20)	0.67 ± 1.15	(-9-9)	12.00 ± 1.24	(3-21)
Chin Resting	Nonlame	**36.27 ± 5.57**	(16-78)	14.40 ± 10.39	(5-34)	4.00 ± 1.51	(-9-12)	14.40 ± 1.32	(3-24)
	Lame	**24.39 ± 3.64****	(0-59)	10.39 ± 1.34	(0-23)	2.29 ± 1.37	(-12-12)	12.00 ±1.33	(0-21)
Flehmen	Nonlame	1.48 ± 0.53	(0-7)	0.80 ± 0.28	(0-3)	0.86 ± 2.51	(-6-12)	3.60 ± 1.38	(0-18)
	Lame	1.33 ± 0.46	(0-6)	1.06 ± 0.35	(0-4)	0.63 ± 2.21	(-9-9)	2.33 ± 0.09	(0-15)
Restlessness	Nonlame	3.00 ± 0.29	(1-5)	---------------[c]	--------	-------------------	----------	10.80 ± 1.27	(3-21)
	Lame	2.94 ± 0.383	(0-6)					10.17 ± 1.24	(0-18)

Significantly lower values in lame cows (n=18) compared to nonlame (n=15) are shown in bold (*p<0.05**0<0.10)

[a] Maximum number of events (average ± SEM; range) observed in one 30-minute observation

[b] Hours from first STBM

[c] Assessed only once in one 30-minute observation period

Chapter 3

Chapter 3

Lameness, time budgets, social behaviours and oestrus in dairy cattle

Walker SL, Smith RF, Routly JE, Jones DN, Morris MJ and Dobson H

Key words: lameness, oestrus, time budgets, order, social rank, bite rate, rumination rate, dairy cow, lying, standing, grazing

Abstract

Lameness is an important welfare issue in cattle and is related to poor oestrus expression and poor fertility. Using behavioural scan and focal sampling following oestrus synchronization, 59 milking cows (52.0 ± 1.95 days postpartum) were monitored to achieve the aim of determining the effect of lameness on daily activity budgets, including behavioural signs of oestrus, bite/rumination rates and social behaviours. Cows were scored weekly for lameness for 4 weeks (lame, n=39; nonlame, n=20) and clinical treatments for lameness continued as usual.

Overall, lame cows spent proportionately less time 'elevated' on their feet (49.9 ± 1.3% *versus* 56.2 ± 1.7%) and more time lying down (50.1 ± 1.3% *versus* 43.8 ± 1.7%) compared to nonlame cows (p=0.003). This is reflected in lame cows spending less time walking (2.1 ± 0.3% *versus* 2.8 ± 0.2%; p=0.007) or standing (11.8 ± 0.8% *versus* 14.2 ± 1.1%; p=0.036). There was no difference between lame and nonlame cows in the time spent grazing (33.8 ±1.0% *versus* 35.5 ± 1.5%), drinking (0.81 ± 0.1% *versus* 0.93 ± 0.2%) or ruminating (35.8 ± 0.9% *versus* 33.4 ± 1.9%). Lame cows had a slower bite rate than nonlame cows (lame 53.07 ± 0.30; nonlame 54.86 ± 0.42 bites/min; p=0.000) but there was no difference in rumination rate (nonlame, 59.37 ± 0.33 versus lame, 59.01 ± 0.38 eructations/rumination bout). Equally for lame and nonlame cows, grazing and drinking both steadily increased throughout the day (p=0.000, p=0.000, respectively), lying and ruminating decreased in the evening (p=0.006 and p=0.043, respectively) and standing decreased in the afternoon and evening compared with the morning period (p=0.000). Walking did not vary during the day for lame or nonlame cows. There was no association between social rank (high/medium/low) and lameness (lame n= 3/23/13; nonlame n=3/11/6 cows respectively); however, cows of higher social rank tended to be nearer to the front of the group walking from the field and prior to milking (r^2= -0.261; p=0.077 and r^2=-0.251; p=0.058, respectively). In contrast, lame cows were near the rear of the herd as they left the field later (p=0.000) and entered the milking parlour later (p=0.000) compared to nonlame cows. Of the 59 dairy cows, 37 were seen in oestrus. There was no association between social rank (high/medium/low) and the incidence of

oestrus (not seen in oestrus n=2/14/6; seen in oestrus n=4/20/13, respectively) or intensity of oestrus expression (total points for, low 1428 ± 738, medium 1792 ± 236, high 1820 ± 287 ranking cows). However, cows with a higher social ranking were more restlessness during oestrus (r^2 0.342; p=0.039). Overall, the total proportion of scans in which an oestrous behaviour was observed was very small (<3%) and tended to be smaller for lame (1.75 ± 0.37%) compared to nonlame cows (2.76 ± 0.57%; p=0.090). During the day, the proportions of scan samples in which oestrous behaviours were observed were the same in the afternoon and evening for lame and nonlame cows; however in the morning, lame cows had a lower proportion of observed oestrous behaviours compared to nonlame cows (p=0.002).

In conclusion, although cause and effect are not clear, alterations in time budgets, in particular 6% less standing, 6% more lying, a 3% reduction in bite rate plus the impact on other social and oestrous behaviours highlights the extent to which lameness is detrimental to the welfare of dairy cattle.

Introduction

Lameness in dairy cows is a welfare issue, which is costly for the dairy industry and to the individual cow (Greenough et al., 1997). The mere presence of lameness suggests an individual is not coping successfully with its environment and this is reflected in the poor reproductive performance that is associated with lameness (Collick et al., 1989). Additionally, lame cows have diminished oestrus intensity (Chapter 1, Chapter 2). A less intensive oestrus in lame cows could result in missed or inappropriately timed insemination and poor fertility.

There are many physiological, psychological and environmental factors that influence the intensity of oestrus expression (Orihuela, 2000). In general, cows in oestrus are restless and spend less time lying down (Roelofs et al., 2005a). Some studies have considered the effect of lameness on the individual activity budgets, including the proportion of time spent lying, standing, walking or feeding in lame and nonlame cows (Hassall et al., 1993; Galindo & Broom, 2002; Singh et al., 1993). However, whether such behavioural changes influence oestrus expression in lame cows has yet to be clarified. Thus, one aim of the present study is to determine if lame and nonlame cows differ in their daily activity budgets during oestrus.

In cattle, previous studies suggest a relationship between oestrous expression and social dominance, when higher ranking individuals initiate more mounts and monopolise oestrus cows, preventing other cows from mounting (Hurnik et al., 1975; Wagnon et al., 1966; Kabuga et al., 1992). (Galindo et al., 2000) demonstrated that cows that became lame had a lower rank or index of displacement compared to cows that did not become lame. This suggests there may be a relationship between lameness, low social rank and poor oestrous expression and is another focus of the present study.

Oestrus may also be indirectly influenced by daily management factors. Lameness may have an impact on daily routines such as the order cows are milked, which could influence the time spent waiting to be milked, the time spent standing, and decreasing feeding time following milking. Nutrition may also influence oestrous expression in cows (Orihuela, 2000). Increasing levels of nutrient intake and/or cows that maintain body condition are associated with shorter intervals to the first postpartum oestrus (Rutter & Randel, 1984; Hurnik, 1987). In a previous study, we have shown that lameness is associated with a low body condition score (Chapter 2). This suggests an association between lameness, nutrient intake and oestrous expression.

The aim of the present study was to determine the impact of lameness on the daily movements, social and feeding activities of dairy cows at pasture to explain the reduction in oestrous intensity in lame cows.

Materials and Methods

Experimental design, animals, feeding and housing

The study was conducted on postpartum Holstein-Friesian cows (n = 59) on a UK commercial dairy farm comprising a total of ~200 year-round calving cows. The parity and days postpartum of the study cows was 3.9 ± 0.2 (range 2-10) and 51.8 ± 1.4 (range 30 – 75), respectively. Cows were part of a parallel study (Chapter 2) and were selected based on lameness score (see below) and days postpartum. Oestrus was synchronized in 5 groups of ~12 animals (including at least 4 nonlame) between May and September 2005 (month 1-5; temperatures ranged 5 to 28 °C; Coastal Observatory, Liverpool Bay) using a 100 µg im injection of a gonadotrophin releasing hormone (GnRH) analogue (Buserelin, 2.5ml Receptal®, Intervet Ltd. Bucks, UK) followed by a single 500 µg im injection of a prostaglandin $F_{2\alpha}$ (PG) analogue (cloprostenol, 2ml, Estrumate® Schering-Plough Animal Health, Uxbridge, UK) 7 days later. Animals were at pasture (seasonal ryegrass, Italian ryegrass and white clover) for the duration of the study with additional access to total mixed rations (TMR) inside at a feed-fence after milking twice a day. The average rolling milk yield per cow in the herd was 8500 litres/year.

Time budget and oestrous behaviours

Time budget behavioural observations were conducted while the cows were in the paddocks with the aid of binoculars, plus flashlights for night observations. To ease identification of the cows, identity labels made from waterproof paper (The Waterbook ™, Stowmarket, UK) and black waterproof pen were attached above each shoulder with Kamar® glue (Kamar® Products, Inc. Steamboat Springs, CO, USA).

Behavioural scan samples (Martin & Bateson, 1986) every 15 min were made daily for 5 days following PG injection. Scans were carried out around milking, which took place twice daily at ~6:30am and ~4:30pm and were classed as morning (~3:00 am to ~6:00 am), afternoon (~9:00am to ~4:00 pm) or evening (~6:00 pm to 12:00am). The following 8 behavioural states were recorded: lying down with or without ruminating, standing up with or without ruminating, walking, grazing, drinking or expressing a behavioural sign of oestrus. Oestrus signs included: mounting the rear or head of another cow, receiving mounts but not standing, standing to be mounted (STBM), chin resting on another cow, sniffing the vulva of another cow or Flehmen. In a concurrent study (Chapter 2), the occurrence (yes/no) of oestrus, the total frequency of each behavioural sign of oestrus and the intensity of oestrus were recorded [total points; based on a weighted scoring method (Van Eerdenburg et al., 1996)] were recorded for 30 min every 3 hours for 7 days following PG.

Bite and rumination rate

Focal behavioural observations (Martin & Bateson, 1986) were conducted during the afternoon for 3 days following PG. Observations were carried out in close proximity to the animals, and because of previous studies conducted on the farm the animals were undisturbed by the presence of observers (Chapter 1). Grazing cows were observed for bite rate, calculated as the number of bites per minute recorded during continuous 1-min periods (n=30 observations/cow). Intermittent breaks in bite rate > 5 seconds were considered not to be continuous and interrupted data were discarded. The order in which bite rate was recorded was randomised so that no two observations were made consecutively in one cow. Similarly, rumination rate was recorded in a randomised fashion. Rumination rate was calculated as the number of chews per rumination bout (n=30 observations/cow).

Social order and rank

Observations for social order were calculated in two situations 1) as cows left the field for milking and 2) entered the milking parlour. Chi-Square analysis revealed that there was no association between month (1-5) and the number of lame and nonlame cows, i.e. there were similar proportions of lame and nonlame cows in each month (p=0.714). Therefore, order (on a scale of 0-1) was standardized across studies by calculating the position (first to last) of a cow's order in relation to other cows within the group divided by the total number of cows in that month's group. An average field or milking order was then calculated for each cow. Consequently, cows with a high order were at the rear of the group. Observations (n=10/cow) for field order were carried out for 5 days following PG by an observer who stood at end of the track as the cows passed on their way to the parlour for milking. Observations (n=17/cow) for milking order were collected by an observer standing inside the milking parlour once a day for one week prior to PG as well as twice a day for 5 days following PG.

Focal behavioural observations for social rank within the group were conducted for a 3-day period following PG. Cows were observed while eating a 'buffer' ration at a housed feed fence following morning milking for 12 two-minute intervals per day (n=36 observations/cow). The two-minute observations were randomised so no two observations were made consecutively in one cow. This randomisation allowed study cows to move and re-position themselves between other members of the herd. Interactions were recorded as either a 'win' (a study cow challenged another cow and successfully displaced that cow, or she herself was challenged and was not displaced from feeding) or a 'loss' (a study cow challenged another cow and was not successful at displacing that cow, or she herself was the recipient of a challenge and was displaced from feeding). Social rank was based on an 'index of displacement' as described (Galindo & Broom, 2000) and was calculated as the total number of times a cow displaced another individual (total 'wins') divided by the total number of interactions recorded (total 'wins + losses') for the same study cow. Based on the calculated index of displacement, cows were retrospectively grouped as low (0 - 0.49), medium (0.5 - 0.69) or high (0.7 – 1.0) social rank.

Lameness scoring

In the concurrent study (Chapter 2) individuals were scored for lameness (score 1-3) for 4 weeks prior to the commencement of the study, based on gait and posture while walking and standing, using methods from (Sprecher et al., 1997) and defined in Table 1. Clinical treatment of lameness continued as usual on the farm. Retrospectively, ninety-five percent of individuals had the same or one ± 1 lameness score for the duration of the study and were therefore grouped based on an average lameness score. Any cow with average score of ≥ 2 was considered to be lame and animals were grouped as either nonlame (score of 1; n = 20) or lame (score of 2 or 3; n = 39).

Data analysis

All data are presented as mean ± SEM and were analysed using Minitab 14®. General Linear Model (GLM) ANOVA Post-hoc comparisons were made with Tukey's 95% Post-hoc pairwise comparisons and statistical differences were reported when $p<0.05$; with a tendency being considered as a difference when $0.06>p<0.10$.

Total time spent in a behavioural state in the morning/afternoon/evening period or in total, was calculated as the number of times a behavioural state was observed divided by the total number of observations recorded for each cow and is presented as the proportion of scans (%). Proportion data was then normalized using arcsine–square root transformation (Martin & Bateson, 1986). Comparison of total time spent in the different behavioural states between lame and nonlame cows were compared with a GLM ANOVA. The model included the fixed factors of lameness, month (1-5) and the interaction between lameness and month. Comparisons of time spent in the different behavioural states in different periods (morning, afternoon and evening) and lameness were analysed with a GLM ANOVA with the fixed factors of lameness, month, period and the interaction of lameness and period.

Associations between lameness (nonlame/lame), social rank (high/medium/low) and oestrus (yes/no) were analysed using Chi-square tests. Pearson's correlation was used to assess the relationship between: 1) milking and field order 2) social rank (index of displacement value) and milking or field order and 3) social rank (index of displacement value) and the total frequency of behavioural signs of oestrus recorded. To compare the effect of social rank (high/medium/low) on oestrus intensity (total points), a GLM ANOVA with the fixed factor of social rank was used.

A GLM ANOVA was used to compare bite/ruminations rates and field/milking order between lame and nonlame cows. The model included the fixed factors of lameness and month and cow ID with lameness and month also nested within cow ID (as each cow was only present in one category within each factor) and the interaction of lameness and month.

Results

Time budgets

Activity budgets comparing the total time spent performing each of the eight behavioural states recorded outside in the field are presented in Figure 1. Cows spent a large proportion of time grazing and this was the same for lame and nonlame cows ($p=0.388$;

Figure 1). Similarly, there was no difference in the time spent drinking between lame and nonlame cows (p=0.885; Figure 1). The total time spent ruminating (includes: standing or lying ruminating) did not differ between lame (35.8 ± 0.9%) and nonlame (33.4 ± 1.9%) cows (p=0.270). Lame cows spent a smaller proportion of time walking compared to nonlame cows (p=0.007; Figure 1). Lame cows spent a smaller proportion of time standing (includes: standing with or without rumination; lame 11.8 ± 0.8%; nonlame 14.2 ± 1.1%; p=0.036). Lame cows tended to spent less time standing ruminating (p=0.080) and standing without ruminating (p=0.083; Figure 1). Lame cows lay down for longer (includes: lying with or without rumination; lame 50.1 ± 1.3%; nonlame 43.8 ± 1.7%; p=0.003; Figure 2) and lame cows tended to lie down with (p=0.059) or without (p=0.051) ruminating more than nonlame cows (Figure 1). Overall, lame cows spent less time 'elevated' on their feet (includes the behavioural states: standing with or without ruminating, drinking, oestrous behaviour, grazing or walking; lame 49.9 ± 1.3%; nonlame 56.2 ± 1.7%; p=0.006; Figure 2) compared to nonlame cows.

The activity budgets of the various behavioural states over the course of the day (morning, afternoon and evening) for lame and nonlame cows are presented in Figure 3. There was no difference in the proportion of scans between lame and nonlame cows that were seen grazing or drinking (p=0.732, p=0.794, respectively). Grazing and drinking both steadily increased throughout the day (p=0.000, p=0.000, respectively) and this was similar for lame or nonlame cows (p=0.340 and 0.776, respectively). Similarly, there was no difference in the average proportion of scans for lame and nonlame cows that were seen ruminating (p=0.913); however, the proportion of time spent ruminating decreased in the evening compared to the morning (p=0.001) and afternoon (p=0.040) for both lame and nonlame cows (p=0.468). The overall proportion of scans lying (including with or without ruminating) was greater in lame cows (p=0.036); and there was less lying time in the evening compared to the morning (p=0.023) and afternoon (p=0.016). The overall proportion of scans in which standing (including with or without ruminating) was observed was lower in lame cows (p=0.036); and was lower in the afternoon (p=0.000) and evening (p=0.000) compared to the morning for both lame and nonlame cows (p=0.433). Lame cows tended to walk less (p=0.050) but the proportion of time spent walking did not change throughout the day (p=0.524) for both lame and nonlame cows (p=0.245).

Oestrous behaviour and time budget

Proportionately, equal numbers of cows were seen in oestrus between lame and nonlame cows (lame 22/39; nonlame 15/20; p=0.162). Compared to the other seven behavioural states that were recorded, the total proportion of scans in which an oestrous behaviour was observed was very small (<3 %; Figure 1) and tended to be lower in lame compared to nonlame cows (lame 1.75 ± 0.37%; nonlame 2.76 ± 0.57%; p=0.090; Figure 1).

The expression of oestrous behaviour during the day (morning, afternoon and evening), revealed that the proportion of scans in which an oestrus behaviour was observed was lower in lame compared to nonlame cows (p=0.003). Nonlame cows expressed oestrus more frequently in the morning compared to the nonlame cows (p=0.002). During the afternoon and evening, the proportion of scans in which an oestrous behaviour was observed was similar in lame and nonlame cows (p=0.999 and p=0.500, respectively; Figure 4).

Social order, rank and oestrous behaviour

Lame cows left the field later (p=0.000) and entered the milking parlour later (p=0.000) than nonlame cows (Table 2). There was a high correlation within cows (n=59) between average order out of the field and average milking order (r^2= 0.704; p=0.000; Figure 5).

There was no association between social rank (high/medium/low) and the occurrence of lameness (lame n= 3/23/13; nonlame n=3/11/6 cows respectively; p=0.691). Cows with a higher social rank (index of displacement) tended to be nearer to the front of the group for both milking order (r^2=-0.251; p=0.058) and order out of the field (r^2= -0.261; p=0.077).

There was no association between social rank group (high/medium/low rank) and the observation of oestrus (high/medium/low rank; not seen in oestrus n=2/14/6; seen in oestrus n=4/20/13 respectively; p=0.768) or intensity of oestrus expression (total points for, low 1428 ± 738, n=6; medium 1792 ± 236, n=34; high 1820 ± 287, n=19 ranked cows; p=0.808). However, cows with a higher social ranking (index of displacement) were more restless during oestrus (r^2 0.342; p=0.039; Figure 6). Other behavioural signs of oestrus were not correlated with social rank (p>0.05; Table 3).

Bite and rumination rate

Lame cows had a lower bite rate than nonlame cows (lame 53.07 ± 0.30 *versus* nonlame 54.86 ± 0.42 bites/min; p=0.000). With the exception of September, there was also a gradual increase in bite rate over the summer months (p=0.000; Figure 7). Conversely, there was no difference in rumination rate between groups (nonlame, 59.37 ± 0.33 *versus* lame, 59.01 ± 0.38 chews/rumination bout; p=0.117).

Discussion

The present study demonstrates that lame cows spend less time elevated on their feet. This is due in part to spending less time standing and walking compared to nonlame cows. Contrary to this, (Hassall et al., 1993) reported lame and nonlame cows at pasture did not differ in the amount of time spent standing or walking. The difference between the current study and (Hassall et al., 1993) is important, as cows in the present study were more likely to be restless as observations were done during the week of oestrus synchronization. The differences observed between lame and nonlame cows is probably the result of lame cows not being as motivated as nonlame cows to be up on their feet and restless. We have already shown in this same group of cows that the lame cows had a shorter duration of elevated restlessness associated with oestrus (Chapter 2). Thus indirectly lameness has an impact on oestrus by reducing the amount of time spent standing and walking.

Similar to the results of (Hassall et al., 1993), lame cows in the present study spent more time lying down compared to nonlame cows. Again, in this same group of cows, we have demonstrated that lame cows have a less intense oestrus (Chapter 2). The present study confirms that lame cows lie down longer and therefore is the likely reason for the observation of a low intensity oestrus. However, the underlying causes, pain and/or an altered physiological control of overt oestrous behaviour, still require further investigation.

Different to the lame and nonlame cows at pasture in the present study, cubicle-housed cows had similar total amount of lying and standing time (Galindo & Broom, 2002; Singh et al., 1993). Although these differences may have been exacerbated by oestrus synchronization in the present study, daily activities such as standing, walking and lying differ in different environments. Indeed, (Singh et al., 1994; Singh et al., 1993) reported that cows in straw yards lie down for longer compared to cows housed in cubicles. Therefore, the environment where observations are conducted will have an impact on daily activity budgets and should, therefore, be taken into account when making comparisons between studies.

Similar to cubicle-housed cows, lame and nonlame cows at pasture spent a similar proportion of time ruminating (Singh et al., 1993). There was no difference between lame and nonlame cows in the proportion of time spent drinking while at pasture and the results were comparable to (Hassall et al., 1993). Lame and nonlame cows also grazed for the same proportion of time; however, lame cows had a lower bite rate. (Hassall et al., 1993) also reported a lower bite rate for lame cow but found that lame cows grazed for a shorter amount of time compared to nonlame cows. In cubicle-housed cows (Galindo & Broom, 2002) and (Singh et al., 1993) found no difference between lame and nonlame cows in the proportion of time spent feeding inside. In the present study, bite rate tended to increase throughout the summer, but this variation did not affect the results as nonlame cows ate faster (with the exception of September) than the lame cows. The impact of a slower bite rate suggests that lame cows are less likely to reach an efficient level of reproductive and/or milk productivity (Hassall et al., 1993). The cows in the present study were scored for body condition in a concurrent study (Chapter 2) and more lame cows had a lower BCS. Poor body condition score could be related to the reduced oestrus expression observed in lame cows. Nutrition and reproduction are interrelated and the loss of body fat or negative energy balance result in poor reproductive efficiency (Lucy, 2003; Ferguson, 2005). A negative energy balance affects LH pulse frequency and/or ovarian steroid synthesis (Butler, 2000), both of which could have a negative impact on oestrous expression (Caraty et al., 2002). Exactly how these mechanisms interact in relation to oestrus intensity in dairy cows has not yet been addressed.

Lameness had no impact on the patterns of grazing, drinking, ruminating, standing or lying throughout the day. Lameness, however, did have an impact on the daily pattern of oestrous expression as nonlame cows expressed oestrus more in the morning compared to lame cows. This was associated with less time lying down, less time grazing and more time standing up compared to lame cows. Although some studies suggest no variation in oestrus during the day (Esslemont & Bryant, 1976; Xu et al., 1998) oestrous behaviours have been reported to be more frequent during the nocturnal period and early morning (Van Vliet & Van Eerdenburg, 1996; Hurnik et al., 1975). The difference between studies is more likely related to difference in farm management practices than a true diurnal rhythm. Even so, the difference reported in the present study suggest that lameness has a negative impact on oestrous behaviours in the early morning on this particular farm, a key time when the herdsman was watching for oestrus.

Overall, the proportion of behavioural scans that accounted for oestrous expression in all cows was very low (<3%) and tended to be lower in lame compared to nonlame cows. In the present study many behavioural signs of oestrus were considered including primary signs of oestrus such as mounting activities and secondary signs of oestrus such as chin resting, sniffing and flehmen. In a concurrent study on the same animals, lame cows did not differ in the overall duration of oestrus but had a less intense oestrus (Chapter 2). Therefore, the lack of a strong difference between lame and nonlame cows in the proportion of scans in which oestrus was observed is more likely due to similar oestrus duration rather than intensity.

In the present study, and similar to the results of (Galindo & Broom, 2002), high-ranking cows were just as likely to be lame as low-ranking cows. However, using the same index of displacement, (Galindo & Broom, 2000; Galindo et al., 2000) described how behaviour and social rank prior to lameness influences the occurrence of lameness. Cows of lower social rank spend more time standing still and standing half inside the cubicles and have a lower survival rate to lameness. Using an index based on displacements, it is understandable how cows that displace other cows may win the preferred lying and standing locations. However, after a cow has become lame, as in the present study and as suggested by (Galindo et al., 2000), an index that bases success on displacement (ie during competitive feeding) may not be the most appropriate method to investigate interactions between social factors and lameness. Studies on various behavioural coping strategies may lead to a better understanding of the association of lameness and social relationships (Mendl & Deag, 1995; Gonzáles & Galindo, 2000).

Although no association between social rank and lameness was observed in the present study, cows of a higher social rank tended to be nearer to the front of the group when leaving the field and entering the parlour. In contrast, lame cows were near the rear of the group as they left the field later and entered the milking parlour later compared to nonlame cows confirming (Hassall et al., 1993). (Sauter-Louis et al., 2004) also demonstrated that low-ranking cows left the field later and were milked later compared to high-ranking cows; and cows that were milked in the last quarter of the herd were at an increased risk of lameness. This implies that lame cows may have a lower ranking status. High-ranking cows in the present study were also more restless during oestrus, similar to nonlame cows described in Chapter 2. However, unlike lameness, social rank had no impact on oestrus intensity in the present study and was not related to (other than restlessness) other primary or secondary behavioural sign of oestrus. Although the present study was unable to demonstrate a strong relationship between dominance and oestrous behaviour some authors suggest that dominance is important in the intensity oestrus expression (Hurnik et al., 1975; Wagnon et al., 1966; Kabuga et al., 1992; Weibold et al., 1983). If lame cows do have a lower ranking social status, as evident in the order that lame cows leave the field and enter the parlour, and lame cows have a less intense oestrus (Chapter 2) this suggests that there is a possible relationship between lameness, social factors and oestrus expression.

In conclusion, lameness negatively impacts on oestrous expression by altering feeding behaviour (i.e. by decreased bite rate) and by influencing daily activities (increased amount of time spent lying down and decreased the time spent walking and standing). The alterations in daily activities and the impact on social and oestrus behaviours highlight the costs and the detriment to welfare that lameness imposes on dairy cattle.

Acknowledgements

The authors wish to thank farm staff, Hilary Pursell for lab technical assistance and Donald Tyson and Paul Fox for their assistance and support with the project. Scholarships (to SLW) were provided by Natural Sciences and Engineering Research Council of Canada (NSERC), Universities UK and University of Liverpool and MJM is Wellcome Trust Clinical Fellow.

Table 1. Lameness scoring scale[1]

Lameness Score	Description	Assessment Criteria		
		Stands	Walking	Gait
1	Nonlame	Level back posture	Level back posture	Normal
2	**Moderately Lame**	Level back posture	Arched back	Normal to short-striding
3	**Severely Lame**	Arched posture	Arched back	Takes one step at a time/reluctance to bear weight on one or more limbs/feet

[1] modified after (Sprecher et al., 1997) 5 point scale, in which scores 1 and 2 are comparable and 3-5 are grouped and equivalent to score 3 as defined above

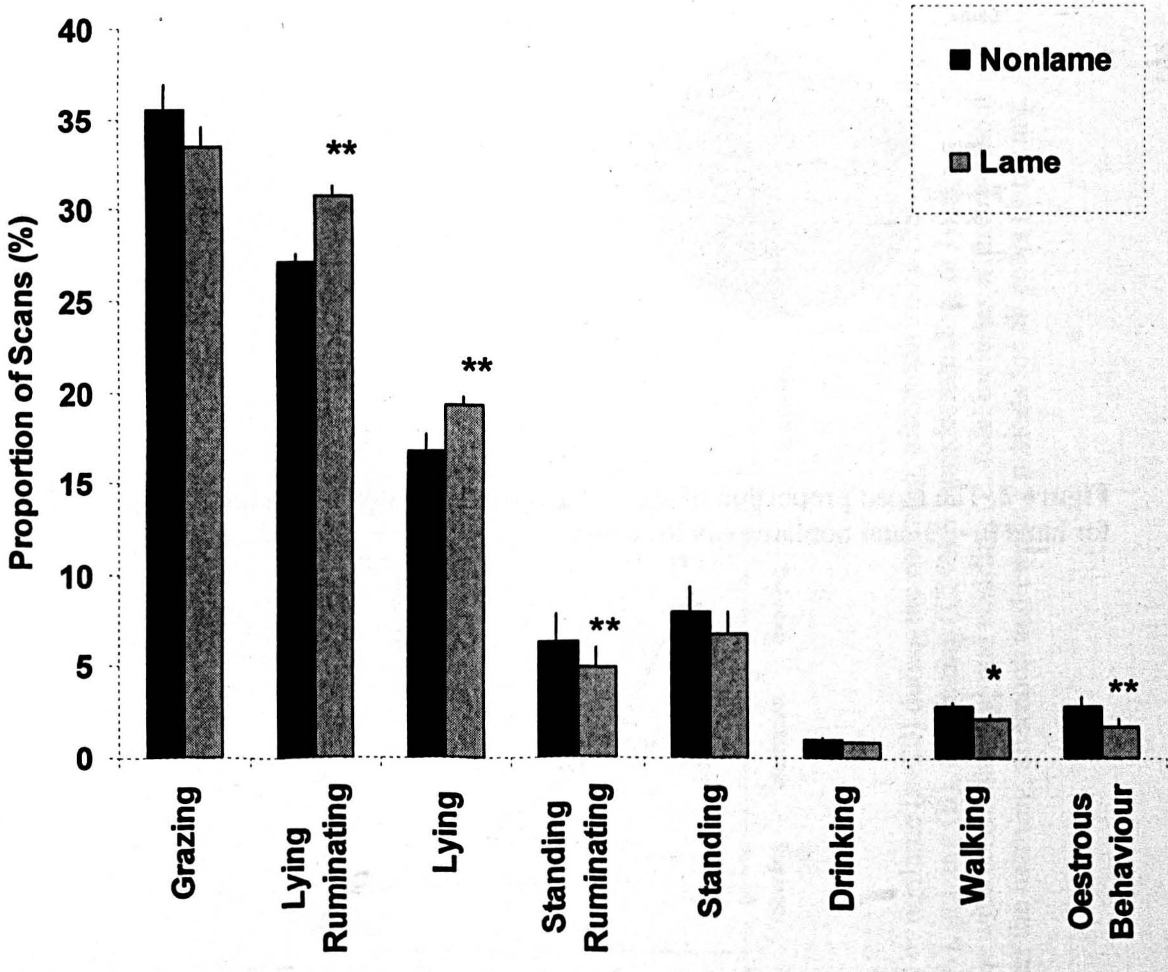


Figure 1. The activity budget of lame (n=39) and nonlame (n=20) dairy cows presented as the total proportion of scans (%) in which a behavioural state was observed. Differences for lame cows are represented by *p<0.05 and **p<0.10

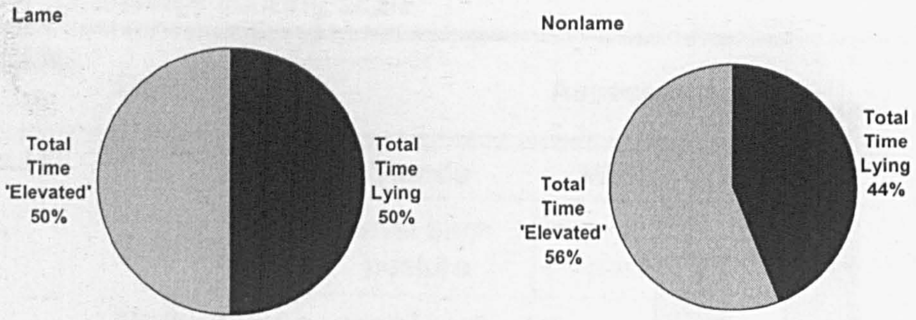


Figure 2. The mean proportion of scans (%) spent lying down or 'elevated' on the feet for lame (n=39) and nonlame (n=20) cows.

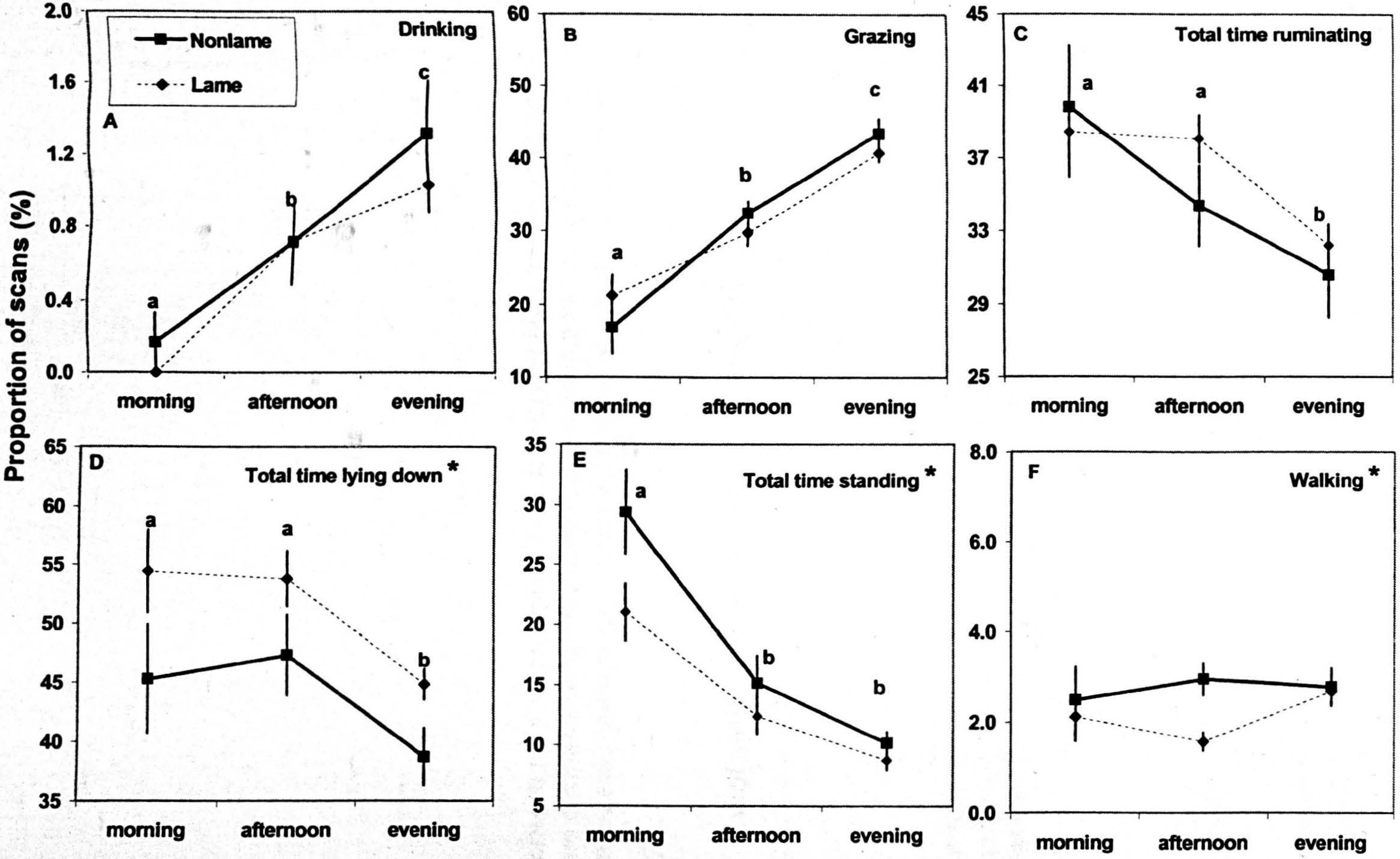


Figure 3. Daily activity budgets for lame (n=39) or nonlame (n=20) during the morning, afternoon and evening including proportion of scan samples (%) spent drinking (A), grazing (B), ruminating (C: total includes standing or lying ruminating), lying down (D: total includes with or without ruminating) and standing (E: total includes with or without ruminating) and walking (F). Differences between the morning, afternoon and evening periods for all cows (lame and nonlame) are shown by ^{abc} p<0.05.

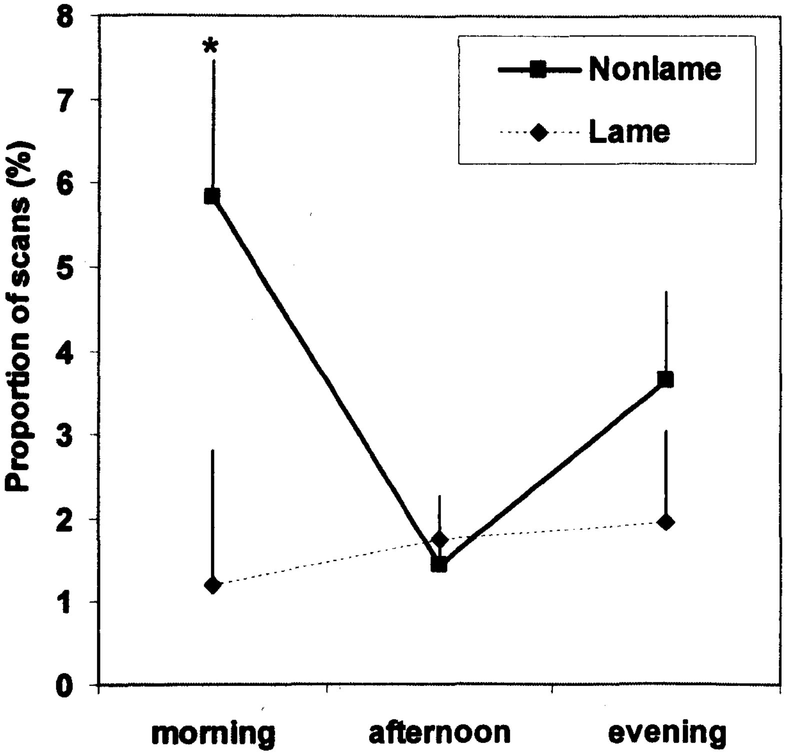


Figure 4. The proportion of scan samples (%) an oestrus behaviour was observed during the morning, afternoon and evening periods in lame (n=39) and nonlame (n=20) cows. Significant differences between lame and nonlame cows at different periods are indicated by * $p<0.05$.

Table 2. Order of lame or nonlame cows from the field or into the parlour

	Field Order	Milking Order
Lame	0.58 ± 0.02	0.58 ± 0.01
Nonlame	0.46 ± 0.02*	0.47 ± 0.02*

Data are presented as average order (scale 0-1) ± SEM. Difference for lame cows are marked with * $p<0.05$. A higher order represents a position nearer to the rear of the group.

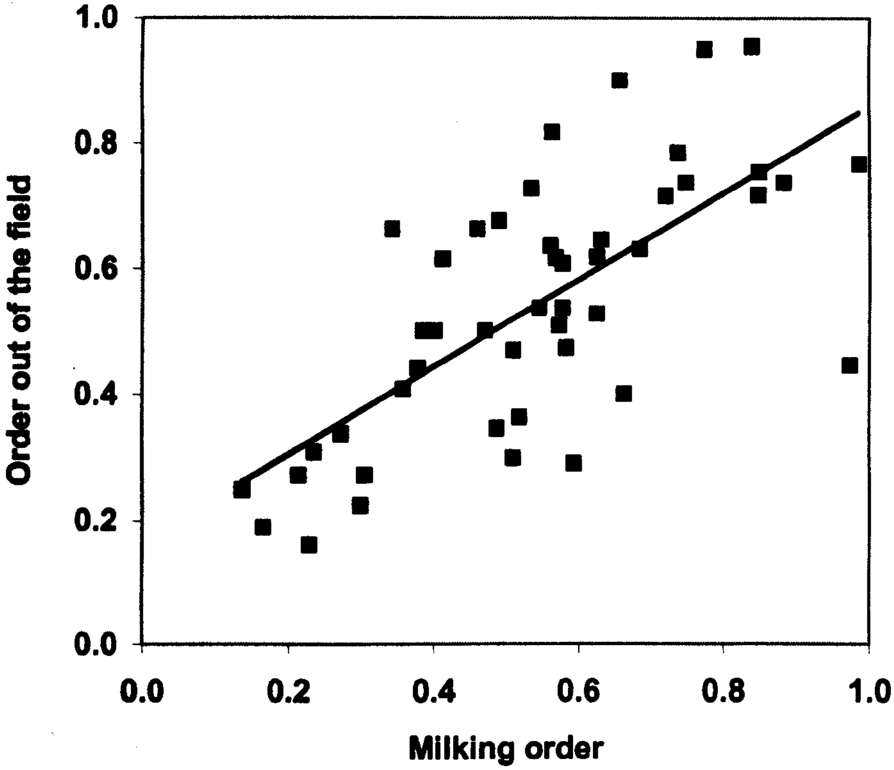


Figure 5. Scatterplot with Pearson's correlation (n=59 cows) of the average milking order and average order out of the field (r^2= 0.704; p=0.000). The higher the order (scale 0-1) the nearer to the rear of the herd.

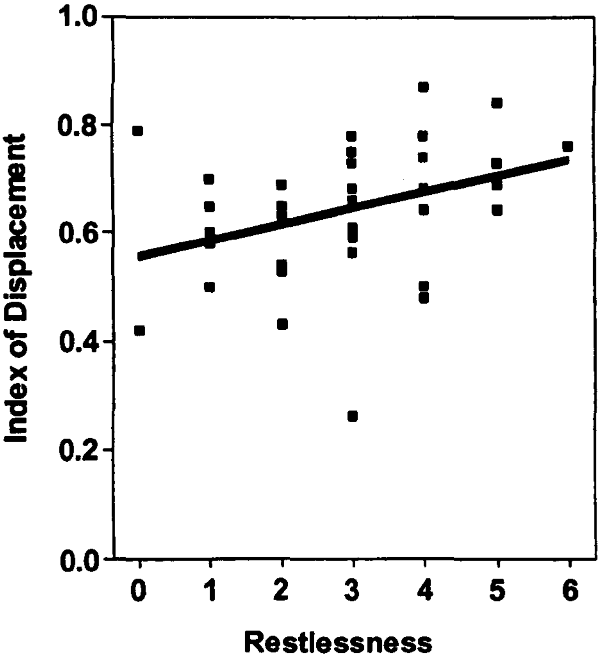


Figure 6. Scatterplot and Pearson's correlation of social rank (index of displacement) and restlessness during oestrus (n=59 cows). A score of '1' is given during each observation period (conducted every 3 h for 30 min) when a cow was in oestrus and was considered to be restless (r^2=0.342, p=0.039).

Table 3. Pearson's correlations between social rank[1] and total frequency of different behavioural signs of oestrus

Behavioural Signs of Oestrus	R^2	P value
Total oestrus score[2]	0.135	p=0.425
Restlessness	**0.342**	**p=0.039**
Sniffing the vulva of another cow	0.246	p=0.143
Flehmen	0.423	p=0.900
Chin resting	0.153	p=0.367
Mounting rear other cows	0.118	p=0.487
Mounting head of other cows	0.129	p=0.446
STBM	0.014	p=0.933
Mounted but did not STBM	0.186	p=0.271
Total mounting activity (TMA)[3]	0.110	p=0.519
Total mounts received (TMR)[4]	0.065	p=0.703

Significant relationships are shown in bold

[1] Index of displacement value

[2] A measure of oestrus intensity based on a weighted scoring method (Van Eerdenburg et al., 1996)

[3] TMA (includes: mounting the rear or head of another cow plus STBM)

[4] TMR (includes: STBM and mounted but did not STMB)

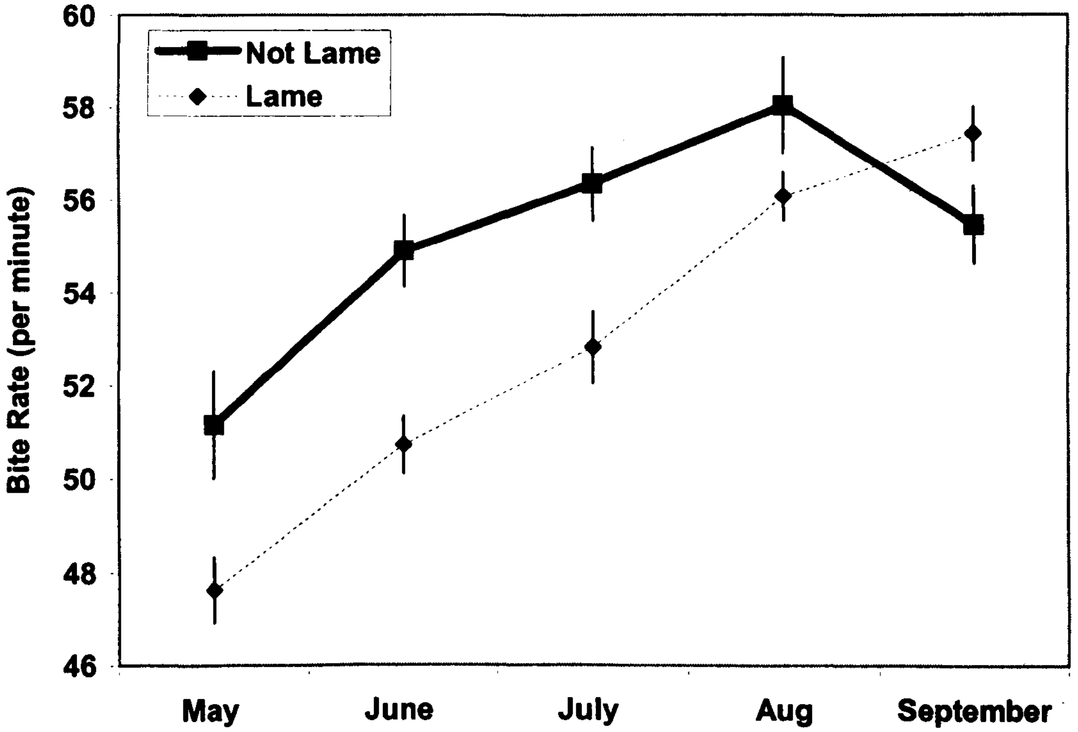


Figure 7. Bite rate (per minute) for lame (n=39) and nonlame (n=20) cows during the summer months while grazing

Chapter 4

Chapter 4

Adrenal and behavioural responses to an acute stressor in chronically lame dairy cattle

Walker SL, Smith RF, Jones DN, and Dobson H

Key words: lameness, acute stress, cortisol, stress behaviours, fearful, progesterone, coping, welfare, chronic stress, oestrus, habituation, facilitation

Abstract

Stress has a negative impact on reproduction and a chronic stressor such as lameness reduces oestrus expression and decreases fertility in dairy cows; however, lameness does not affect long-term resting cortisol concentrations. The main objective of the present study is to determine if lameness, as a chronic stressor, alters behavioural responses and/or cortisol to an acute stressor.

Lame or nonlame postpartum dairy cows (n=44) were exposed to a challenge stimulus (loud band) on two occasions ~50 days apart (Test 1: 23.5 ± 0.3 days postpartum and Test 2: 77.4 ± 3.3 days postpartum) and milk samples for cortisol and stress-related behaviours were monitored at the time of challenge (0 min) and every 5 min for 75 min. Milk samples prior to the Test were also taken to determine progesterone concentrations on the day of the Test.

For Tests 1 and 2, lame cows had a greater cortisol response to the acute stressor and moved less during the Tests compared to nonlame cows (p=0.046 and p=0.028, respectively). Lame cows had fewer gentle head movements during the Tests (p=0.001) and tended to have fewer aggressive head movements (p=0.067). Lame cows also vocalized less compared to nonlame cows (p=0.000). Other behaviours (gentle shifts, aggressive body movements, tail flicks, defaecations and urinations) did not differ between lame and nonlame cows. There was a possible association between lameness and overall behaviour (low/moderate/strong) during the Test (p=0.079). Proportionately, more lame cows had a low reaction to the Test (p=0.028). Cows that had a low reaction had a greater cortisol response than those with a moderate reaction (p=0.052).

Eighty-four percent of all cows were classified with the same overall behavioural reaction in their first and second test. Indeed, comparisons between Test 1 and 2 revealed overall that there was no difference in several behaviours between Test 1 and Test 2 for either lame or nonlame cows. However, the number of tail flicks decreased in both groups in Test 2 (p=0.031) and in nonlame cows, there were fewer aggressive head and body

movements between Test 1 and Test 2 (p=0.000 and p=0.000, respectively). In lame cows aggressive movements were similar in each Test. Gentle shifting increased in Test 2 (p=0.030); however, pairwise comparisons reveal that this only tended to occur in lame cows (p=0.069) and not in nonlame cows (p=0.871). The cortisol response in Test 2, diminished for both lame and nonlame cows (p=0.000). However, the cortisol response to the acute stressor in Test 2 was greater in lame compared to nonlame cows (p=0.039).

Progesterone concentration at the time of the Test also explained some variation in the data. Cows with luteal (>0.17 ng/ml) concentrations of progesterone at the time of the Test had a higher cortisol response and more movement mainly as a result of more gentle head movements compared to cows with basal concentrations (p=0.042, p=0.000, and p=0.000, respectively). Progesterone concentration at the time of the test did not account for variations in all other stress-related behaviours.

In summary, lame cows responded in a more fearful style to an acute stressor compared to nonlame cows. Lame cows had a lower behavioural response and greater cortisol response to an acute stressor. When confronted with the same acute stressor on a second occasion ~ 50 days later, nonlame cows suppressed aspects of their behavioural responses and had a lower cortisol response but lame cows did not alter their behaviour and had a higher cortisol response. In conclusion, chronically stressed lame cows do not cope as well as nonlame cows with a repeated acutely stressful situation.

Introduction

We are interested in responses to both acute and chronic stressors because they have an impact in fertility in cattle. The response of the hypothalamus-pituitary-adrenal axis (HPA) to a stressful situation disrupts normal functioning of hypothalamus-pituitary-ovarian axis (Rivier & Rivest, 1991; Dobson et al., 2003; Moberg, 1985). The degree of disruption may depend on the nature of the stressor and/or the individual, however, the hormonal mechanism for affecting fertility is probably common, irrespective of the stressor involved (Dobson & Smith, 2000). The chronic pain and stress associated with lameness impairs proper endocrine function which ultimately has detrimental effects on ovarian cyclicity (Dobson & Alam, 1987; Li & Wagner, 1983; Dobson et al., 2003). Thus, lameness is associated with poor reproductive performance (Collick et al., 1989) and decreased oestrus intensity (Chapter 1, Chapter 2, Walker et al., 2005).

There are many factors that affect oestrous intensity (Orihuela, 2000). The exact mechanism by which 'stress' alters oestrous behaviour in cattle is not yet clear. However, oestrous behaviour is particularly vulnerable to the effects of acute stress during the pre-ovulatory period when dramatic shifts in hormone concentrations occur.

Disruption in this period disturbs the carefully synchronized sequence of endocrine events that are responsible for oestrous behaviours (Walker et al., 2006; Moberg, 1985; Liptrap, 1993). Chronic stress also alters oestrous expression by either delaying and/or impairing oestrus postpartum or altering oestrous intensity (Chapter 1, Chapter 2, Walker et al., 2005; Petersson et al., 2006). Indeed, in previous studies we have shown that a reduced intensity of oestrous expression is associated with lameness and low progesterone exposure prior to oestrus (Chapter 1, Chapter 2, Walker et al., 2005; Walker et al., 2006). Furthermore, lameness disrupts time budgets, feeding strategies and other social behaviours (Chapter 3). However, nothing is known about the effects of lameness on coping style, especially in response to novel situations. Dairy cows face numerous possible acute stressors on a daily basis and these can have a negative impact on reproduction and oestrous behaviours. Hence, one of the aims of the present study is to examine how lame cows cope in an acutely stressful situation.

Stress has been defined by the inability of an animal to cope with its environment and this phenomenon is revealed by a failure to reach genetic potential (Dobson & Smith, 2000). The mere occurrence of lameness itself suggests that a cow is not coping with its environment. Chronic stress may alter hypothalamic-pituitary-adrenal axis function and change the way an individual copes with a stressful situation. The style or degree to which an individual copes with a stressful (i.e. chronic) and/or fear-related (i.e. psychological acute stress) situation can be measured by examining the magnitude of the psychobiological responses, comprised of behavioural changes to counteract the effects of the stimulus (e.g. movement or vocalization), and neuroendocrine adjustments that are needed to maintain homeostasis (Boissy, 1995). The endocrine stress response is mediated via the HPA, which involves the sequential release of corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) from the hypothalamus, adrenocorticotropic hormone (ACTH) from the anterior pituitary and glucocorticoids, such as cortisol, from the adrenal glands. However, we have already shown that resting cortisol profiles do not differ between lame and nonlame postpartum dairy cows (Chapter 1, Chapter 2). The unchanged resting cortisol concentrations may be a result of adaptation to the stress and pain of lameness (Kant et al., 1983; Tache et al., 1976; Kant et al., 1987). Chronic stress and pain are often associated with adapations of the hypothalamic-pituitary-adrenal axis, resulting in both functional and structural modifications and long-term alterations in neuroendocrine reactions to subsequent stressors (Ostrander et al., 2006; Ulrich-Lai et al., 2006b). Therefore, although cortisol concentrations did not differ with lameness, dysfunction in the hypothalamic-pituitary-adrenal is a possibility and is another focus of the present study.

Additionally, the hormonal milieu of an individual may impact on coping responses to an acute stressor. Gonadal steroids influence behavioural responses to a stressful stimulus.

Testosterone-treated cows, ewes or chickens exposed to various fear-eliciting tests exhibit lower behavioural fear reactions than controls (Boissy & Bouissou, 1994; Archer, 1973b; Archer, 1973a; Bouissou & Vandenheede, 1996; Vandenheede & Bouissou, 1993). Oestradiol attenuates stress-induced hormone responses in cows (Hollenstein et al., 2006) and in pregnant ewes, there is a negative correlation between plasma progesterone and stress or fear-related behaviours (Vierin & Bouissou, 2001).

Thus, the aims of the present study are to investigate if lame cows differ in their behavioural and hormonal coping style to an acutely stressful situation, and to investigate if these responses are influenced by prevailing endogenous progesterone concentrations.

Materials and Methods

Experimental design, animals, feeding and housing

The study was conducted on postpartum Holstein-Friesian cows (n = 44) on a UK commercial dairy farm comprising a total of ~200 year-round calving cows. The average rolling milk yield per cow in the herd was 8300 litres/year. Cows randomly entered into the study at Day 20 postpartum; cows were part of a parallel investigation (Chapter 1, Walker et al., 2005) and at any one time only 12 cows were studied. Cows were monitored from Day 20 postpartum until the first observed oestrus postpartum. The parity of the study cows was 3.6 ± 0.2 (range 1-7). During the winter months (February to April 2004), animals were housed in a cubicle shed with concrete flooring or were at pasture during the summer (April to November 2004). The temperatures during the study period ranged 0.2 to 27 °C. Milking took place twice a day starting at 6:30am and 4:30pm. All year round animals had access to a total mixed ration (TMR) at a housed feed-fence after milking. Pastures were of seasonal ryegrass, Italian ryegrass and white clover.

Adrenal challenge

Adrenal responses to an acute stressor were examined on two occasions ~50 days apart. Test 1 was on Days 20-26 postpartum (23.5 ± 0.3 days postpartum) and Test 2 was on the seventh day after the first observed postpartum oestrus (77.4 ± 3.3 days postpartum; range 45-133; a Chi-square test determined that there were equal proportions of lame and nonlame cows that had Test 2 >80 or <80 days postpartum, p=0.757). Immediately following milking on a Test day (1 and 2), cows were individually diverted into a holding area and placed in a crush within minutes of entering the holding area. The holding area was separated from the main corridor by a small wall, therefore, cows could smell and hear, but not see, other cows leaving the milking parlour. Most cows entered the crush with little persuasion as going in the holding area and crush were familiar processes; and were chosen for this reason. Throughout, there were no sudden movements and no shouting. Once individuals were restrained in the crush, a milk sample was taken at time 0 (and was considered to be the resting cortisol sample) then every 5 min from 10 - 75 min and at 90 min. After 5 min in the crush, the acute stressor was given. The stimulus was the quick and sudden movement of the observer in front of the cow's head to make a loud bang and swiftly moving away again. During the remainder of the Test, the observer stood 1 metre behind the cow and made no movements remaining quiet. In parallel to the collection of milk samples, continuous behavioural focal samples were taken, blocked into 5-minute intervals (10-75,90 min). Table 1 defines the stress-related behaviours that were recorded (includes: aggressive/gentle head/body movements, tail flicking, vocalization, urination and defaecation). Following the test, the observers recorded an overall subjective behaviour reaction score to the as either low, moderate or strong test adapted from (Grandin, 1993) and defined in Table 2. Behavioural data were collected by one of two observers and Pearson's correlation for inter-observer reliability (n=700 pairs of scores) was r^2 =0.998, p=0.000.

Lameness scoring

Individuals were scored for lameness (score 1-3) prior to Test 1 and Test 2, based on gait and posture while walking and standing using methods adapted from (Sprecher et al., 1997) and defined in Table 3. Cows were grouped retrospectively as either nonlame (score 1) or lame (score 2 or 3). Clinical treatments for lameness continued as usual. A number of cows (10/44) had a different lameness score prior to Test 1 than Test 2 (7 increased and 3 decreased). Therefore, the score given immediately prior to each Test was used to group cows.

Hormone Assays

In addition to the milk samples taken for cortisol analysis during Tests 1 and 2, milk samples were taken immediately prior to milking to determine progesterone metabolite concentrations. Milk progesterone metabolite concentrations [hereafter referred to as progesterone as it is the predominant progestagen compound in bovine milk (Purdy et al., 1980)] on the day of Test were classed as basal (below baseline progesterone concentrations) or luteal (above baseline progesterone concentrations). Baseline progesterone was calculated as the mean basal progesterone concentration in all cows during potential oestrus periods + 2SD and was equal to 0.17 ng/ml milk.

Milk progesterone and cortisol were analysed by previously described (Chapter 1, Young et al., 2004) enzyme immunoassay's (EIA) using methods adapted from (Munro & Stabenfeldt, 1984). Briefly, the EIA utilized an antibody (monoclonal antiserum progesterone metabolite Quidel Clone #425, polyclonal cortisol antiserum R4866; supplied by CJ Munro, University of California, Davis, CA), horseradish peroxidase conjugated label (progesterone and cortisol) and standard (progesterone, hydrocortisone; Sigma-Aldrich, UK). In general, assay procedures were as follows: 1) antiserum was diluted at 1:10,000 for progesterone, and 1:8500 for cortisol 2) standards (progesterone, 4-200 pg/well and cortisol, 3.9-1000 pg/well) and samples (ran undiluted for progesterone and cortisol) were loaded (50 μl/well for progesterone and cortisol) onto the plate; and 3) the horseradish peroxidase conjugate was used at a dilution of 1:33,000 for progesterone and 1:40,000 for cortisol. The progesterone antiserum cross-reacts 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). The cortisol antiserum cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (C.J. Munro, pers. comm.). Parallel displacement curves were obtained for serial diluted pooled milk samples and the progesterone and cortisol standard curves. The intra- and interassay coefficients of variations were <5% and <15% for both assays, respectively.

Data analysis

All data are presented as mean ± SEM and were analysed using Minitab 14®. Statistical differences were reported when $p<0.05$; with a tendency considered as a difference when $0.06>p<0.10$. Significant differences detected by General Linear Model (GLM) ANOVA were compared using Tukey's 95% Post-hoc pairwise comparisons.

The cortisol and behavioural responses to the acute stressor were analysed using a GLM ANOVA with repeated measures. Cortisol concentrations were normalized using logarithmic transformation. Behavioural responses were normally distributed and did not require transformation. Behavioural responses included: 1) vocalization 2) tail flicking 3) gentle head movements 4) gentle body movements 5) aggressive head movements 6) aggressive body movements 7) total movements (includes tail flicking and all head and body movements). The model included the random factor of cow ID and the fixed factors of Test (1/2), lameness (lame/nonlame), time (10-75, 90 min), progesterone (basal/luteal) and the interactions of Test with lameness and Test with time.

The total number of defaecations and urinations per Test and the resting cortisol concentrations immediately prior to the acute stressor were analysed using a GLM

ANOVA with repeated measures with random factor Cow ID and the fixed factors of lameness, Test and the interaction of Test with Lameness.

The relationship between the overall behavioural score (low/moderate/strong) and cortisol or behavioural responses were analysed with a GLM ANOVA with repeated measures. The model included the random factor Cow ID and the fixed factors of overall behavioural score, time and the interaction between overall behavioural score with time. A Chi-square test was employed to assess the relationship between overall behavioural rating score and lameness.

Results

Resting milk cortisol concentrations immediately prior to the acute stressor (time 0) were the same for lame and nonlame cows (1.10 ± 0.09 and 1.07 ± 0.08 ng/ml, respectively; p=0.550). Following the acute stressor, there was a gradual increase in cortisol concentration (from 10-90 min) in lame and nonlame cows (p=0.000; Figure 1ab). Overall, for Tests 1 and 2 combined, the average cortisol concentration over the 10-90 min period demonstrated that lame cows had a greater cortisol response to the acute stressor compared with nonlame cows (p=0.046). The overall mean cortisol value in Test 1 was similar for lame and nonlame cows (p=0.792; Figure 1c). The overall mean cortisol value in Test 2, diminished for both lame and nonlame cows (p=0.000; Figure 1c) but post-hoc pairwise comparisons revealed that overall mean cortisol values to the acute stressor in Test 2 was less in nonlame compared to lame cows (p=0.039; Figure 1c).

Assessment of stress-related behaviours revealed that total movement (total number of all head and body movements plus tail flicking) increased with time following the acute stressor in both lame and nonlame cows (p=0.002; Figure 1de). However, lame cows exhibited less total movement during the Tests than nonlame cows (p=0.028; Figure 1f). Overall, there was no difference in total movement between Test 1 and Test 2 for either lame or nonlame cows (p=0.970; Figure 1f).

Analysis of individual stress-related behaviours revealed that the number of gentle head movements increased over time during each Test period (p=0.054; Figure 2ab). Overall, lame cows had fewer gentle head movements during the Tests (p=0.001; Figure 2c). The average number of gentle head movements was the same between Test 1 and Test 2 for both lame and nonlame cows (p=0.137; Figure 2c).

The number of gentle body shifts increased with time (p=0.000; Figure 2de). Overall, lame and nonlame cows exhibited a similar number of gentle shifts during the Test (p=0.796; Figure 2f). Gentle shifting seemed to increase in Test 2 (p=0.030); however, post-hoc pairwise comparisons revealed the number of gentle shifts in Test 1 and Test 2 only tended to increase in lame cows but was the same in nonlame cows (p=0.069 and 0.871, respectively; Figure 2f).

Overall, the number of aggressive head movements did not change with time (p=0.129; Figure 3ab). Lame cows tended to have fewer aggressive head movements during the Tests (p=0.067; Figure 3c). Post-hoc pairwise comparisons between Tests revealed that the number of aggressive head movements in lame cows did not change from Test 1 to Test 2 (p=0.997; Figure 3c). However, in nonlame cows, there was a decrease in the number of aggressive head movements between Test 1 and Test 2 (p=0.000; Figure 3c).

The number of aggressive body movements did not change with time (p=0.628; Figure 3de). Overall, lame cows and nonlame cows exhibited the same number of aggressive body movements (p=0.270; Figure 3f). Post-hoc pairwise comparisons between Tests revealed that the number of aggressive body movements in lame cows did not change from Test 1 to Test 2 (p=0.986; Figure 3f). However, in nonlame cows, there was a decrease in the number of aggressive body movements observed between Test 1 and Test 2 (p=0.000; Figure 3f).

There was a gradual increase in vocalization during the Tests (p=0.000; Figure 4ab). Lame cows vocalized less compared to nonlame cows (p=0.000; Figure 4c) and the number of vocalizations did not change from Test 1 to Test 2 for either lame or nonlame cows (p=0.824; Figure 4c).

The number of tail flicks did not change with time (p=0.285; Figure 4de) nor was there a difference between lame and nonlame (p=0.297). However, in Test 2, tail flicking decreased in both groups (p=0.031; Figure 4f).

The total number of defaecations did not differ between lame and nonlame cows (p=0.505; data not shown) or Test 1 and 2 (p=0.189; lame Test 1: 1.056 ± 0.171; Test 2: 0.864 ± 0.190; nonlame Test 1: 1.296 ± 0.149; Test 2: 1.045 ± 0.180). Similarly, there was no difference in the number of urinations recorded between lame and nonlame cows (p=0.795; data not shown) or between Tests (p=0.420; lame Test 1: 1.222 ± 0.367; Test 2: 1.000 ± 0.316; nonlame Test 1: 0.926 ± 0.118; Test 2: 0.682 ± 0.102).

Categorization of animals, into those with basal or luteal (>0.17 ng/ml) concentrations of progesterone at the time of each Test revealed that cows with luteal progesterone concentrations had a higher mean cortisol response compared to cows with basal concentrations (p=0.042; data not shown). Additionally, cows with luteal progesterone concentrations had a greater number of total movements (p=0.000, basal 17.1 ± 0.5, luteal

21.2 ± 0.4) and gentle head movements (p=0.000; basal 7.6 ± 0.3, luteal 9.1 ± 0.2). There was no difference in all other stress-related behaviours and progesterone concentration (p>0.05; data not shown).

Eighty-four percent of cows (37/44) had the same overall behaviour reaction in their first and second test. There was a tendency for an association between lameness and the overall behavioural reaction (low/moderate/strong) to the Test (p=0.079; lame 10/33/5 cows, nonlame 17/21/2 cows, respectively). Only 7/88 overall reactions were considered to be 'strong' reactions to the Test. Proportionately, more lame cows had a low reaction to the Test, compared with moderate and strong reactions (p=0.028, nonlame 10/38, lame 17/23).

Regardless of lameness, there was a step-wise increase in the total number of movements (including all head and body movement plus tail flicking) and frequency of each stress-related behaviour (gentle head/body movements, vocalization, aggressive head/body movements and tail flicking) with an increasing behavioural reaction (low/moderate/high) during the Tests (p>0.05; Figure 5). Cows that had a low reaction had a higher cortisol response than those with a moderate response (p=0.051; data not shown). Although lower than cows with low behavioural reaction, there was no difference in cortisol concentration between cows with a strong behavioural response and any other category possibly due to the fewer cows in this category (n=7; p=0.424; data not shown).

Discussion

The hypothalamic-pituitary-adrenal axis (HPA) is a vital regulator of homeostasis in vertebrates and increased concentrations of glucocorticoids are important for survival during acute stress. Dairy cows face numerous possible acute stressors on a daily basis, e.g. social stimuli (constant re-grouping based on milk yield, social isolation), physical restraint (for artificial insemination, foot trimming), novelty (introduction of new herd-mates, handlers, changed management routines). All of which have the potential to induce fear and activate the HPA in cattle (Boissy, 1995; Hopster & Blokhuis, 1994; Grignard et al., 2001). The results from the present study are the first to demonstrate that in a real-life situation, spontaneous chronically stressed (lame) cows have a greater cortisol response to an acute stressor compared to normal healthy herd-mates. In general, the HPA response is related to acute stimulus intensity i.e. the stronger the perceived stressor the greater the amplitude of the HPA response (Garcia et al., 2000; Smith et al., 2003a). Furthermore, in contrast with lame cows, nonlame cows had a lower cortisol response after exposure to the same acute stressor a second time ~ 50 days later, suggesting that nonlame cows 'coped' better and had habituated to the acute stressor.

Lameness is a painful and stressful process characterized by hyperalgesia (Whay et al., 1997). In humans, chronic pain along with other stress-related disorders are accompanied with disturbances in the HPA axis (Blackburn-Munro & Blackburn-Munro, 2001; Boyer, 2000; Yehuda, 2001). In rodents, long-term or repeated activation of the HPA, results in functional and structural adaptations within the HPA (Ostrander et al., 2006; Ulrich-Lai et al., 2006a). Indeed, chronic stress results in adrenal hypertrophy (Armario et al., 1985) and is associated with elevated ACTH and glucocorticoid concentrations (Kiss & Aguilera, 1993; Marti et al., 1994; Herman et al., 1995). Glucocorticoids, the final output of the acutely stimulated HPA, normally inhibit stress-induced CRH and ACTH secretion through negative feedback actions in the brain and anterior pituitary. However, during chronic stress, glucocorticoids act on the brain in a excitatory rather than inhibitory fashion (Dallman et al., 2004; Makino et al., 2002). Although not always the case (Ostrander et al., 2006), chronically stressed individuals characteristically exhibit facilitated HPA responses to acute novel stressors (Dallman et al., 2004; Armario et al., 1985; Kiss & Aguilera, 1993; Bhatnagar & Dallman, 1998; Ma & Morilak, 2005; Marti et al., 1994). The facilitory response maintains HPA responsiveness to acute novel stressors. The results from the present study therefore suggest, that similar to rodents, chronically stressed lame cows exhibit a facilitated response to a repeated acute stressor. Similarly in another study, chronically stressed cattle, induced by 3-weeks of isolation or deprivation of lying down, had an increase in cortisol when exposed to a novel object (Munksgaard & Simonsen, 1996). Cows that do not successfully cope with acutely stressful situations may have poor fertility as activation of the HPA has negative effects on hypothalamic-pituitary-ovarian axis (Dobson et al., 2003).

Chronic stress is normally associated with elevated glucocorticoid concentrations (Sapolsky, 1992). However, in previous studies we have shown that lameness was not associated with elevated 'resting' or unstimulated cortisol concentrations (Chapter 1 and Chapter 2). Similarly, (Ley et al., 1996) found no difference in resting cortisol concentrations between lame and nonlame cows. Likewise, in the present study, resting cortisol concentrations, prior the acutely stressful challenging test, were the same for lame and nonlame cows. In other studies, chronically stressed cattle, induced by 3-weeks of isolation or deprivation of lying down, had similar baseline cortisol and ACTH concentrations (Munksgaard & Simonsen, 1996). Models for chronic stress in rodents usually involve a highly predictable repetitive regimen of intermittent stressors (Cole et al., 2000; De Boer et al., 1990a; Spencer & McEwen, 1990). The hormonal response in animals exposed to a repeated stressor is termed adaptation or habituation, such that exposure to the same stressor evokes less of a hormonal response to each successive stress session (Tache et al., 1976; Kant et al., 1985; Garcia et al., 2000; Hauger et al., 1990; Cole et al., 2000). Habituation to a chronically applied stressor occurs so that the

HPA response to a novel stressor can be preserved (Marti & Armario, 1998). Thus, during the chronic stress of lameness, habituation occurs resulting in similar cortisol concentrations to nonlame cows but the response to novel stimuli is facilitated.

In the present study, in addition to a physiological response (cortisol concentrations), behavioural responses to the acute stress were simultaneously recorded. Proportionately more lame cows had a 'low' reaction (related to little gentle or aggressive movement) to the acutely stressful situation and exhibited less total movement compared to nonlame cows. In general, the diminished amount of movement resulted from a decrease in gentle and aggressive head movements. During the acutely stressful situation, lame and nonlame cows exhibited a similar number of gentle shifts; therefore, an increased behavioural response was not related to lame cows shifting more within the crush to alleviate pressure on their feet. The aggressive stress-related behavioural responses were affected by the second exposure to the challenging situation. Nonlame cows decrease aggressive related movements (i.e. strong movements relating to trying to escape from crush) whereas there was no change in these behaviours in lame cows. This suggests that lame cows did not adjust or adapt their behavioural reaction when exposured to the same acutely stressful situation for a second time.

Overall in the current study, lame cows exhibited fewer movements (including fewer aggressive head movements associated with trying to escape from the crush) and a higher cortisol response to the acutely stressful situation. A similar psychobiological response has been observed in birds, rodents and pigs when an active (e.g. confrontation, fight, escape) behavioural response is associated with low corticoid concentrations and high corticoid concentrations are associated with a passive behavioural response (e.g. quiescence, immobility, low levels of aggression) (Jones & Satterlee, 1996; Beuving et al., 1989; De Boer et al., 1990b; Mendl & Deag, 1995; Koolhaas et al., 1999; Korte et al., 1997).

A passive behavioural response is believed to indicate a more fearful reaction (low levels of aggression and increased immobility) to a stressful situation (Koolhaas et al., 1999). Fear is defined as an emotional state induced by the detection of danger threatening the well-being of an individual (Boissy, 1995). This is related to elevated corticosteroids and inactivity in cattle (Boissy & Le Neindre, 1997; Van Reenen et al., 2005; Boissy & Bouissou, 1995). The acute stress cows were exposed to in the present study contained components (isolation, novelty and surprise) that are classically reported to induce fear in cattle (Hopster & Blokhuis, 1994; Van Reenen et al., 2004; Boissy & Bouissou, 1995). Thus, the higher cortisol response and fewer movement in lame cows observed in the present study suggest that, lame cows are more fearful of an acutely stressful situation.

Lame cows also vocalized less during the acutely stressful challenging test. This could be related to a passive coping response to the test (Erhard et al., 1999; Jones & Satterlee, 1996). Pigs that exhibit passive coping responses in restraint episodes also vocalize less (Geverink et al., 2002; Ruis et al., 2000a; Koolhaas et al., 1999). In cattle, less frequent vocalization is associated with higher levels of fear in open-field tests (Boissy & Bouissou, 1995) also suggesting lame cows were more fearful of the challenge in the current study. However, contrary to this, (De Passillé et al., 1995) suggest vocalization may represent a fearful response to novelty and social separation. Nevertheless, in other studies frequent vocalization was not considered to be a defining stress-related behaviour in a fearful situation (Prelle et al., 2004; Grignard et al., 2001). It is possible that the more frequent vocalization from nonlame cows in the present study was related to other factors, e.g. response to vocalizations coming from other animals leaving the milking parlour (Kilgour, 1975).

Nevertheless, the results from the current study suggest that lame cows were more fearful during the challenging test and dealt with the stressful situation by expressing a passive coping response.

We have also produced evidence that the hormonal status of a cow when challenged with an acute stressor influences HPA and behavioural responses. Cows with luteal concentrations of progesterone at the time of the acute stress had higher cortisol responses. This on its own, suggests a more fearful reaction to the acute stressor. However, high concentrations of progesterone at the time of the challenge were also associated with more gentle movements of the head and body in the crush. In the present study, gentle movements, as opposed to fighting reactions to get out of the crush, were related to more investigative behaviours (sniffing the air or trying to see the human observer). In other studies where cows explore or investigate novel environments or objects, these types of behaviours are associated with low levels of fear (Herskin et al., 2004). In humans and rodents, exogenous progesterone has anxiolytic properties, relieving anxiety (Dennerstein et al., 1985; Mora et al., 1996). Additionally, pregnancy, and thus high concentrations of progesterone, are associated with low fear-related behavioural reactions to novel stimuli in sheep (Vierin & Bouissou, 2001). Learning about a new situation can lead to an increase in cortisol (Rushen, 1986). (Manteca & Deag, 1993c) suggest that active animals that are exploring their environment are probably learning more, thus leading to an increase in cortisol concentrations. Thus, it is possible that in the present study, the higher frequency of gentle movements associated with higher cortisol concentrations in cows with high progesterone concentrations at the time of the challenging test could be related to low levels of fear and increased in familiarization with the challenging situation.

In summary, progesterone concentration at the time of an acute stressor influences the psychobiological response. Overall, lame cows have a lower behavioural response and greater cortisol response to an acutely stressful situation. When confronted with the same acute stressor on a second occasion ~ 50 days later, nonlame cows decreased behavioural and cortisol responses. In contrast, lame cows did not alter their behavioural response and had a higher cortisol response compared to nonlame cows. In conclusion, lame cows do not cope as successfully as nonlame cows in a repetitive acutely stressful situation.

Acknowledgements

The authors would like to thank farm staff for their cooperation with the study. Thank you to Hilary Pursell for technical assistance and Coralie Munro for supplying the EIA assay reagents. A very special thank you to Donald Tyson and Paul Fox for their assistance with the project. Scholarships (to SLW) were provided by Natural Sciences and Engineering Research Council of Canada (NSERC), Universities UK and University of Liverpool. This work was presented in part at the annual meeting of the European Society of Domestic Animal Reproduction, 2005 (Walker et al., 2005).

Table 1. Definitions of stress related behaviours

Head Movements	
Gentle	Gentle horizontal or vertical head movements corresponding to sniffing the air and looking around. The beginning and end of movement was considered to be the break in movement that was greater > 2 seconds
Aggressive	Aggressive horizontal or vertical head movements corresponding to an attempt to escape from the bars restraining the head. There was no 'aggressive body' movement associated with this behaviour.
Body Movements	
Gentle	Gentle shifting of body weight from side to side. The beginning and end of shifting was considered to be the when one foot was lifted off the ground or weight was shifted to the opposite foot and back again[a]
Aggressive	Strong whole body (including the head) movements corresponding to attempts to escape from bars by pulling whole body in backward and forward motion
Tail flicking	A single back and forth sharp motion of the tail
Other Behaviours	
Urination	A mark was recorded for every urination
Defaecation	A mark was recorded for every defaecation
Vocalization	A mark was recorded for every vocalization

[a] The multiple shifts that were observed with defaecation and urination were considered as a single shift.

Table 2. Definitions of overall behaviour reaction[a]

Low	Calm, hardly any gentle or aggressive movement
Moderate	Slightly restless, squirming and occasionally shaking the crush
Strong	More continuous vigorous movement and aggressively shaking the crush

[a] Definitions are adapted from (Grandin, 1993) 5 point scale, in which scores 1 is comparable to low, 2/3 and 4/5 are grouped and equivalent moderate and strong responses as defined above, respectively

Table 3. Lameness scoring scale[1]

Lameness Score	Description	Assessment criteria		
		Stands	Walking	Gait
1	**Normal**	Level back posture	Level back posture	Normal
2	**Moderately Lame**	Level back posture	Arched back	Normal to short-striding
3	**Severely Lame**	Arched posture	Arched back	Takes one step at a time/reluctance to bear weight on one of more limbs/feet

[1] modified after (Sprecher et al., 1997) 5 point scale, in which scores 1 and 2 are comparable and 3-5 are grouped and equivalent to score 3 as defined above

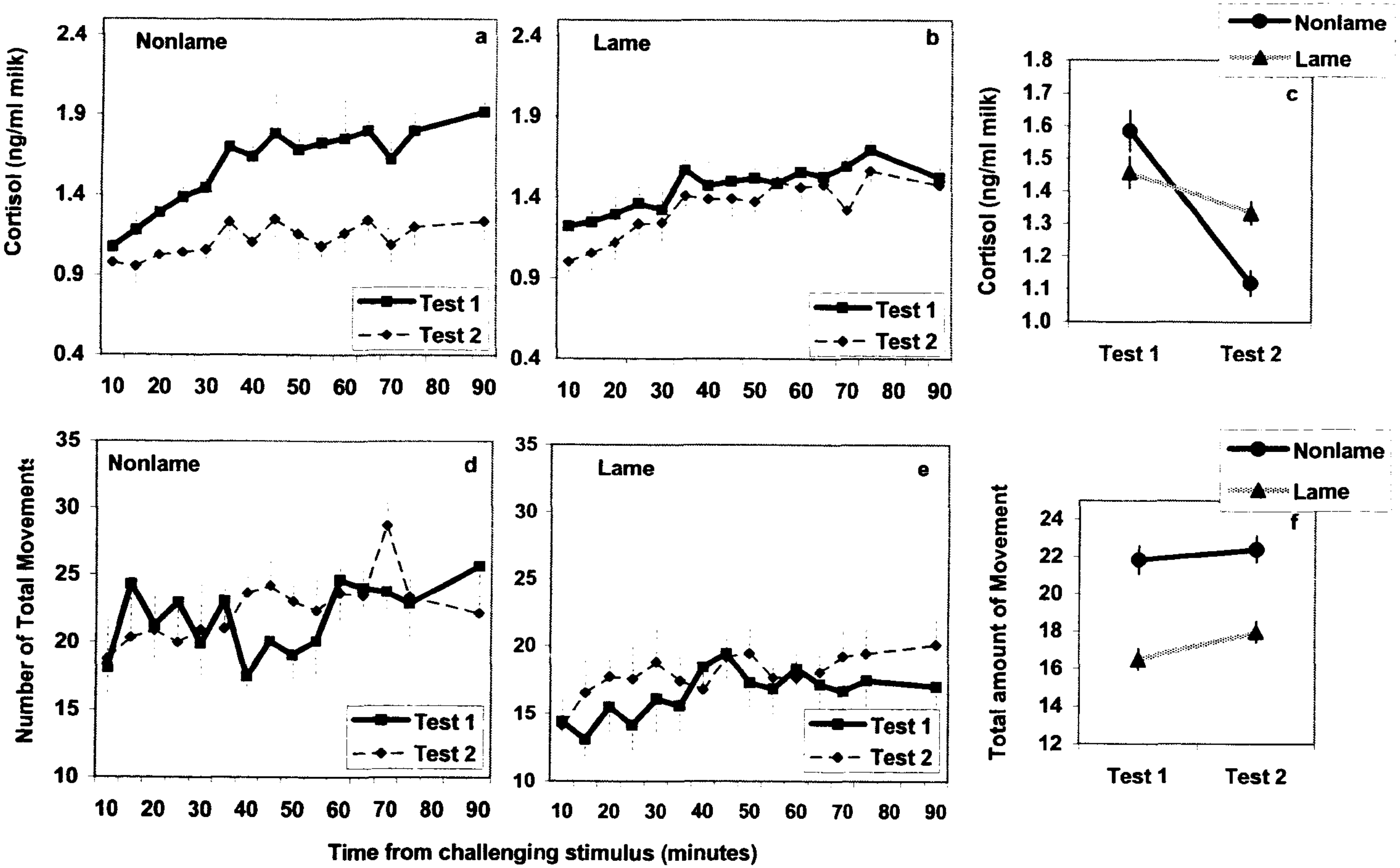


Figure 1. Mean ± SEM cortisol (ng/ml milk) **(a,b)** or total movement **(d,e)** profiles following an acute stressor in nonlame (Test 1 n=26, Test 2 n=22) or lame cows (Test 1 n=18, Test 2 n=22) and the overall mean (10-90 min) ± SEM values from Test 1 and Test 2 for nonlame or lame cows **(c,f)**.

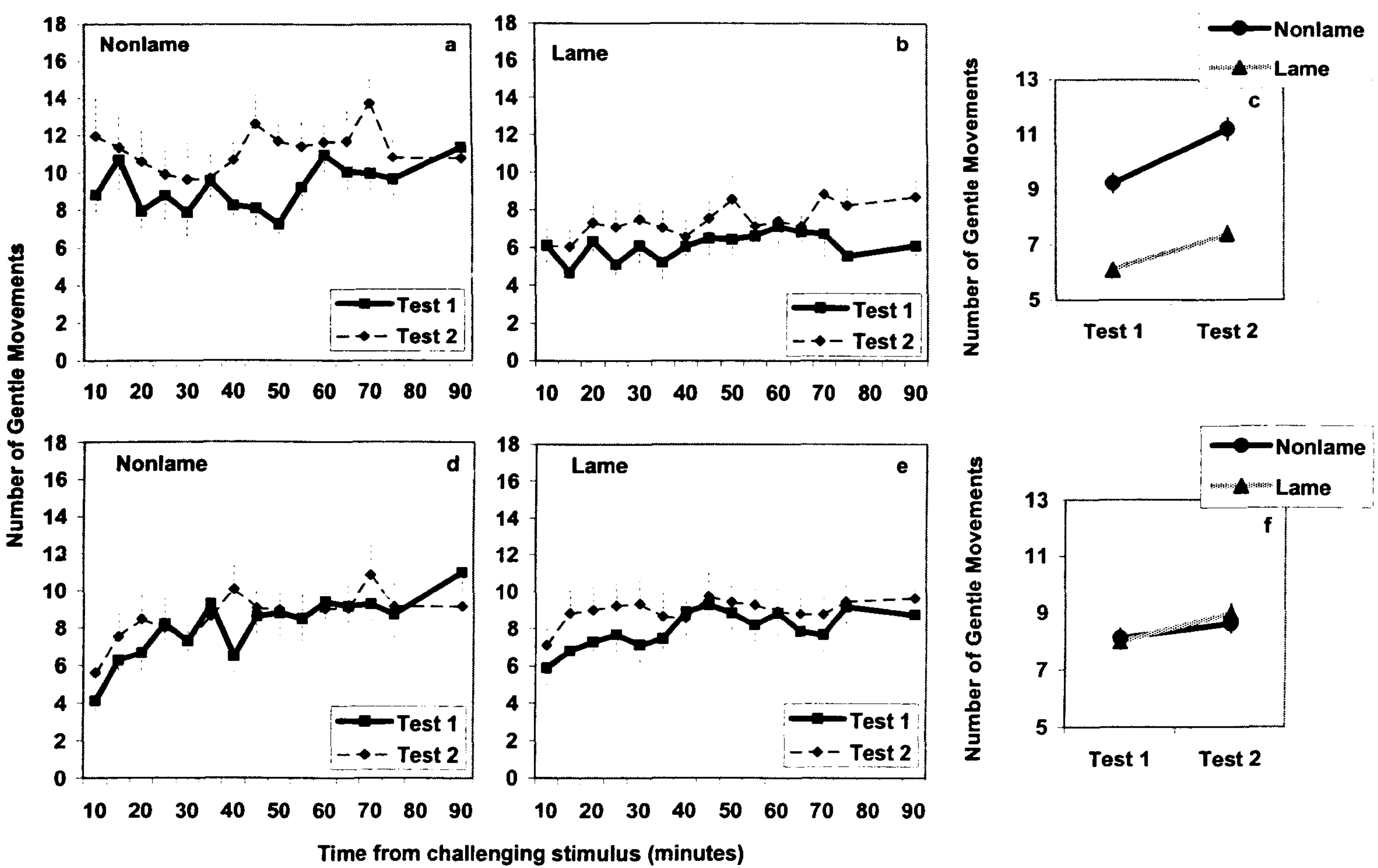


Figure 2. Mean ± SEM profiles of the number of gentle head **(a,b)** or gentle body shifting **(d,e)** movements following an acute stressor in nonlame (Test 1 n=26, Test 2 n=22) or lame cows (Test 1 n=18, Test 2 n=22) and the overall mean (10-90 min) ± SEM values from Test 1 and Test 2 for nonlame or lame cows **(c,f)**.

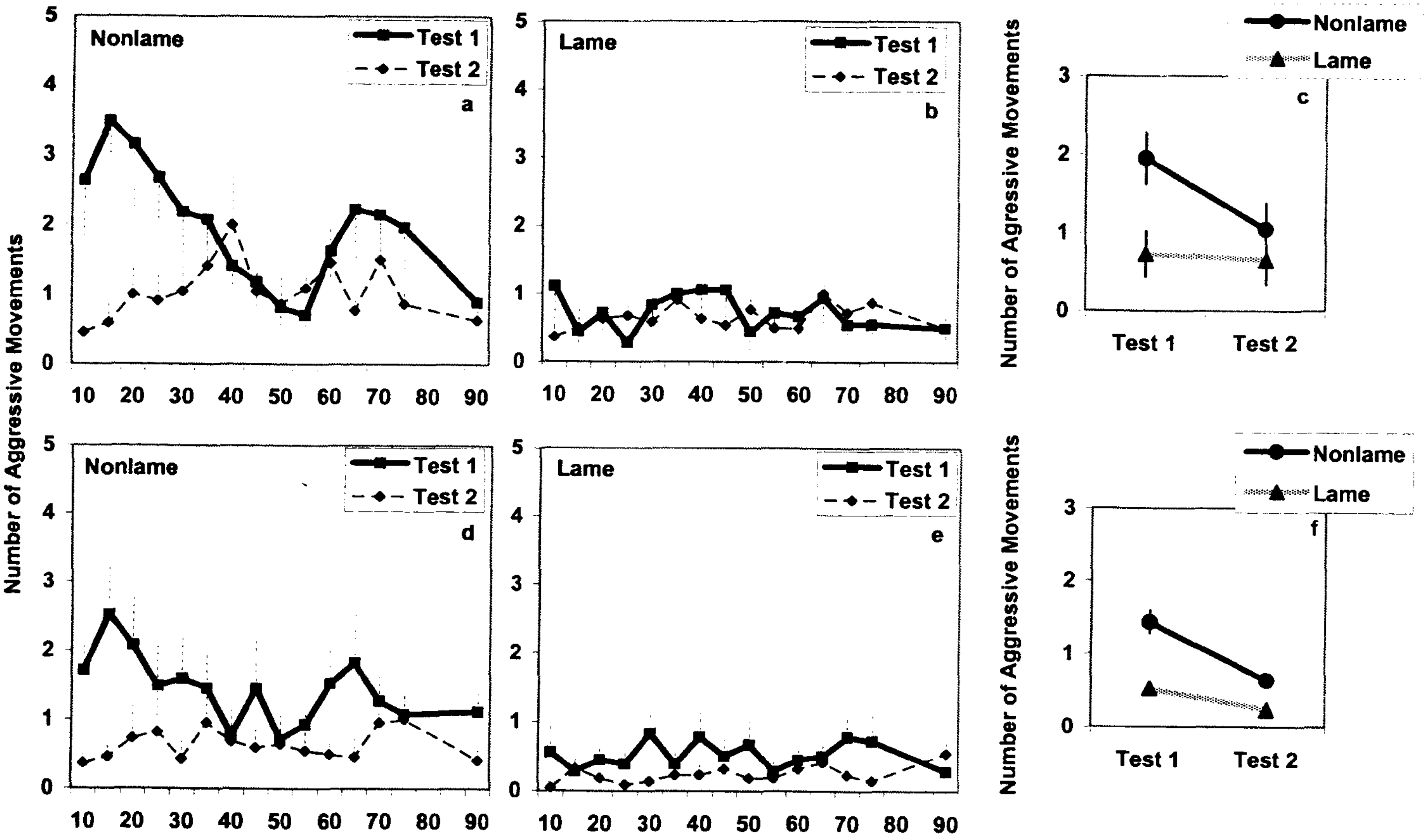


Figure 3. Mean ± SEM profiles of the number of aggressive head **(a,b)** or body **(d,e)** movements following an acute stressor in nonlame (Test 1 n=26, Test 2 n=22) or lame cows (Test 1 n=18, Test 2 n=22) and the overall mean (10-90 min) ± SEM values from Test 1 and Test 2 for nonlame or lame cows **(c,f)**.

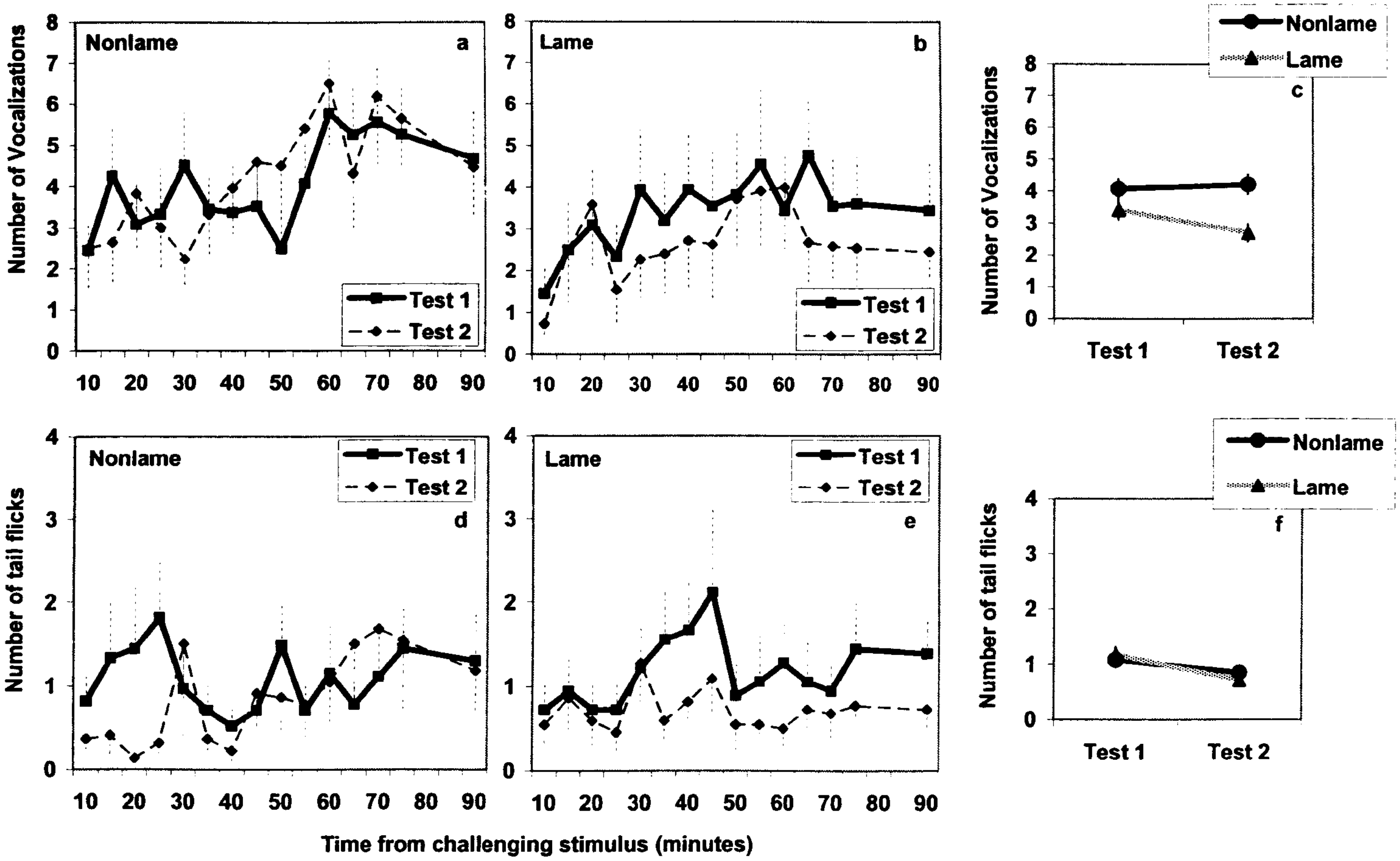


Figure 4. Mean ± SEM profiles of the number of vocalizations **(a,b)** or tail flicks **(d,e)** following an acute stressor in nonlame (Test 1 n=26, Test 2 n=22) or lame cows (Test 1 n=18, Test 2 n=22) and the overall mean (10-90 min) ± SEM values from Test 1 and Test 2 for nonlame or lame cows **(c,f)**.

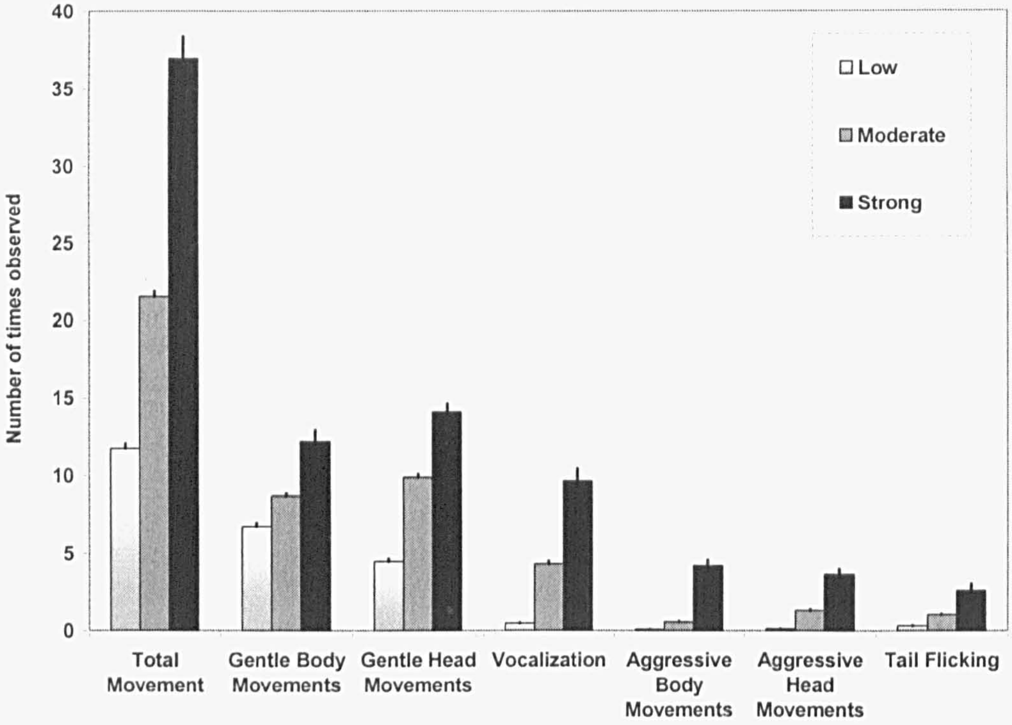


Figure 5. The average (±SEM) total number of all movements and of gentle head/body movements, vocalization, aggressive head/body movements and tail flicks in subjective behavioural reaction categories that were considered to be a 'low' (n=27), 'moderate' (n=54) or 'strong' (n=7) to an acute stressor. Increasing step-wise differences ($p<0.05$) were observed between low, moderated or strong reactions for all seven categories.

Chapter 5

Chapter 5

Coping strategies and adrenal responses in lame dairy cattle

Walker SL, Smith RF, Jones DN and Dobson H

Key words: lameness, acute stress, milk cortisol, coping, predictability, control

Abstract

We have already shown that lame cows exhibit fear-related coping responses to an acute stressor. The objective of the present study was to determine if lame cows have an increased need for predictability and/or control over their environment as measured by the consistency of side preference when entering the milking parlour and, if so, does this need for predictability and/or control explain the adrenal response to an acute stressor in lame cows.

Groups of ~12 lame or nonlame postpartum dairy cows (total 59 cows) were exposed an acute stressor over three consecutive days (Test Day 1,2,3). Milk cortisol was assessed immediately prior to milking and then every 5 min from 40 to 65 min after a challenge with an acute stressor (a loud bang).

Cows were scored for lameness and consistency of parlour side preference [low (<70%) 8=nonlame, 17= lame; medium (70-89%) 10= nonlame, 17=lame; high (>90%) 2= nonlame, 5= lame].

Resting cortisol concentrations were similar for all groups of cows (p>0.05). A GLM ANOVA revealed that the cortisol response following an acute stressor, increased with time (p=0.025) and varied with Test Day (p=0.002). There was a trend for lame cows to have higher cortisol responses compared to nonlame cows; however, this was not statistically significant (p=0.150). Post hoc pairwise comparisons revealed that the cortisol response increased, in both nonlame and lame cows, when challenged on a second occasion with the same stressor (p=0.024 and p=0.021, respectively), the cortisol response then remained the same on the third occasion (p=0.753 and p=0.528). There was a trend for high side-consistency cows to have lower cortisol responses but this was not statistically significant (p=0.170). There was also an interaction between side-consistency and Test Day (p=0.000). Lastly, there was no association between lameness and side-consistency (p=0.884).

Therefore, although there were trends towards differences in adrenal responses within lameness and side-consistency for milking, predictability and/or control, as measured by consistency of side preference in the milking parlour, appears not to be a behavioural trait or coping strategy associated with lameness.

Introduction

Coping is a behavioural reaction that aims to reduce the effect of aversive stimuli (Wechsler, 1995) and coping successfully in a social environment requires adopting certain behavioural strategies (Prelle et al., 2004). When environmental stressors are too demanding and an individual cannot cope, health is in danger (Koolhaas et al., 1999). Therefore, the presence of lameness itself, suggests an inability to cope with the demands of the surrounding environment. The risk of becoming lame may be related to certain social and behaviour strategies. Cows that become lame, stand for longer half inside a cubicle and are of lower social rank as they are less likely to displace other cows (Galindo et al., 2000). Regardless of cause and effect, lameness is also associated with modifications in routine behaviours such as daily time budgets, feeding, milking order, social interactions and oestrous behaviours (Chapter 1,2,3 Hassall et al., 1993; Galindo & Broom, 2002; Singh et al., 1993; Sauter-Louis et al., 2004).

Once lame, cows may adopt a different coping response to a stressful situation. Dairy cows face numerous acute stressors on a daily basis, e.g. social stimuli (constant re-grouping based on milk yield, social isolation), physical restraint (for artificial insemination, foot trimming), novelty (introduction of new herd-mates, handlers, new management routines). All of which have the potential to induce fear and activate the hypothalamic-pituitary-adrenal (HPA) axis in cattle (Boissy, 1995; Hopster & Blokhuis, 1994; Grignard et al., 2001). Chronic stress and pain, such as lameness, is also often associated with disturbances of the HPA axis, resulting in both functional and structural modifications and long-term alterations in neuroendocrine reactivity to subsequent stressors (Ostrander et al., 2006; Ulrich-Lai et al., 2006b). We have shown that lame cows cope differently to an acute stressor compared to nonlame cows, by altering cortisol and behavioural responses to deal with the situation (Chapter 4). Lame cows have higher cortisol responses and move less, suggesting a more passive (or fear-related) coping response to an acute stressor (Koolhaas et al., 1999; Wechsler, 1995). This demonstrates that once lame, a cow reacts differently to an acute stressor compared to nonlame cows.

Successfully coping with an aversive situation highly depends on the predictability and/or control of a situation (Ursin & Olff, 1993; Weiss, 1968; Boissy, 1995). In general, the prediction and/or control of a threatening situation results in a dampened anxiety-related response (Zvolensky et al., 2000). Anxiety suggests an animal is fearful and not coping well with the situation at hand. It has been suggested that coping strategies could be interpreted as differences in the need for predictability and control (Hansen & Damgaard, 1993) so that animals with high levels of anxiety and lower thresholds in eliciting stress responses, have an increased need for predictability and control of their environment (Prelle et al., 2004). Therefore, the attenuated cortisol responses and fear-related behavioural responses we observed in lame cows in Chapter 4 suggests that lame cows may have an increased need for predictability and control in their environment. (Prelle et al., 2004) have suggested that side preference in the milking parlour might be a way of assessing individual behavioural characteristics (such as anxiety), as a cow that creates a routine may be a cow that has an increased need to control the environment. As inability to cope with the environment is detrimental to welfare (Broom & Johnson, 1993) and reproduction (Dobson & Smith, 2000), it is important to understand the mechanisms and factors underlying an individual's ability to cope with the surrounding environment. Therefore, the aim of the present study was to investigate whether predictability or control, measured as side-consistency in the milking parlour, are behavioural character traits associated with adrenal responses to an acute stressor in lame cows.

Materials and Methods

Experimental design, animals, feeding and housing

The study was conducted on postpartum Holstein-Friesian cows (n = 59) on a UK commercial dairy farm comprising a total of ~200 year-round calving cows. The parity and days postpartum of the study cows was 3.9 ± 0.2 (range 2-10) and 51.8 ± 1.4 (range 30 – 75), respectively. Cows were part of a corresponding study (Chapter 2 and Chapter 3) in which ovarain cycles were synchronized in 5 groups of ~12 animals between May and September 2005 (temperatures ranged 5 to 28 °C) using a 100 µg im injection of a gonadotrophin releasing hormone (GnRH) analogue (Buserelin, 2.5ml Receptal®, Intervet Ltd. Bucks, UK) followed by a single 500 µg im injection of a prostaglandin F2α (PG) analogue (cloprostenol, 2ml, Estrumate® Schering-Plough Animal Health, Uxbridge, UK) 7 days later. Animals were at pasture (seasonal ryegrass, Italian ryegrass and white clover) for the duration of the study with additional access to total mixed rations (TMR) inside at a feed-fence after milking twice a day. The average rolling milk yield per cow in the herd was 8500 litres/year.

Behavioural observations and milk sampling

Adrenal responses to an acute stressor were tested on three occasions in 5 groups of ~12 cows. 'Tests' were carried out on 3 consecutive days prior to PG injection. Following milking, groups of cows were collected in a holding area which was separated from the main corridor by a small wall therefore cows could smell and hear, but not see, other cows leaving the milking parlour. Once milking was finished, all cows were moved to the entrance of the parlour and as a group were exposed to a challenging stimulus. The stimulus was the quick and sudden movement of two observers moving towards the group of cows to make a loud bang and swiftly moving away again. Cows remained in the entrance of the parlour undisturbed for 35 minutes. Cows were then moved into the parlour and milk samples were taken by hand every 5 min between 40 and 65 min following the challenging stimulus. To compare cortisol concentrations prior to Tests, milk samples were taken in the parlour prior to milking for 5 days, including the three Test days and two days following the Tests.

Side consistency

Observations (n=17/cow) for side-consistency (which was a measure of a cows' consistency to visit the same side of a two-sided parlour) were collected by an observer standing inside the milking parlour once a day for one week prior to PG injection and twice a day for 5 days following PG injection. For each cow, side-consistency (%) was calculated as the number of times a cows was milked on each side divided by the total number of observations (n=17). A cows' side-consistency was then retrospectively grouped as either low (<70%, n=25 cows), medium (70-89%, n=27 cows) or high (>90%, n=7 cows). Side-consistency was intended to reflect a cows predictability and/or control over of a particular situation, in this case, which side to be milked from.

Lameness scoring

In a parallel study, Chapter 2, individuals were scored for lameness (1-3) for 4 weeks prior to the commencement of the study, based on gait and posture while walking and standing, using the methods adapted from of (Sprecher et al., 1997) and previous described in (Chapter 2). Clinical treatment of lameness continued as usual on the farm. Retrospectively, ninety-five percent of individuals had the same or ± 1 lameness score for the duration of the study and were therefore grouped based on an average lameness score. Any cow with average score of ≥ 2 was considered to be lame and animals were grouped as either nonlame (score of 1; n = 20) or lame (score of 2 or 3; n = 39).

Enzyme immunoassays

Milk cortisol were analysed by previously described (Chapter 1,Young et al., 2004) enzyme immunoassay's (EIA) using the modified methods of (Munro & Stabenfeldt, 1984). Briefly, the EIA utilized an antibody (polyclonal cortisol antiserum R4866; supplied by CJ Munro, University of California, Davis, CA), horseradish peroxidase conjugated label and cortisol standard (Sigma-Aldrich, UK). In general, assay procedures were as follows: 1) antiserum was diluted at 1:8500 2) standards (3.9-1000 pg/well) and samples (ran undiluted) were loaded (50 µl/well) onto the plate; and 3) the horseradish peroxidase conjugate was used at a dilution of 1:40,000. The cortisol antiserum cross-reacts with cortisol 100%, prednisolone 9.9%, prednisone 6.3%, cortisone 5% and <1% with corticosterone, desoxycorticosterone, 21-desoxycortisone, testosterone, androstenedione, androsterone, and 11-desoxycortisol (C.J. Munro, pers. comm.). A parallel displacement curve was obtained for serial diluted pooled milk samples and the cortisol standard curve. The intra- and interassay coefficients of variations were <5% and <15%, respectively.

Data Analysis

All data are presented as mean ± SEM and were analysed using Minitab 14®. Minitab 14® restricts post-hoc comparisons on factors nested within random factors therefore any significant differences detected by a model with factors nested within random factors were analysed using GLM ANOVA comparing within and between factors and Tukey's 95% post-hoc pairwise comparison. Statistical differences were reported when $p<0.05$.

Cortisol concentrations were normalized using logarithmic transformation. Resting cortisol concentrations prior to milking were compared using a General Linear Model (GLM) ANOVA with repeated measures with the random factor of cow ID and the fixed factors of lameness (not lame/lame), side consistency (low, medium, high), Day (1-5), with lameness and side consistency nested within cow ID and the interaction of side consistency x lameness.

To determine if lameness or side-consistency were predictors of cortisol response following an acute stressor a GLM ANOVA with repeated measures was performed including the random factor of cow ID and the fixed factors of lameness (not lame/lame), side consistency (low, medium, high), Time (40-65), Test (Day 1,2,3) with lameness and side consistency nested within cow ID and the interactions of side consistency x Test, lameness x Test and side consistency x lameness.

Chi-Square tests were employed to assess the relationship between side consistency and lameness.

Results

Resting cortisol concentrations before cows were milked and prior to each Test and 2 days following the Tests did not differ between lame and not lame cows (lame 2.54 ±

0.09 ng/ml milk; not lame 2.40 ± 0.17 ng/ml milk, p=0.289) or cow with high, medium or low side consistency (2.37 ± 0.01, 2.68 ± 0.15 and 2.25 ± 0.22 ng/ml milk respectively, p=0.233).

The GLM used to determine if lameness or side-consistency were predictors of the cortisol response following an acute stressor showed that cortisol concentrations varied significantly with time (p=0.025) and between Test Days (p=0.002). Although there was a trend towards lame cows having high cortisol responses there was no statistical significance (p=0.150; Figure 1a) and this trend did not vary between Test Day (p=0.484; Figure 1b). Although there was no overall affect, post hoc pairwise comparison within nonlame and lame cows revealed, there was a significant increase in cortisol concentrations from Test Day 1 to Test Day 2 (p=0.024 and p=0.021, respectively; Figure 2) and cortisol concentrations did not change from Test Day 2 to Test Day 3 (p=0.753 and p=0.528; Figure 1b). There was also a trend for cows with a high side-consistency to have lower cortisol; however, the difference was not statistically significant (p=0.170; Figure 2a). There was an interaction between Test Day and side consistency (p=0.000; Figure 2b) with a trend of high side-consistency having low consistent cortisol responses compared to moderate and low side-consistency cows.

The incidence of lameness was not associated with low, medium or high side-consistency (not lame 8/10/2 and lame 17/17/5 cows respectively; p=0.884).

Discussion

In previous studies we have shown that lame cows have a higher cortisol response and expressed less movement during an acutely stressful situation (Chapter 4), suggesting a passive coping response to an acutely stressful stimulus (Koolhaas et al., 1999). A passive coping response, with low levels of aggression and immobility and high cortisol response, is believed to indicate a more fearful response to a stressful situation (Jones & Satterlee, 1996; Koolhaas et al., 1999; Beuving et al., 1989; De Boer et al., 1990b; Mendl & Deag, 1995; Korte et al., 1997). In the present study, we observed a similar trend in the cortisol response, in that lame cows had higher (but not significant) cortisol concentrations compared to nonlame cows. In Chapter 4, cows were challenged with an acute stressor (loud bang) when restrained and isolated in a crush, whereas, in the present study cows were challenged with the same acute stressor but in groups of ~12 cows. Social buffering is known to decrease the cortisol response, especially in gregarious animals like cows. Rats, fowl and monkeys exposed to a novel environment or fear-producing stimuli have stronger behavioural and physiological responses when tested alone than when tested in the presence of conspecifics (Taylor, 1981; Coe et al., 1982; Jones & Merry, 1988; Hennessy et al., 1982). In heifers, the mere presence of conspecifics is sufficient to lessen the threatening effect of an unexpected event (Boissy & Le Neindre, 1990). Perhaps this explains why in the present study lameness was not an explanatory variable in the model.

Models for chronic stress in rodents usually involve a highly predictable repetitive regimen of chronic intermittent stressors (Hauger et al., 1990; Bhatnagar & Meaney, 1995; De Boer et al., 1990a; Dobrakovova et al., 1993; Spencer & McEwen, 1990). The hormonal response in animals exposed to a repeated stressor is adaptation or habituation so that exposure to the same stressor evokes less of a hormonal response to each successive stress session (Tache et al., 1976; Kant et al., 1985; Garcia et al., 2000; Hauger et al., 1990; Cole et al., 2000). In the present study, there was an increase in cortisol concentrations in both lame and nonlame cows on the second day the cows were challenged and the values remained the same on third day. In the present study, if the Test had continued for >3 days this may have resulted in a decreasing trend in the cortisol response over a longer duration. In studies with rodents and pigs, several days or weeks of chronic intermittent stress are used to detect differences in HPA activity (Otten et al., 2004; Bhatnagar & Meaney, 1995; Ostrander et al., 2006). In rats repeated exposure for up to 7 days is required before corticosterone concentrations decrease to concentrations similar to non-stressed controls (Kant et al., 1987).

In Chapter 4, challenges were separated by ~50 days, whereas in the present study cows were challenged on consecutive days. Comparisons between the present study and Chapter 4 reveal that the timing between acutely stressful situations appears to have an impact on adrenal cortisol responses in lame cows. If the repeated stressor is daily, lame cows appeared to cope with situation in a similar manner to nonlame cows (i.e. pattern of the cortisol response from day to day was similar); however, if the stressor is separated by a period of time lame, as in Chapter 4, lame cows do not adjust to the acute stressor as well, as there was no change in stress-related behaviours and a higher cortisol response was observed compared to nonlame cows. Comparisons between Chapter 4 and the present study are difficult as cows in the present study cows faced an acute stressor in the presence of ~12 conspecifics, which may have provided social buffering and decreased anxiety. However, the practical implications are, if lame cows are faced with challenges on a daily basis (i.e. re-grouping based on milk yield, physical restraint for artificial insemination, foot trimming or introduction of new herd-mates etc.) and are not isolated from their herd-mates they may be able to cope with the situation at hand; however, if isolated and challenged less frequently lame cows may perceive this as more stressful and are unable to cope with the situation. The consequences of not coping with stressful stimuli may result in a decrease in oestrus intensity (Chapter 1 and 2, Walker et al., 2005) and poor reproductive performance (Moberg, 1985; Collick et al., 1989; Dobson & Smith, 2000; Rivier & Rivest, 1991; Liptrap, 1993).

In the present study, there was a trend in high side-consistency cows to have low consistent cortisol responses to an acute stressor compared to moderate or low side-consistency cows. A lower cortisol response to the repeated presentation of the same acute stressor in high consistency cows suggests a reduction in fear of novelty (Boissy, 1995). Fear is defined as an emotional state induced by the detection of danger threatening the well-being of an individual and this in cattle is related to elevated corticosteroids and inactivity (Boissy & Le Neindre, 1997; Boissy, 1995; Boissy & Bouissou, 1995; Van Reenen et al., 2005). Based on behavioural observations, cows that consistently enter the parlour on the same side (>90 % of the time) are less active when introduced to novel stimuli, which could be interpreted as high levels of fear to novel stimuli (Prelle et al., 2004). However, there was substantial evidence in that study that cows that are consistently milked on the same side were dominant over other cows in challenging situations, suggesting reduced fearfulness of others (Boissy, 1995). Although, different stimuli trigger different neuroendocrine changes (Mormede et al., 1990; Pacak & Palkovits, 2001) and comparisons between the studies should be interpreted with caution, it is suggested that cows with the behavioural trait of predictability and control as measured by side preference in the milking parlour are less fearful and cope better with stressful stimuli.

Nevertheless, in the present study, there was no association between lameness and the repeatability of side-consistency for milking. This suggests that, although there were trends towards differences in adrenal responses within lameness and side-consistency for milking, the need for control was not behavioural trait associated with lameness. Therefore, coping strategies to acute stressors that are adopted by lame cows requires further investigation. Understanding how and what coping strategies an individual uses will lead to improved welfare and reproductive efficiency.

Acknowledgements

The authors would like to thank farm staff for their cooperation with the study. Thank you to Hilary Pursell for technical assistance and Coralie Munro for supplying the EIA assay reagents and a very special thank you to Donald Tyson and Paul Fox for their assistance with the project. Scholarships (to SLW) were provided by Natural Sciences and Engineering Research Council of Canada (NSERC), Universities UK and University of Liverpool.

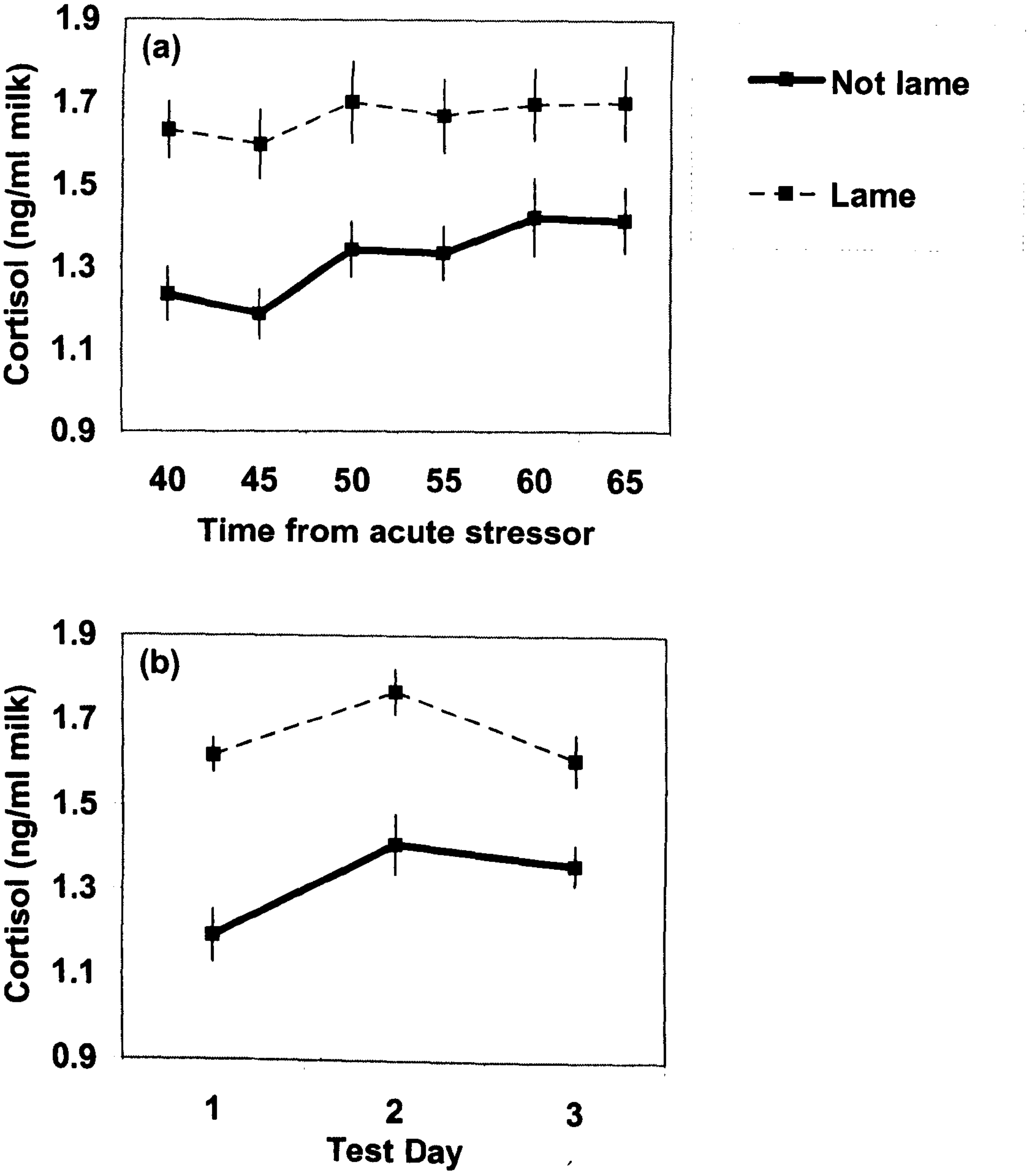


Figure 1. Mean cortisol ± SEM concentrations at 40 to 65 mins (from Test Day 1,2 and 3) following an acute stressor in lame (n=39) or not lame (n=20) cows (a) and overall mean cortisol ± SEM concentrations on Test Day 1,2 and 3 following an acute stressor in lame (n=39) or not lame (n=20) cows (b).

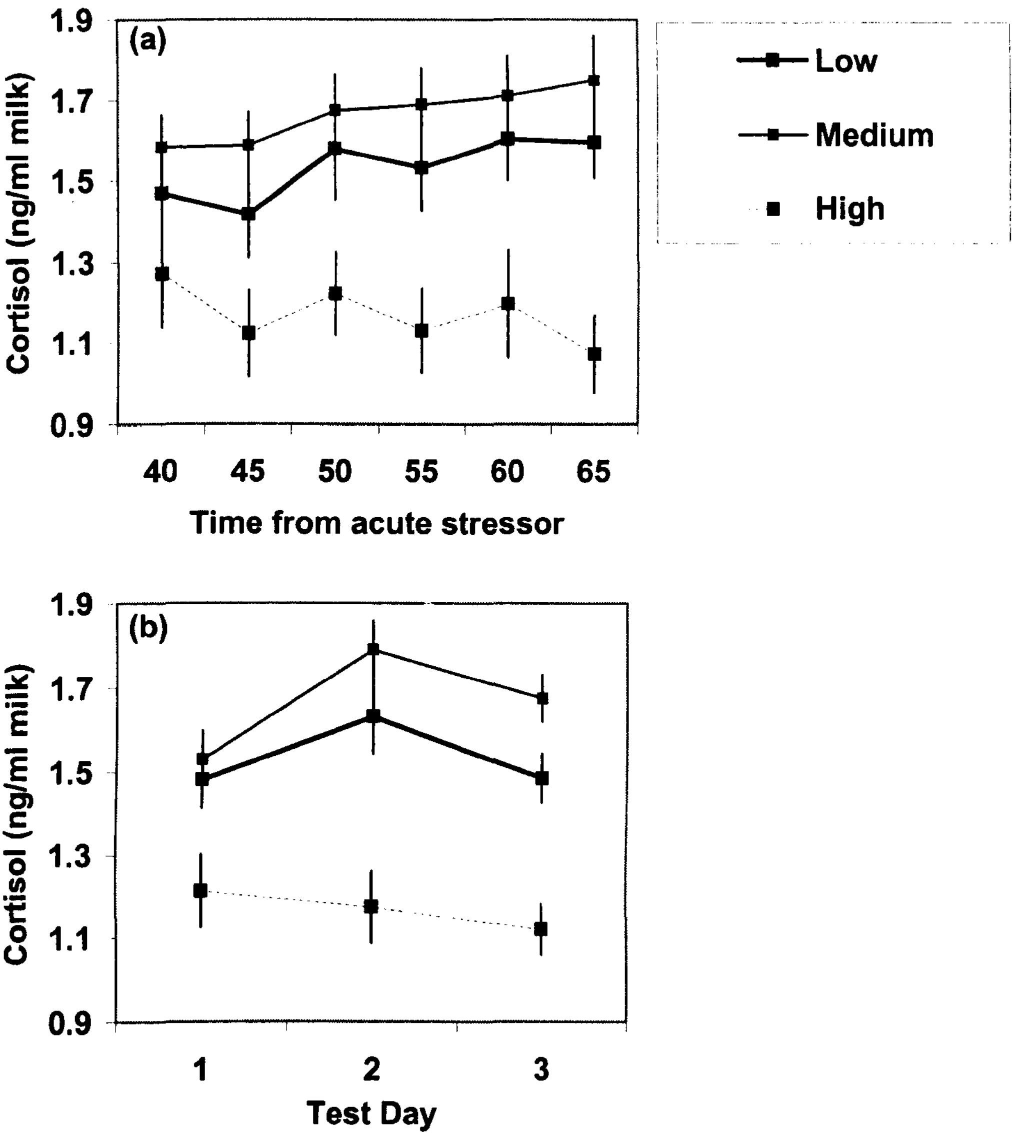


Figure 2. Mean cortisol ± SEM concentrations at 40 to 65 mins (from Test Day 1,2 and 3) following an acute stressor in low (n=25), medium (n=27) or high (n=7) side-consistency cows (a) and overall mean cortisol ± SEM concentrations on Test Day 1,2 and 3 following an acute stressor in low (n=25), medium (n=27) or high (n=7) side-consistency cows (b).

General discussion and conclusions

General discussion and conclusions

One of the aims of the present study was to examine the relationship between lameness and intensity of various behavioural signs of oestous and the incidence of oestrus on a commercial dairy farm.

From Chapters 1 and 2 of this thesis we conclude lame cows express oestrus with less intensity. In brief, lameness impacts on primary and secondary signs of oestrus to varying degrees. Lameness, however, does not affect the incidence of oestrus or total duration of oestrus; therefore, a missed or inappropriately timed insemination in lame cows is most likely related to a decrease in oestrus intensity rather than a decrease in oestrus incidence or duration.

In Chapters 1 and 2 it was demonstrated that lame cows sniff and chin rest less compared to nonlame cows. The purpose of sniffing and chin resting may be to permit the transmission of chemical signals or pheromones between oestrous herd-mates. The receipt of pheromones may be important and drive oestrous behaviour in a positive feedback fashion. Lame cows may not be receiving the same level of pheromonal positive feedback due to an inability to keep up with the sexually active group and therefore, express a less intense oestrus. From the behavioural observations in Chapter 2 it was also revealed that lameness affects the amount of mounting by fellow herd-mates. This raises the question that, although a lame cow is less likely to stand when mounted as a result of pain, it may also be possible that lameness may diminish how 'attractive' the cow is (i.e. pheromone production). These suggestions provide an alternative explanation for why lame cows are mounted less than nonlame cows and exhibit a less intense oestrus. Investigation into chemical communication between cows during oestrus and the impact lameness or stress may have on chemical communication will be a fruitful future area of research.

A second aim of the present study was to elucidate progesterone, oestradiol and cortisol profiles that accompanied unobserved and observed oestrus.

In Chapter 1 and 2 of this thesis, after the start of postpartum ovarian cyclicity or following oestrous synchronization, reduced intensity of oestrus in lame cows was associated with lower progesterone prior to oestrus but not with abnormal cortisol or oestradiol values. In the ewe, prior progesterone priming is not only essential for the display of oestrus but increased concentrations increase the intensity of oestrus expression (Fabre-Nys & Martin, 1991a). Therefore a possible explanation for a decrease in oestrus intensity in lame cows could be related to poor progesterone exposure prior to oestrus. Research with replacement progesterone treatment prior to oestrus in lame cows should be investigated to determine if progesterone is a determining factor in oestrus intensity in cattle.

The 'silent' or unobserved oestrus in Chapter 1 and 2 was associated with continued elevated oestradiol following progesterone exposure around the expected time of oestrus (and ovulation). This presumably representing continued follicle growth and a delay or impairment of ovulation [which was confirmed during Chapter 2 in a parallel study (Morris et al., 2006)]. However, in both Chapters 1 and 2 there were similar numbers of lame and nonlame cows with continued elevated oestradiol around the expected time of oestrus (i.e following prior progesterone exposure lame and nonlame cows had an equal chance of being observed in oestrus). In Chapter 1, clinical fertility treatments continued as usual on the commercial dairy farm; therefore, cows (>20 days postpartum) came into oestrus naturally or oestrus was the as a result of a fertility treatment. In the naturally occurring oestrus events, lameness was not associated with the incidence of oestrus (i.e it took lame cows just as many luteal cycles following partuition as nonlame cows to be seen in oestrus).

In Chapter 2, the ovarian activity of cows (30 to 70 days postpartum) was synchronized using GnRH and prostaglandin injections. In this scenario, lameness was associated with low incidence of oestrus. The difference between these studies (Chapter 1 and 2) was the presence of cows with continued low progesterone (i.e. did not respond to the synchronization regime); there were 10 lame cows and 2 nonlame cows. When these cows were removed from the analysis lameness had no effect on the incidence of oestrus (as seen in Chapter 1). Additionally, of the cows that did reponse to the GnRH/PG injections, more lame cows had baseline progesterone concentrations following the GnRH injuection (i.e. more nonlame cows possessed luteal structures at the time of the GnRH injection). These observations, along with the observed lower progesterone concentrations in lame cows prior to observe in oestrus, suggest an effect on ovarian activity in lame cows. Therefore, further investigations in the possible disruptive mechanisms in ovarian cyclicity in lame cows should be undertaken.

A third aim of the present study was to determine what impact lameness had on social behaviours, the daily feeding and activity budget and body condition in relation to oestrus expression.

In Chapter 3 of this thesis it was revealed that lame cows lay down more and spent less time elevated on their feet (stand and walk less). Additionally, lameness tended to decrease the total proportion of time spent expressing an oestrous behaviour and varied the expression of an oestrous behaviour in relation to time of day. In general, cows in oestrus are restless and tend to form very mobile sexually active groups; indeed in

Chapter 2 nonlame cows were more restless than lame cows. Expressing behavioural signs of oestrus requires cows to be in close contact with female herd-mates. It is possible that lame cows are not motivated or rewarded sufficiently to remain with the sexually activie group and, therefore, exhibit fewer behavioural signs of oestrus. Thus, the results from this thesis suggest that lameness reduces oestrus intensity by hampering the number of interactions between cows in oestrus.

In Chapter 3 it was also demonstrated that although lameness had no effect on social rank, lameness did affect social activities such as movement out of the field and the order cows were milked. Additionally, lame cows had a lower bite rate when grazing, and were more likely to have a low body condition score and less likely to be seen in oestrus following oestrus synchronization. Poor nutrition is related to poor reproductive performance and could provide another explanation for the diminished oestrus expression in lame cows.

The alterations in time budgets demonstrated in this thesis, in particular the reduction in time spent standing and an increased time spent lying, the reduction in bite rate and the impact on social and oestrus behaviours highlight the detriment to welfare that lameness imposes on dairy cattle.

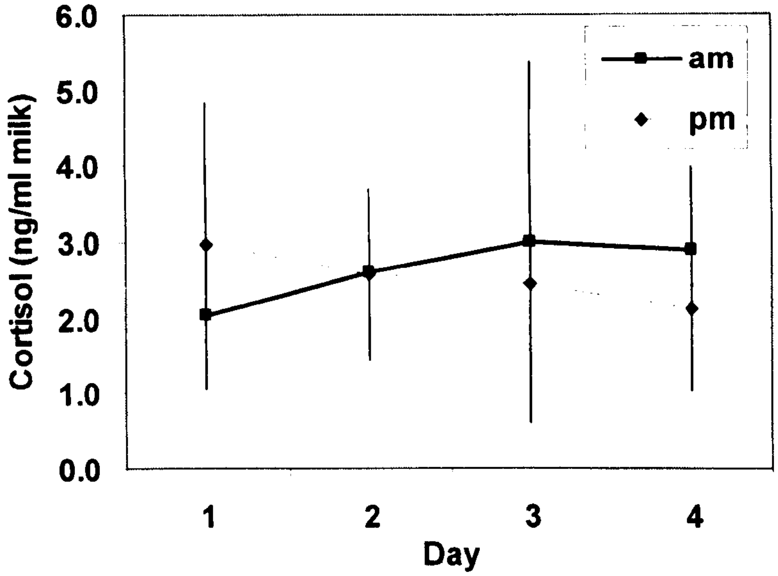
The final aim of the present study was to determine if lame cows differed in their behavioural and hormonal coping style to an acute stressor and investigate whether predictability and control are behavioural character traits that are associated with adrenal response to an acute stressor in lame cows.

In Chapter 1 it was demonstrated the chronic stress imposed by lameness was not associated with alterations in resting cortisol concentrations. However, in Chapter 4 lame cows coped differently to an acute stressor with a lower behavioural response and greater cortisol response to an acute stressor, suggesting a passive (fearful) coping reaction. This implies that lame cows do not cope as well as nonlame cows in a repetitive acutely stressful situation. The facilitated response to novel stimuli in lame cows could be one explaination for the low progesterone concentrations prior to oestrus. Alternatively, the low progesterone concentrations could also be explained by the low BCS in lame cows and/or an alteration in sensitivity or pattern of pulsaltile GnRH release (i.e resulting from the chronic pain/stress of lameness). Both of which could affect gonadotrophin support for ovarian structures (i.e. follicles and corpus luteum). This is a major area that requires further research.

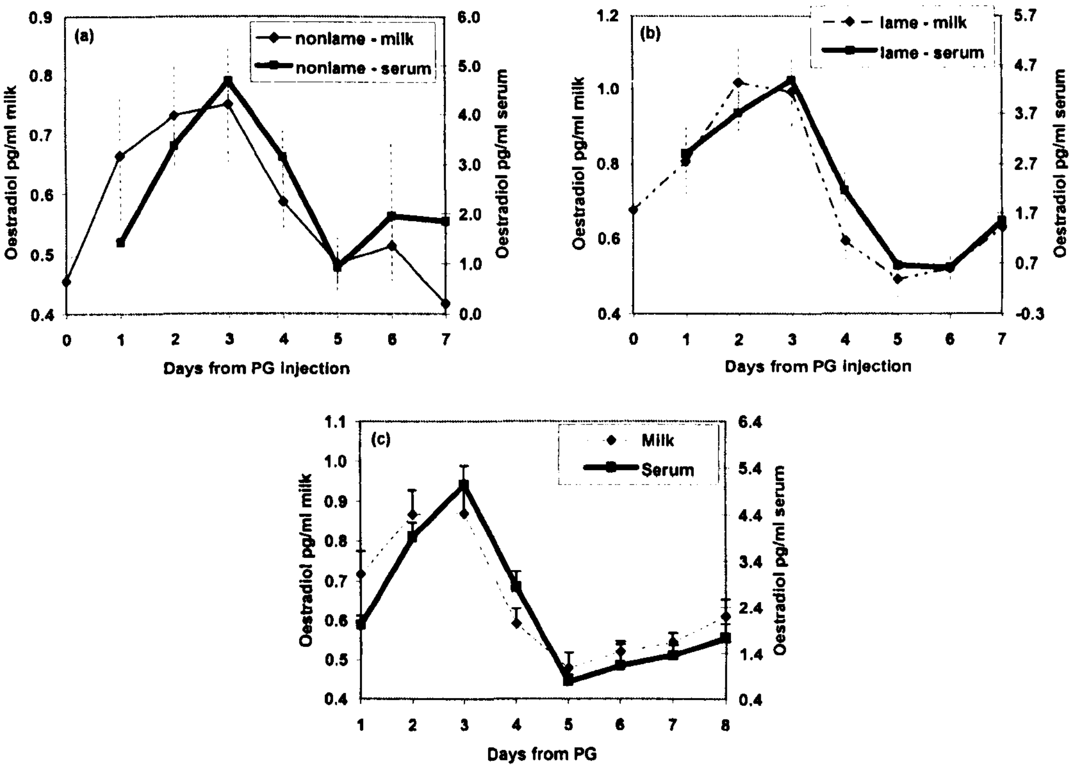
In Chapter 5 the consistency of side preference for milking was used as a measure of a cows' need for predictability and control of their environment. Although there were trends towards differences in adrenal responses within lameness and side-consistency, predictability and control were not behavioural traits associated with lameness. Understanding how an individual copes with a stressful situation and what factors determine coping strategies is important because this can lead to improved welfare and reproductive efficiency and this deserves further investigation.

Appendix

Appendix I – Milk cortisol diurnal samples



Milk Cortisol Figure. Milk samples were taken from 12 cows immediately prior to the morning and afternoon milking over a 4-day period and analyzed in a cortisol EIA, as described in Chapter 1. The data was anaylzed with a GLM ANOVA with repeated measures. The model included the random factor of cow ID with the fixed factors of day of sample (1-4), time (am/pm) and the interaction between day and time. The random factor of cow ID was significant (p=0.002, r2 adj=19.1%). No other factors were significant; day (p=0.858), time (p=0.845) and the interaction of day and time (p=0.103).



Milk and Serum Oestradiol Figure. Samples for oestradiol were taken from 37 cows (nonlame n=15 and lame n=22) observed in oestrus (see Chapter 2 for details). Milk samples were taken twice a day (immediately prior to the morning and evening milking) following PG injection. The two milk samples were averaged for a daily average. Corresponding serum samples were taken once a day following PG injection. Pearsons correlations were made between milk and serum daily averages for (a) nonlame p=0.073 and r^2= 0.711 and (b) lame cows p=0.001 and r^2= 0.959 and for (c) all cows seen in oestrus p=0.002 and r^2= 0.906

Appendix III – Fertility data

Year 1 (Chapter 1 n=44 cows)	*Nonlame*	***Moderately lame***	***Severely Lame***	P value
Calving to first insemination (days)	80.0 ± 6.1	71.2 ± 5.0	65.3 ± 6.1	p=0.263
Calving to pregnancy (days)	161.2 ± 26.0	89.6 ± 15.9	72.4 ± 5.4	p=0.072
Calving interval (days)	447.0 ± 28.1	376.9 ± 22.9	355.0 ± 4.1	p=0.111
Number of insemination required for pregnancy	2.0 ± 0.2	1.4 ± 0.3	1.2 ± 0.2	p=0.106
Number of cows that were not inseminated during the study and were culled /not culled	0/0	0/0	1/0	99% served
Of the cows that were inseminated (n=43) the number of cows that became pregnant at first insemination (yes/no; % of cows that became pregnant)	6/16 (27.3%)	6/5 (54.5%)	4/6 (40.0%)	p=0.305
Of the cows that were inseminated (n=43) the number of cows that became pregnant on all inseminations (yes/no; % of cows that became pregnant)	20/2 (90.1%)	9/2 (81.2%)	5/5 (50.0%)	
Of the n=44 cows the number of cows that were culled at the end of the study (yes/no # cows; % of cows culled)	2/20 (9.1%)	3/8 (27.3%)	6/5 (54.5%)	***p=0.017****

Year 2 (Chapter 2 n= 59 cows)	***Nonlame***	***Lame***	
Calving to first insemination (days)	72.3 ± 6.9	72.8 ± 6.2	p=0.996
Calving to pregnancy (days)	107.1 ± 14.9	131.8 ± 11.3	p=0.192
Calving interval (days)	397.1 ± 13.5	414.0 ± 11.2	p=0.345
Number of insemination required for pregnancy	2.0 ± 0.3	2.0 ± 0.2	p=0.898
Number of cows that were not inseminated during the study and were culled /not culled	1/1	5/1	86% served
Of the cows that were inseminated (n=51) the number of cows that became pregnant at first insemination (yes/no; % of cows that became pregnant)	6/12 (33.3%)	6/27 (18.2%)	p=0.223
Of the cows that were inseminated (n=51) the number of cows that became pregnant on all inseminations (yes/no; % of cows that became pregnant)	32/9 (78.0%)	7/3 (70.0%)	p=0.591
Of the n=59 cows the number of cows that were culled at the end of the study (yes/no # cows; % of cows culled)	1/19 (5.0%)	8/31 (20.5%)	p=0.117

Year 1 and 2 combined (n=103 cows)	***Nonlame***	***Lame***	
Calving to first insemination (days)	76.6 ± 4.6	71.0 ± 4.0	p=0.368
Calving to pregnancy (days)	138.0 ± 16.6	114.0 ± 8.9	p=0.195
Calving interval (days)	425.0 ± 17.1	398.0 ± 9.4	p=0.163
Number of insemination required for pregnancy	2.0 ± 0.2	1.8 ± 0.2	p=0.342
Number of cows that were not inseminated during the study and were culled /not culled	1/1	6/1	91% served
Of the cows that were inseminated (n=94) the number of cows that became pregnant at first insemination (yes/no; % of cows that became pregnant)	12/28 (30.0%)	16/38 (29.6%)	p=0.969
Of the cows that were inseminated (n=94) the number of cows that became pregnant on all inseminations (yes/no; % of cows that became pregnant)	52/11 (82.5%)	21/10 (67.8%)	p=0.105
Of the n=103 cows the number of cows that were culled at the end of the study (yes/no # cows; % of cows culled)	3/39 (7.1%)	17/44 (27.9%)	***p=0.009****

Fertility Data. Fertility data were collected retrospectively following studies (2004 Year 1 Chapter 1; 2005 Year 2 Chapter 2) using the farm's NMR database and compiled using InterHerd © (PAN Livestock Services Limited). **Data analysis**: For years 1 and 2 and the combined data, the calving to first service, calving to conception, calving interval and number of inseminations required for pregnancy were analysed using a GLM ANOVA with the fixed factor of lameness. The conception at first service (was either yes or no), conception from any service (was either yes or no) and culled at the end of study (was either yes or no) and were analysed with Chi-square tests. Significant differences ($p<0.05$)* are in bold. **Results**: Lame cows were more likely to be culled at the end of study 1 ($p=0.017$). Analysis using a Chi-square between each group revealed the following; nonlame and moderately lame ($p=0.170$), moderately lame and severely lame ($p=0.193$) and nonlame and severely lame ($p=0.004$), suggesting severely lame cows are more at risk of being culled. The combined data also indicated a relationship between lameness and culling ($p=0.009$). The associated between culling and lameness has been shown by others (Collick et al., 1989). No significant differences were found with any other parameter, this is in disagreement with others who have demonstrated a relationship between lameness and several reproductive parameters (Table 5 in Literature Review). This may have been due to an insignificant number of cows in the study (~100). The work by (Collick et al., 1989) was conducted on >400 case/control pairs of cows.

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