DAIRY COW BEHAVIOUR AND ESTROUS EXPRESSION:

EFFECTS OF DISEASE AND MANAGEMENT

by

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Abstract

Management of modern dairy farms spend a considerable amount of time ensuring that every cow produces a calf every year, that all cows stay healthy. Despite these laudable goals, fertility and disease continue to be two of the major challenges facing the dairy industry. In this thesis I address both of these areas in an attempt to improve the health and welfare of dairy cattle and to aid farmers in the early identification of sick cows and identifying ways of improving fertility on their farms. My first aim was to assess changes in rumination and feeding behaviour associated with diseases common after calving (Chapter 2). The results indicate that monitoring rumination and feeding behaviour during the time around calving is helpful in the detection of cows with metabolic problems. The second aim focused on understanding how stocking density affects estrous expression and biomarkers of stress (Chapter 3). The results from Chapter 3 showed only mild effects of short-term exposure of lactating dairy cows to housing conditions where there is insufficient lying space for all cows to lie down at the same time, suggesting that many dairy cows are relatively resilient to short-term sub-optimal housing conditions. Lastly, I examined how standing and lying time, in general and around estrus, are affected by stocking density (Chapter 4). This chapter showed that even a short-term increase in stocking density to 133% (cow to stall ratio of 4:3) can have detrimental effects on the standing behaviour of healthy lactating dairy cattle and that individual standing times can be an indicator for the onset of estrus, particularly in understocked cows. In summary, this body of work shows how an improved understanding of behaviour can identify cows at risk for disease, that the choices made by farmers regarding how much lying space cows are given can affect standing and lying behaviour, and that changes in standing behaviour can be used detect estrus in dairy cows.

Lay Summary

Dairy farms require cows to stay healthy and reproduce successfully. Identifying behaviours that are indicative of cows at risk of becoming ill, and behaviours associated with estrus, is therefore of great importance, especially if this allows farmers to change management in ways that facilitate these assessments. For this thesis I investigated the effects of health and estrus on several behaviours, such as feeding, rumination, standing and lying behaviours, as well as how these behaviours are affected by common management practices on farms. My results show that dairy cows alter their behaviour according to health status and according to where they are in their reproduction cycle, and that these behaviours vary depending upon the conditions under which cows are housed.

Preface

The data for my thesis was derived from two separate studies conducted at the UBC Dairy Education and Research Centre in Agassiz, British Columbia, Canada. Animals were cared for following the guidelines of the Canadian Council on Animal Care (2009). All procedures were approved by the UBC Animal Ethics Committee (Protocols A 05-0660 and A 10-0290). Throughout the thesis, I refer to the first person plural when referring to research developed and conducted in collaboration with co-authors. When expressing my own personal opinions, I use the first person singular.

A version of chapter 2 has been published. Schirmann K., D. M. Weary, W. Heuwieser, N. Chapinal, R. L. A. Cerri, and M. A. G. von Keyserlingk. 2016. Short communication: Rumination and feeding behaviours differ between healthy and sick dairy cows during the transition period. J. Dairy Sci. doi:10.3168/jds.2015-10548. K. Schirmann, M. A. G. von Keyserlingk, W. Heuwieser, and D. M. Weary developed the main ideas for the manuscript. K. Schirmann collected and analyzed the data and wrote the manuscript. Co-authors assisted with interpretation of the material and with editing drafts.

K. Schirmann and R. L. A. Cerri developed the main ideas for the study presented in Chapters 3 and 4. R. L. A. Cerri, D. M. Weary and M. A. G. von Keyserlingk assisted with the study design. K. Schirmann collected the data. K. Schirmann and R. L. A. Cerri analysed the data. K. Schirmann is writing the manuscript and R. L. A. Cerri, M. A. G. von Keyserlingk and D. M. Weary assisted with the interpretation and with editing the drafts. A version of Chapters 3 and 4 is under preparation for submission for publication.

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List of Abbreviations

AAM = Automated Activity Monitor

BCS = Body Condition Score

BHB = β-Hydroxybutyrate

CL = Corpus Luteum

DIM = Days in Milk

DM = Dry Matter

DMI = Dry Matter Intake

FSH = Follicle-Stimulating Hormone

GnRH = Gonadotrophin Releasing Hormone

IGF = Insulin-like Growth Factor

LH = Luteinizing hormone

LH-r = Luteinizing hormone receptors

PGF = Prostaglandin $F_{2\alpha}$

SCK = Subclinical Ketosis

TGF = Transforming Growth Factor beta

TMR = Total Mixed Ration

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Chapter 1: Introduction

1.1 General Introduction

Estrous behaviour has long been the focus of research by those interested in the natural world (Heape, 1900). Although early interest focused on areas such as species differences between how the female and male interacted with one another (e.g. sexual behaviour) the field of reproductive physiology in farm animals has gained much traction with the introduction of artificial insemination. In the case of farm animals much of this interest has focused on the dairy cow whose ability to produce milk is dependent on her becoming pregnant and delivering a calf.

Over the past decades researchers specializing in dairy cattle estrous behaviour have gained further insights on the specific reproductive behaviours expressed, but also on their occurrence, in relation to ovulation (Walker et al., 1996; Roelofs et al., 2005a). Furthermore knowledge regarding the cyclic recurrent physiological changes on organs and at the hormonal level has resulted in major advances in this area (Sirois and Fortune, 1988; Battaglia et al., 1997; Walker et al., 2008). This interest in reproductive behaviour in dairy cattle has also likely been spurred in part by the growing recognition that fertility rates of dairy cows have been declining since the 1970's (Butler, 1998). Considering that fertility problems are long known it seems that, just like in the past, we need to expand our knowledge base on dairy cow estrous behaviour under today's housing conditions if we are to overcome the challenges associated with reduced fertility (Cole and Winters, 1939; Aschbacher et al., 1956).

Concurrent with the growing knowledge on the estrus related behaviours in dairy cattle, technologies for automatic detection have been introduced to decrease labour requirements associated with visual heat detection but also to increase detection rate per se (Kiddy, 1977). It is

the development of these new technologies that has enabled today's researchers to generate even more detailed knowledge of the reproductive behaviour of dairy cattle (Nebel et al., 2000). However, despite the introduction of numerous technologies designed to automatically detect estrus in dairy cattle this is still an evolving area in contemporary research (Madureira et al., 2015a).

Concurrent with the onset of these new technologies a tremendous amount of effort has been placed on gaining additional insights on hormonal changes in the female dairy cow. In essence, given that the goal has been to avoid having to rely on the visual observation of sexual behaviours in female dairy cattle, researchers have focused their efforts on developing estrus synchronization protocols that involve the injection of specific hormones at specific times that allow for prediction of when ovulation will occur and thus enables them to determine the best time to breed (Driancourt, 2001; Stevenson, 2008; Pulley et al., 2015).

Improved genetics has resulted in an annual increase of ~2% in milk production each year in countries such as the US and Europe (Buckley et al., 2000); with the increased metabolic demands placed on the cow as a result of increasing milk production it is not surprising that new problems, such as increased health and reproductive challenges, have developed. Also, over the last 5 decades there has been increased intensification of the dairy industry (see reviews by von Keyserlingk et al., 2013 and Barkema et al., 2015) that has resulted in dramatic changes in how dairy cattle are housed. These changes have resulted in fewer and fewer cows having access to pasture. Zero grazing systems such as the freestall or tie stall housing require cows to stand on alternate flooring such as concrete or slatted floors, which have been shown to also reduce the expression of estrous behaviours (Platz et al., 2008; Palmer et al., 2010). Not surprising,

decreasing detection of behavioural estrous and fertility problems have been reported (Lucy, 2001).

To counteract these challenges, as well as to facilitate breeding in larger scale dairy farms (see Barkema et al., 2015 for description of changes in herd sizes), an increasing number of producers are implementing hormonal protocols as standard operating procedure (DeJarnette et al., 2007). Since the development and first introduction of these hormone injection protocols in the 1970's (Roche, 1977) they have become increasingly popular and are now widely adopted, especially in the case of early postpartum breeding programs (Caraviello et al., 2006; Miller et al., 2007).

There is a large amount of research available that focuses on hormonal pathways and the associated changes on the ovarian structures throughout the estrous cycle (Roche 1996; Melvin et al. 1999; Macmillan et al. 2003). Also, the literature on hormonal protocols that facilitate breeding has surged over the last two decades (e.g. Kasimanickam et al. 2005; Cerri et al. 2009; Carvalho et al. 2015). However, despite the increased interest in this specific research area, literature on the effect of housing and health on estrous behaviour in lactating dairy cattle is scant (e.g. Gwazdauskas et al., 1981; Platz et al., 2008; Palmer et al., 2012).

Clearly, the increased implementation of hormone injection protocols on North American dairy farms over the last 5 decades has affected the management associated with estrus detection on farms but despite these advances the industry continues to face challenges regarding fertility. Thus, the overall aim of this review is to describe the naturally occurring estrous cycle and the corresponding behaviours in dairy cattle and how human intervention has manipulated this cycle and the behaviours related to it. To address this aim I have divided this literature review into two parts. In Part I (section 1.2), I briefly describe the natural estrous cycle, the behaviours related to

it, and natural situations where a cow may stop cycling, as this will serve as a platform for the following sections. I will then go on to highlight the impact of parturition and lactation on the cyclic activity of the dairy cow. This will be followed by the effect of illness and metabolic problems on the estrous cycle and its behaviours. Part II (section 1.3) of this review focuses on the direct and indirect effects of human intervention on the estrous cycle and behaviour of dairy cows. To address this section I will take a two-pronged approach. First, I describe the major hormonal protocols currently in place that alter (and arguably allow us to control) the dairy cows estrous cycle and secondly, I describe the indirect effects of the artificial housing conditions on dairy cow estrous behaviour. Where possible I will also identify gaps in the literature where further research is needed.

1.2 Natural estrus

1.2.1 Natural estrus, related behaviours and physiological anestrus

According to Merriam-Webster Encyclopaedia estrus is defined as:

> "A regularly recurrent state of sexual excitability during which the female of most mammals will accept the male and is capable of conceiving."

Like humans, cows are polyestrous animals, meaning that under normal physiological conditions these animals will experience repeated estrous cycles throughout the entire year with no effect of season. However, in contrast to the bovine cow, the canine bitch and ursine sow experience only a single estrus in a year and are classified as monoestrous while the equine mare and the ovine doe experience multiple estrous episodes during a defined period of the year and thus belong to a third group, that are referred to as seasonal polyestrous.

1.2.1.1 The estrus cycle

The estrous cycle covers the physiological changes (e.g. hormonal) that occur between two successive ovulations. The normal length of an estrous cycle in cattle is 21 d (ranging from 18 to 24 d) (Forde et al., 2011). The estrous cycle is naturally divided into two phases: the luteal and the follicular. Depending on the formation, presence and regression of the active structures (follicles, corpora lutea) on the ovaries, their corresponding hormonal production and behaviours expressed, the estrous cycle can be further subdivided into met-estrus, di-estrus (both luteal phase), pro-estrus and estrus (both follicular phase) (Heape, 1900).

General overview. A new estrous cycle starts immediately after ovulation and marks the beginning of the luteal phase (met-estrus). During d 1 - 3 the ovulated follicle initially becomes a corpus haemorrhagicum, a consequence of the ruptured blood vessels after ovulation, but then is morphologically remodelled into a corpus luteum (**CL**). The second part of the luteal phase, the di-estrus (approximately d 4 to 18 of the estrous cycle), is dominated by the presence of a progesterone secreting CL. Approximately between d 16 and 18 of the estrous cycle the CL undergoes luteolysis (is regressing) a period where its progesterone secretion is simultaneously decreasing. This marks the beginning of the follicular phase, the pro-estrus period. During pro-estrus, selection and deviation of the dominant follicle take place and estradiol is the dominant hormone instigating the cows behavioural expression of estrus (Wettemann et al., 1972; Hodgen, 1982). This preovulatory follicle, also called a Graafian follicle, will then ovulate at the end of this estrous event. In dairy cows we typically see only one dominant follicle under natural conditions; in farm animals ovulation rate is closely tied to the numeric capacity of the mother to gestate and subsequently nurture her young (Lucy, 2007).

Important hormones. The main regulatory hormones of the estrous cycle are gonadotrophin releasing hormone (**GnRH**; released from the hypothalamus' tonic center or surge center), follicle-stimulating hormone (**FSH**) and luteinising hormone (**LH**; both released from gonadotroph cells of the anterior pituitary), progesterone (released from the CL), estradiol (secreted from follicles) and prostaglandin $F_{2\alpha}$ (**PGF**; released from the uterus).

All the above mentioned hormones are interlinked via positive and negative feedback mechanisms and are involved in regulating the estrous cycle (Roche, 1996). During the luteal phase of the estrous cycle, when circulating progesterone concentrations are high (approximately 8 ng/ml) (Savio et al., 1990), the tonic center of the hypothalamus releases GnRH in a low and steady pulsatile secretion. GnRH then binds on its receptor on the gonadotroph cells of the anterior pituitary and causes FSH and LH release. This in turn allows for follicles to grow and reach dominance, but ovulation is inhibited as progesterone levels are still to high. In the absence of pregnancy, the uterus releases PGF causing luteolysis (regression of the CL). This marks the beginning of the follicular phase. PGF eliminates the negative feedback on GnRH secretion from the hypothalamus as previously established by progesterone. Progesterone concentrations now decrease to basal levels (< 0.5 ng/ml; Savio et al. 1990). Simultaneously GnRH pulses increase, resulting in release of increasing amounts of FSH and LH, which stimulate follicular growth and promote estradiol release. The results of this increase in estradiol are twofold: 1) secretion of FSH is down regulated via a negative feedback mechanism on the anterior pituitary and, 2) once estradiol concentrations surpass the threshold level of 6 pg/ml (Reames et al., 2011; Perry et al., 2014a), they cause a positive feedback at the surge center of the hypothalamus. This positive feedback results in GnRH pulses of high amplitude and high frequency and triggers a surge in LH

and FSH release (Kulick et al., 1999). This surge is called the preovulatory surge, which is followed by maturation and ovulation of the Graafian follicle.

Also involved in regulating the estrous cycle are a number of locally (intra-ovarian) produced growth factors, especially the insulin like growth factor (**IGF**) super family and the transforming growth factor beta (**TGF**) super family. The IGF super family contributes to the growth, proliferation and steroidogenic capacity of the future dominant follicle and consists of IGF-I and IGF-II, their respective receptors and numerous binding proteins and proteases (Rivera and Fortune, 2003). It has been stated that the IGF family indirectly affects the estradiol induced feedback mechanism to the hypothalamus and the pituitary (see review by Lucy, 2007). The TGF super family includes, amongst other things, activin, follistatin and inhibin. Activin has been described to increase the production of estradiol in the follicular fluid; whereas, follistatin impedes this positive steroidogenic effect (Knight and Glister, 2003; Forde et al., 2011). Inhibins, detected in the granulosa cells of the follicle, play a role in the suppression of FSH secretion from the anterior pituitary by enhancing LH-induced androgen production in larger follicles (Knight and Glister, 2003).

Ovarian dynamics. It has been reported that in > 95% of the estrous cycles cows experience 2 to 3 waves of follicular growth and development during each 21-d estrous cycle (Ireland et al., 2000; Peter et al., 2009a). Following the 2-wave theory this means that after ovulation a cohort (group) of 5 to 20 small follicles is recruited, begins to grow up to 4 to 5 mm in diameter becoming detectable on the ovary. Any growth beyond that size is FSH dependent (Garverick et al., 2002). Hence with increasing circulating levels of FSH a subgroup of the recruited follicles will now continue to grow while the others undergo atresia. The growing follicles start producing small amounts of estradiol and continue to grow until the first follicles

reach a size of approximately 8 mm in diameter. At this point the smaller follicles cease to produce estradiol and luteinizing hormone receptors (**LH-r**) start to appear on the larger follicles (Aerts and Bols, 2010). Typically it is the largest follicle which is now selected to continue to grow, while the other follicles undergo atresia (Hodgen, 1982). This selected follicle is also the only one that is now still producing and secreting estradiol (Ginther et al., 2000). This now dominant follicle is approximately 1 to 2 mm larger in size than the next biggest follicle (also referred to as deviation) (Ireland et al., 2000). It has been stated that at 8.5 mm the largest follicle shows an enhanced growth rate, allowing it to become the dominant follicle (Mihm and Austin, 2002). The increasing amounts of estradiol secreted by the dominant follicle lead to decreasing amounts of circulating FSH via a negative feedback system to the hypothalamus. Thus inducing atresia of the still FSH dependent follicles while the dominant follicle with its LH-r will start to rely on LH. Due to the high circulating concentrations of progesterone the pulsatory release of LH is of lesser amplitude and greater frequency during the early luteal phase (20 to 30 pulses/24 h) and of greater amplitude and lesser frequency during the mid-luteal period (6 to 8 pulses/24 h), which are both insufficient for final maturation and subsequent ovulation of the dominant follicle (Ireland et al., 2000; Forde et al., 2011). Hence dominant follicles developing during the luteal phase of the estrous cycle normally undergo atresia. Once the former dominant follicle becomes atretic and circulating estradiol concentrations are decreasing another cohort of follicles starts to grow and the second follicular wave begins. This wave is similar to the first one, however, the luteolysis results in decreasing progesterone concentrations and peak circulating concentrations of estradiol reaching the threshold leading to a surge in LH release and ultimately causing final maturation and ovulation.

An example of a 3-wave estrous cycle including day of the estrous cycle has been described as follows: first wave appearing at d 4 of the cycle (range 2 to 5), a single follicle reaching dominance by d 6, stable in size until approximately d 10 at which time it regresses and disappears by d 15; the second wave appeared at d 12, the dominant follicle reaching maximum size by d 16 (range d 13 to 18) immediately followed by regression; the third dominant follicle was first detected on d 16 and reached maximum size by d 21 and then ovulating (Savio et al., 1988).

More detailed reviews on the estrous cycle in cattle and the ovarian follicular dynamics in specific have previously been published (Roche, 1996; Lucy, 2007; Peter et al., 2009a; Aerts and Bols, 2010; Forde et al., 2011).

1.2.1.2 Estrus behaviours

In nature the behavioural expression of estrous, signalling the readiness for breeding, is important to ensure copulation at the optimal time to achieve pregnancy. The high estradiol concentrations at estrus induce ovulation as well as promote reproduction related changes in the animals' behaviour (Lyimo et al., 2000). The behavioural response generated by estradiol seems to be 'all or none' (Allrich, 1994), but it is possible that different thresholds of circulating estradiol concentrations are in place for initiating behavioural estrous and the LH surge (Reames et al., 2011).

In the female mammal, high estradiol concentrations are present during the follicular phase of the estrous cycle and reach peak concentrations at estrous; in dairy cows this typically occurs between 24 to 32 h before ovulation (Wiltbank et al., 2002). This results in the cyclic expression of distinct behaviours around the time of estrus. Please note that the male produces

and releases small amounts of testosterone every 4 to 6 h. This near constant supply of testosterone to the hypothalamus, where it is aromatized to estradiol, explains the near always readiness of a male for breeding. Although estradiol may be responsible for the initiation of reproductive behaviours, other – external – stimuli also play an important role especially when it comes to mounting and being mounted (Van der Horst and Holstege, 1998). Visual, olfactory, auditory and tactile stimuli all send neural messages to the hypothalamus and cause it to release neurotransmitters to the midbrain. These neurotransmitters are translated into a fast response and result in neurons from the midbrain to synapse with neurons in the medulla (brain stem). Nerve tracts from the medulla extend to the spinal cord where they synapse with motor neurons. These motor neurons then directly innervate muscular tissue causing the mounting and standing to be mounted response.

Estrous behaviours have been categorized according to one of three types of functions: attractivity, proceptivity and receptivity (Beach, 1976; Katz and McDonald, 1992). *Attractivity* includes the behaviours expressed and signals sent by the female to attract the males' attention and the males' response to them. An example would be the 'flehmen' behaviour (curling of the upper lip) expressed by a bull after a cow in estrus urinated close to him (Beach, 1976). *Proceptivity* enfolds all behaviours that arouse the male and sexually stimulate him to engage in copulation; female-female mounting is also a typical behaviour in this category (Beach, 1976). Lastly, *receptivity* describes the behaviours that ensure insemination, such as mounting (male) and standing immobile (lordosis; female) (Beach, 1976).

Differentiating between primary and secondary estrous signs is a way of categorizing estrus specific behaviours in dairy cows (Glencross et al., 1981; Diskin and Sreenan, 2000; Roelofs et al., 2010). The primary most reliable behaviour identified to describe a dairy cow in

estrus is ‘standing to be mounted’ by another cow. ‘Standing to be mounted’ has also been referred to as, and is interchangeably used with, ‘standing heat’ or ‘standing estrus’ and refers to a cow standing immobile while she is being mounted by another cow (Peter et al., 2009a). Research has shown that this behaviour is exclusively expressed when a cow is in estrus (Glencross et al., 1981; Sveberg et al., 2011). Despite the majority of mounting activity appearing throughout the night time (Hurnik et al., 1975), standing to be mounted has become the single most important sign for the visual detection of a dairy cow in estrous. In the past researchers (and arguably farmers) would rely solely on visual detection of estrous by identifying those cows that were exhibiting standing estrous (Gwazdauskas et al., 1981; Lewis and Newman, 1984). Some researchers even define estrus as the interval from the first to the last mounting or being mounted response (Hurnik et al., 1975; Lopez et al., 2004).

Secondary signs may be indicative of approaching receptivity, however they can occur before, during and after estrus (Peter et al., 2009a; Sveberg et al., 2011). Typical secondary signs include restlessness, sniffing the genital region of another cow, resting with the chin on another cow, mounting another cow, vocalization and mucous vaginal discharge (Van Eerdenburg et al., 1996; Kerbrat and Disenhaus, 2004; Sveberg et al., 2011). The inclusion of secondary estrous behaviours for detection of cows in estrous has been suggested and a scoring system has been proposed (Van Eerdenburg et al., 1996; Kerbrat and Disenhaus, 2004). With reportedly declining numbers of cows expressing standing estrous the detection of secondary estrous behaviours are gaining importance (Van Eerdenburg et al., 2002; Dobson et al., 2008). In particular, a cow mounting other cows has been shown to occur naturally but also able to arouse a nearby bull; displayed in ~90% of all estrous episodes (Roelofs et al., 2005a).

Another important secondary sign observed during pro-estrus and estrus is restlessness, defined as an increase in physical activity (Kiddy, 1977). Cows in estrus have a 4 fold increase in activity compared to their own di-estrous baseline (Kiddy, 1977). Using pedometers, this behaviour was also one of the first to be implemented for automatic estrous detection (Kiddy, 1977; Lewis and Newman, 1984).

Chin resting and sniffing other cows are also common secondary signs and have been reported to occur in 100% of estrus events and are thus reliable indicators for a cow in estrous (Lyimo et al., 2000). The frequency of expressing and receiving chin rests and sniffs increases significantly during the 48 h leading up to standing estrous (Palmer et al., 2012). However, these behaviours also occur, albeit less frequent, when the animal is not in estrous (Roelofs et al., 2005a).

1.2.1.3 Natural reasons for anestrus

Anestrus is described as a period without cyclicity and more specifically without ovulation (Peter et al. 2009; Wiltbank et al. 2002). In dairy cows there are 3 distinct phases of anestrus: 1) before puberty, 2) pregnancy and, 3) during the early postpartum period (puerperium).

Follicular waves with ovulatory size follicles have been reported to occur in heifers before the onset of puberty, starting as early as 2 weeks of age (Evans et al., 1994; Wiltbank et al., 2002). However, it is not until approximately 12 months of age that the first ovulation takes place (Evans et al., 1994). It is assumed that increasing LH pulses, during the last 3 months before first ovulation, stimulate the growth of the dominant follicle to a point where it produces and secrets

enough estradiol to surpass the threshold and initiate the GnRH release via positive feedback (Melvin et al., 1999).

During pregnancy it is the persisting and progesterone secreting CL that maintains the pregnancy and prevents cyclicity via negative feedback on GnRH secretion from the hypothalamus (Bouchard et al., 1988; Wiltbank et al., 2002; Crowe et al., 2014). It has been demonstrated, that even in pregnant animals, emergence of follicles occurs at a regular interval of 7 to 10 d, however they do not ovulate and are entirely absent during the last 21 d of the pregnancy (Ginther et al., 1989, 1996).

Directly after calving, during the puerperium, dairy cows enter a period of postpartum anestrus. The puerperium period lasts approximately 6 weeks during which the uterus returns to a pre-gravid state and the hypothalamus-hypophyseal-ovarian axis resumes cyclical secretions of gonadotroph hormones (Peter et al., 2009b). (For more detail see section 1.2.2.)

In other species there are also natural occurring anestrous periods due to seasonality and photoperiod. Goats, for example, are typical short-day breeders that enter a period of anestrous during the months with greater daylight length (Deveson et al., 1992; Amoah et al., 1996). In contrast, mares are long-day breeders that enter an anovulatory period during the winter months (Gentry et al., 2002; Ginther et al., 2004). In both cases melatonin, produced at night, has been shown to play a role in the inhibition of GnRH release (Deveson et al., 1992; Bazer and Spencer, 2005; Donadeu and Watson, 2007).

1.2.2 Effect of parturition on cyclicity and estrus behaviour

1.2.2.1 Resumption of ovarian activity

Towards the end of the pregnancy, approximately the last 20 to 25 d, the strong negative feedback of progestagens and estrogens inhibit the emergence of follicles by suppressing FSH release (Ginther et al., 1996; Crowe, 2008). This leaves the ovaries at the time of parturition largely quiescent (Crowe, 2008). Consequently the first ovulation postpartum reflects the resumption and completion of preovulatory follicular development and recovery from the hormonal conditions of late pregnancy (Butler, 2003). Typically modern dairy cows experience their first ovulation at 43 ± 5 d (mean ± SD) postpartum (Lucy, 2001).

Directly after calving, during the puerperium, dairy cows enter a period of physiological postpartum anestrus. The puerperium is a period of approximately 6 weeks during which the uterus undergoes histologic and morphologic changes returning to a pre-gravid state (uterine involution) (Peter et al., 2009b). At the same time the hypothalamus-hypophyseal-ovarian axis resumes cyclical secretions of gonadotroph hormones (Peter et al., 2009b). Parturition allows for the removal of the negative feedback of high estradiol. Progesterone and estradiol concentrations are declining to basal concentrations thus allowing for recommencement of FSH and LH synthesis (Crowe et al., 2014). Within 5 d postpartum recurrent transient increases in blood concentrations of FSH *resume and then* subsequently occur at intervals of 7 to 10 d (Crowe et al. 1998). The first of these stimulates the growth of the first follicular wave postpartum that generally produces a dominant follicle by 7 to 10 d postpartum (Savio et al., 1990). However, at this point the LH storages of the anterior pituitary are low and take up to 3 weeks to re-establish, hence the circulating concentrations of LH and LH pulse frequency are low (Griffith and

Williams, 1996). Between 10 to 20 d postpartum the release of pulsatile LH frequency in dairy cows increases (Crowe et al., 2014). If IGF-I availability and LH frequency during the dominant phase of the follicle are sufficient, the dominant follicle will produce and release enough estradiol to pass the threshold and induce the GnRH surge and subsequently ovulate. If, however, the dominant follicle fails to ovulate it will either undergo atresia and a new follicular wave emerges, or it continues to grow and becomes cystic in which case the cow re-enters a state of anestrous (Roche et al., 2000).

1.2.2.2 First estrus postpartum

Multiple studies found that the first postpartum ovulation is often silent, e.g. without exhibiting estrous behaviour (Savio et al., 1990; Shipka, 2000; Johnson et al., 2012). However, the percentage of cows detected in estrus varies greatly, especially when using automatic detection devices. For example, 2 studies using the same automatic detection device reported 42% (Shipka, 2000) and 88% (Johnson et al., 2012) of first ovulations to be silent while 2 other studies that included visual observation found almost 95% of first ovulations to be silent (Savio et al., 1990; Shipka, 2000). However, Shipka (2000) reported that relying only on visual observation approximately 95% of the cows seemed to have a silent heat but that they could reduce this to 42% when visual heat detection was combined with an automatic monitoring system. Similarly another study reported that twice daily 30-min visual observation left 81% of first postpartum ovulations undetected while activity monitors (pedometers) decreased this to 43% (Peter and Bosu, 1986). Although Van Vliet and Van Eerdenberg (1996) were not specifically looking for anestrous these authors did report the only signs expressed by cows during the first postpartum estrous were chin resting and sniffing of the genital region of another

cow. Interestingly Johnson et al. (2012) noted no difference in mean number of days from parturition to first ovulation for cows that exhibited estrus and cows that did not, although the range was narrower for cows that exhibited estrous behaviour (17 to 40 d postpartum) compared to those that had ovulation confirmed by hormone analysis only (10 to 49 d postpartum).

Following the 'all or none' theory proposed by Allrich (1994) it is possible that estradiol concentrations at first ovulation postpartum are high enough to cross the threshold to induce ovulation but not high enough to induce estrous behaviour. A recent study showed that different concentrations of estradiol administered to the same cow elicited different responses regarding ovulation and the concomitant behaviours (Reames et al., 2011). These same authors also reported between cow differences in estradiol concentrations required for initiating estrous behaviour. Given that estradiol concentrations produced by the first dominant follicle postpartum most likely vary greatly future research should investigate the estradiol concentrations at first and subsequent ovulation postpartum and correlate these with behavioural estrous expression.

Time spent on visual estrous detection varies greatly between studies, for example Morris et al. (2013) observed cows 8 times per day for 30 min each while van Eerdenburg et al. (2002) observed twice per day for 30 min. It would be interesting to see the difference in detection rate between visual observation and automatic detection using continuous data (e.g. from video recordings). However, given that continuous observations are time consuming it is not surprising that only one study has attempted to use continuous video recordings to investigate reproductive behaviour in dairy cattle (Sveberg et al., 2015).

Most of the studies use standing estrous or an increase in activity as the main indicator for defining onset of estrus. When considering activity the behaviours used for visual observation (such as frequency of sniffing and chin resting) likely vary greatly from automatic detection

(mainly walking activity) and make comparison between studies and devices difficult as different behaviours are expressed at different time relative to the onset of estrous.

1.2.2.3 Effect of nursing and milking

Beef cows housed under extensive systems normally nurse their offspring until approximately 6 months of age at which time they are weaned to ensure the cows' return to cyclicity (Enríquez et al., 2011). Nursing can cause anovulatory conditions where the follicles grow to deviation but not to ovulatory size, which is likely due to an infrequent release of LH (Griffith and Williams, 1996; Wiltbank et al., 2002). In beef cows it has been demonstrated that an established maternal bond as well as recognition of the own or an accepted offspring are necessary for the inhibition of the LH release in suckling cows (Griffith and Williams, 1996; Lamb et al., 1997). The major physiological difference between milked dairy cows and nursing beef cows at 15 to 20 d postpartum is reportedly the lower frequency of pulsatile LH release in beef cows nursing their own calves (Griffith and Williams, 1996).

In contrast to beef production, on most dairy operations is it standard practice to separate cow and calf within hours after birth (see review by von Keyserlingk and Weary, 2007). This practice prevents suckling and also avoids bonding. In theory this should allow for a faster return to cyclicity. However, milk production in the modern dairy cows has been shown to decrease normal cyclic patterns as well as increase the number of cows with a prolonged luteal phase (Opsomer et al., 1998). Both of these contribute to increased numbers of cows with a prolonged interval to first ovulation postpartum (Opsomer et al., 1998).

This effect of milk production on cyclicity is most likely due to a combination of high milk production, low feed intake and a resulting negative energy balance (Crowe et al., 2014).

Only one recent study has tried to uncouple the effects of BCS and milk production on reproduction (Cutullic et al., 2012). Interestingly these authors found that a low BCS or a high change in BCS impaired cyclicity while high milk production primarily negatively affected estrous behaviour and late embryo mortality. However these results should be viewed with some caution give that they only detected 17% first postpartum estruses and excluded these from their analysis (Cutullic et al., 2012). Increased milk production has been associated with a decrease in estrous duration (Lopez et al., 2004).

Although there is a growing body of work focused on understanding the effects of estrous behaviour the results are not consistent; likely a consequence of differences such as milk production, body condition score and negative energy balance between the various studies but work is required to disentangle these various factors. For example Harrison et al. (1990) reported decreased estrous behaviours for cows with an average 305 milk production of 10,800 kg compared with animals producing only of 6,900 kg; in contrast van Eerdenburg et al. (2002) reported no effect of milk production on estrous behaviour in a study where average 305 d milk yield was approximately 7,800 kg (Harrison et al., 1990; Van Eerdenburg et al., 2002). Thus it is possible that level of milk production in the latter study was not high enough to impair estrus. However, comparison between studies is further complicated as not all authors report 305-d milk yield. For example Lopez et al., (2004) used a 5-d average of the days prior to estrus and found that cows with an average production of $\geq$ 35.1 kg/d had lower behavioural estrous duration than cows with a production of $\leq$ 35.1 kg/d.

Most modern dairy cows today produce on average $\geq$ 9,000 kg/ 305 d compared with 4,400 kg/ 305 d in 1970 (Bello et al., 2012). The high milk production of the modern dairy cow likely affects resumption of postpartum cyclicity as well as behavioural expression. Nevertheless

more research on estrous behaviours expressed at first and second estrus postpartum is needed especially in relation to the daily milk production at that time.

1.2.2.4 Voluntary waiting period (VWP) – to wait or to breed

The voluntary waiting period (VWP) has been defined as the time period (day or week postpartum) at which the cow is first eligible for insemination (Inchaisri et al., 2011). Basically it describes the period during which the farmer refrains from breeding the cow, even if she is expressing estrous behaviour. This time of recovery between calving and breeding is much needed for the cow to finish the morphologic and histologic changes in the uterus (Peter et al., 2009b). Early work indicated that the uterus needs close to 7 weeks to recover from the previous pregnancy (Willett, 1956). However, in the past half century much has changed for the dairy cow included dramatic increases in milk production, increased rates of dystocia and postpartum health events; coinciding with a move away from pasture-based systems to indoor zero grazing housing systems (e.g. freestall, tie stall, dry lot – see Barkema et al., 2015) which some argue require cows to have a longer voluntary waiting period before breeding begins.

In dairy cattle farming the general rule is to aim for one calf per cow per year to obtain a 305 d milk production period (Zobel et al., 2015). Considering a gestation length of 280 d this means that the cow needs to be pregnant again no later than 85 d after parturition. Yet, it has been stated that determining the optimal VWP is challenging for dairy producers due to the lack of reproductive, productive and economic data associated with it (Gobikrushanth et al., 2014). Nevertheless a recent survey has shown that farms in the United States use an average VWP of 56 d (ranging from 30 to 90 d) (DeJarnette et al., 2007), very similar to that recommended 50 years ago (Aschbacher et al., 1956). However, 64% of the farms stated that they would alter the

VWP on an individual basis related to postpartum health, season, milk yield or parity (DeJarnette et al., 2007). Interestingly Aschbacher et al. (1956) also stated that breeding could begin again after ~60 d unless abnormalities of the reproductive tract indicated otherwise. It seems that the VWP has not changed over the last decades despite the fact that the dairy cow has changed dramatically in response to intensive genetic selection for specific traits such as higher milk production (Dobson et al., 2008).

Interestingly most of the available information on effects related to changes in VWP is based on simulation models (Inchaisri et al., 2011; Löf et al., 2012) or makes use of a retrospective analysis approach using previously collected data, whereby the VWP lengths are varied according to a pre-set inclusion criteria (Miller et al., 2007; Gobikrushanth et al., 2014). For instance, one study grouped cows according to their production level and then split the set and bred them at different VWP's (Tenhagen et al., 2003). These authors reported that regardless of production level cows that were bred later had higher conception rates (Tenhagen et al., 2003). This is not surprising as it has been shown that number of ovulatory estrous cycles preceding insemination influences conception rate (Butler, 2003).

Breeding the cow after 85 d postpartum would result in longer days open (not pregnant) and consequently result in a longer than average lactation. However, a recent review by Zobel et al. (2015) extrapolated data from Cole et al. (2011) and USDA (2012) showed that the level of milk production of today's dairy cows is considerably higher than the one from cows 40 years ago (see Figure 1.1). Furthermore Tenhagen et al. (2003) reported that by 200 d postpartum fewer high producing cows were pregnant and stated that prolonging the VWP for these cows is of low economic importance (longer days open are balanced with high milk production). It may therefore be beneficial for the cow and the farmer to allow for a longer VWP and lactation length

to allow for the cow to return to cyclicity. However more research in this area is needed to confirm this theory. Any analysis of the costs and benefits should take into account the effects on animal welfare of repeated breeding attempts for animals unready to conceive.

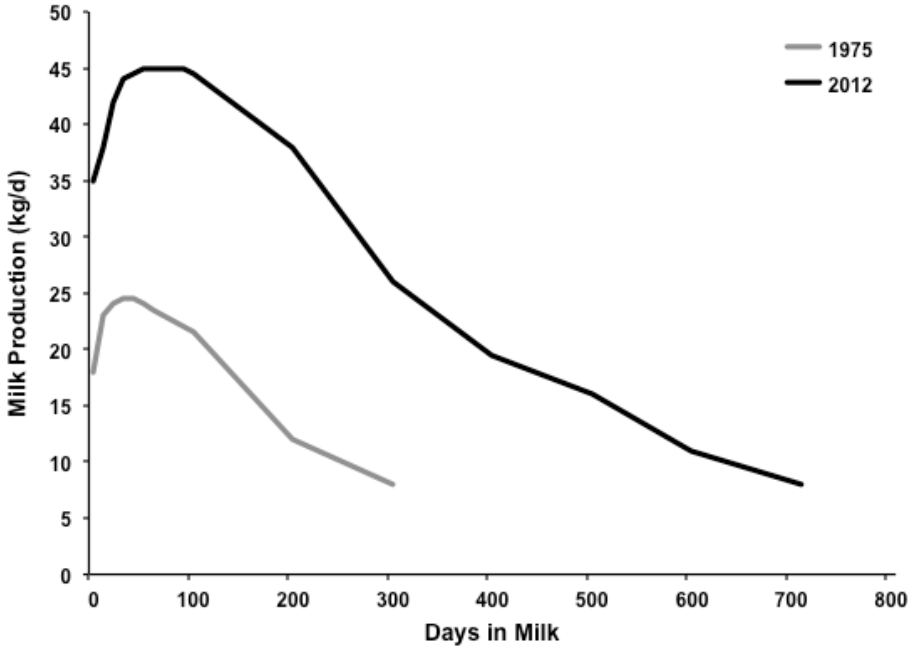

Figure 1.1: Example dairy cow lactation curves for 1975 (gray line) and 2012 (black line). Redrawn from from Zobel et al. (2015).

1.2.3 Effect of postpartum illness on estrus and estrous behaviours

1.2.3.1 Uterine infection

Uterine infections postpartum have been defined as metritis (abnormally enlarged uterus with purulent uterine discharge detectable in the vagina during the first 21 d postpartum), puerperal metritis (like metritis plus symptoms of systemic illness, such as fever), endometritis (abnormal uterine discharge > 21 d postpartum) and pyometra (accumulation of purulent material

in the uterine lumen in presence of a persistent CL and a closed cervix) (Sheldon et al., 2006). These conditions are all caused by bacterial contamination of the uterine lumen after parturition (Sheldon et al., 2008). Identified predisposing factors are retained placenta, the calving environment, twins, dystocia and diet (Roche, 2006; Sheldon et al., 2008).

Subclinical and clinical uterine infections postpartum affect fertility and, more specifically, ovarian activity (Peter et al., 2009b; Crowe et al., 2014). Metritis has been shown to be a major risk factor for prolonged luteal activity after the cows had resumed cyclicity (Opsomer et al., 1998, 2000). Occurrence of first postpartum ovulation before completion of uterine involution leads to bacteria growth and results in a pyometra, which then compromises the uterus' ability to produce or transport sufficient amounts of prostaglandins for luteal regression and causes a persistent CL (Opsomer et al., 1998).

Research has found that cows with uterine disease have smaller follicles and lower estradiol concentrations (Sheldon et al., 2002). Furthermore the estradiol induced LH surge are weakened when a bacterial endotoxin was infused (Battaglia et al., 1997). These lower estradiol concentrations are likely the cause for the lower estrous behaviour expressed by postpartum cows with metritis when compared to healthy animals (Callahan et al., 1971).

In summary, pathological uterine conditions postpartum can cause prolonged luteal activity and irregular estrus cycles by disrupting the endometrial PGF secretion. They can also cause retarded follicular growth resulting in smaller follicles and lower estradiol concentration and alter estrous behaviour (Callahan et al., 1971; Sheldon et al., 2008; Crowe et al., 2014).

1.2.3.2 Lameness

Lameness is the disability of a cow to move and walk freely (Flower and Weary, 2006). A sound cows' movement typically is smooth and free moving, while lame cows, depending upon the severity, range from having imperfect locomotion to the inability to bear weight on one or more limbs (Flower and Weary, 2006). Lameness is a multifactorial condition (Espejo and Endres, 2007) and one of the most important welfare and production concerns facing the industry (Barkema et al., 1994; von Keyserlingk et al., 2009). The incidence of lame animals in a herd varies greatly between farms as well as between regions (von Keyserlingk et al., 2012). Detection is also greatly observer dependent (Espejo et al., 2006).

With regards to reproduction, it has been acknowledged that lameness has a negative impact on fertility (Garbarino et al., 2004; Hernandez et al., 2005; Morris et al., 2011). More specifically, research has shown that lameness can delay the resumption of cyclicity (Opsomer et al., 2000; Garbarino et al., 2004). Lame animals that fail to ovulate have been shown to have a low LH pulsatility as well as lower circulating estradiol concentrations (Morris et al., 2011). Therefore it has been proposed that lameness in postpartum cows is associated with a graded reduction of ovarian hormone production. It was further proposed that the lower LH pulse frequency in lame cows that do not ovulate reduces the ability of selected follicles to produce sufficient estradiol to induce intense estrous behaviour, an LH surge and ovulation (Morris et al., 2011).

A negative impact on intensity of estrous behaviour as a consequence of lameness has also been reported (Walker et al., 2008; Morris et al., 2011). Given that lameness typically means that the animal is experiencing pain when walking it is not surprising that these animals have decreased estrous intensity, especially when it comes to mounting activity (Walker et al., 2008).

Interestingly, Morris et al. (2011) reported that all of the lame animals that ovulated expressed some form of estrous behaviour, indicating that when lameness is not inhibiting ovulation it is also not inhibiting the expression of related behaviours. I strongly encourage further work in this area, not only because lameness is a serious welfare problem (von Keyserlingk et al., 2009) but also because there is a growing body of evidence that it likely has a profound impact on reproduction.

1.2.3.3 Mastitis

Mastitis is an infection of the udder (intramammary infection) and its incidence in dairy cows is at the highest level around parturition (Pyörälä, 2008). This is not surprising given that close to calving the teat canal starts to open and leaks mammary secretion, facilitating intrusion of bacteria (Oliver and Sordillo, 1988). Typical mastitis symptoms are swelling of the udder and abnormal milk (Klaas et al., 2004; Santos et al., 2004). It has been shown that mastitis impairs fertility (Schrick et al., 2001; Santos et al., 2004; Bijker et al., 2015).

Research has shown that occurrence of mastitis in the early postpartum period can be a cause for delayed resumption of ovarian activity (Opsomer et al., 2000; Huszenicza et al., 2005). Furthermore, mastitis can negatively affect cows which have resumed cyclicity by causing premature luteolysis or a prolonged follicular phase (Hockett et al., 2000; Huszenicza et al., 2005). It is assumed that premature luteolysis is a result of increasing levels of PGF as an immune response to the inflammation and bacterial endotoxins (Hockett et al., 2000; Lavon et al., 2008). Additionally there is evidence that type of bacterial agent also plays a role in effect elicited on the ovaries (Huszenicza et al., 2005; Lavon et al., 2011b). A delay in ovulation has also been reported in connection with mastitis and it has been suggested that low circulating

estradiol concentrations are causing a delayed preovulatory LH surge thereby delaying ovulation (Lavon et al., 2010, 2011a).

Huszenicza et al. (2005) reported that cows diagnosed with mastitis between d 15 and 28 postpartum, expressed delayed estrous behaviour compared to healthy cows. Interestingly, they also reported that almost none of the cows which had resumed cyclicity before getting mastitis expressed behavioural estrous (Huszenicza et al., 2005). Furthermore a decrease in estrous intensity for cows with mastitis compared to healthy cows has been reported (Morris et al., 2013). However, research investigating the effect of mastitis on behavioural estrous expression is limited and more research connecting it to first and following postpartum ovulations is needed to better understand the influence on the mechanisms.

1.2.3.4 Malnutrition and over condition

In dairy cows, nutritional status is typically determined using body condition score (BCS). The scale used to measure BCS differs between countries, but low values always reflect emaciation and high values obesity (Roche et al., 2009).

Emaciation and obesity have both been linked to lower reproductive efficiency. It has been shown that high BCS prepartum leads to a decrease in feed intake in the days before parturition and causes a negative energy balance (**NEB**). In contrast, cows with low BCS prepartum are at great risk of failing to gain weight and may in fact experience longer than normal periods of NEB (Wathes et al., 2007). Decades ago researchers noticed that energy balance postpartum is correlated with delayed resumption of ovarian activity (Butler et al., 1981; Harrison et al., 1990). More recent research has demonstrated that NEB impacts resumption of reproduction postpartum, mainly by increasing the interval to first postpartum ovulation (Roche

et al., 2000; Wiltbank et al., 2002; Crowe et al., 2014). Extreme under-nutrition prevents growth of follicles past emergence (Wiltbank et al., 2002). In comparison, less severe cases of under-nutrition allows follicular growth to resume but inhibits growth to ovulatory size by a greater negative feedback of estradiol on GnRH/LH pulses (Wiltbank et al., 2002). Furthermore it has been described that severe NEB results in lower amounts of circulating IGF-I which is likely to affect ovarian function (Wathes et al., 2007).

No differences in estrous expression between animals with different BCS scores were found in a study including only heifers (Villa-Godoy et al., 1990). However the same authors reported that high and low BCS did impact onset of di-estrous and it was suggested that, based on progesterone profiles, these animals had an impaired luteal development (Villa-Godoy et al., 1990).

Recently it was described that cows with a higher BCS loss postpartum had reduced estrous activity compared to cows that maintained BCS (Lüttgenau et al., 2015). However, information on estrous behaviour in relationship with BCS is scarce and it would be interesting to know if other studies on different farms would confirm these recent findings in behavioural difference related to metabolic changes.

In summary the body of evidence described above suggests that a number of conditions influence reproductive efficiency and possibly alter the hormonal cascade in one or more ways. However, the majority of studies to date have focused primarily on endocrine and metabolic parameters with only a few studies including behavioural responses (e.g. Callahan et al., 1971; Walker et al., 2008; Morris et al., 2013). However, information on behavioural changes associated with estrus are still scarce and more research is needed to gain insight into how behavioural responses relate to endocrine and metabolic findings. Lastly, it is also likely that

cows that experience excessive NEB may also struggle in terms of social behaviour (e.g. dominance relationships) which could further influence behavioural expression and thus worthy of further investigation.

1.2.4 Effect of the social environment on behavioural estrus expression

When primiparous cows (heifers) are housed together with multiparous cows it has been reported that primiparious heifers exhibited mounting activities for a shorter period compared to the multiparous cows (Walker et al., 1996). However in a more recent study primiparous cows were reported to have a higher activity peak when compared to multiparous cows (Madureira et al., 2015a). However, it should be noted that in the Madureira et al. (2015) the authors used an automatic detection device and did not distinguish between specific behaviours such as mounting or standing heat.

When a number of heifers were in estrus simultaneously, the number of attempted mounts increased by six times and successful mounts by 10 times as compared to when only one animal was in estrus (Helmer and Britt, 1985). Number of cows in heat simultaneously also affects estrous intensity (Hurnik et al., 1975; Roelofs et al., 2005b). More specifically, one study found had an overall display of standing heat of 58% for multiple animals in heat in comparison to only 20% when there was only one animal in estrous (Roelofs et al., 2005a). These results agree with the recent suggestion that cows form sexually active groups and such groupings could be used as a novel estrous sign (Sveberg et al., 2013).

Homosexual-like behaviour, such as a female cow mounting another female cow, has been reported to cause sexual arousal in the male. Other secondary signs of estrous detection resemble the behaviours typically expressed by a bull and could be intensified due to the lack of

male presence. It has been stated that exposure to a bull had no influence of estrous behaviours exhibited at first estrus postpartum (Shipka and Ellis, 1998). Nevertheless bulls have been shown to interact with cows coming into estrous as early as 4 d prior to estrous although this did not elicit the same interest from other females (French et al., 1989).

In summary, the number of animals in estrous simultaneously and parity influence the intensity of estrous expression while presence of a bull does not seem to have an influence on female-female interactions.

1.3 Effect of direct and indirect human intervention

1.3.1 Human intervention – Induced estrus

1.3.1.1 Why and when is estrus induced

With the introduction of artificial insemination to dairy farming (Cole and Winters, 1939; Foote, 1979) the identification of estrous behaviours for dairy cows has become highly relevant for the industry. In particular, timely detection of cows in estrous is important for breeding at the right time to ensure fertilization (Aschbacher et al., 1956).

Over the past decades researchers have reported a decrease in percentages of cows that are seen in standing estrus as well as a decrease in the frequency. The average duration of standing estrous has reportedly decreased from 17 h in the 1950's (Aschbacher et al., 1956; Willett, 1956) to 15 h in the 1970's (Esslemont and Bryant, 1976), to an average of 7 h (range of 2 of 32 h) in the 2000's (Yoshida and Nakao, 2005). Furthermore it appears that the number of cows expressing standing estrus is declining; from 90% reported in the 1970's (Hurnik et al.,

1975) to ~30 - 50% in the 2000's (Lyimo et al., 2000; Van Eerdenburg et al., 2002; Yoshida and Nakao, 2005). Yet standing heat is still the most reliable behaviour when it comes to predicting ovulation relative to exhibiting this behaviour (Roelofs et al., 2005a).

Given that most cows show standing estrous behaviour during the night time (Esslemont and Bryant, 1976) or early morning hours up until noon (Gwazdauskas et al., 1981), the time of day that visual observations are performed is a critical factor. Clearly, observations at the wrong time of day, and too few observations in general, likely contribute to the lack of estrus detection. This in turn has contributed to a decrease in successful breedings, as timely breeding (relative to ovulation) is needed to ensure fertilisation (Roelofs et al., 2004). The old AM/PM rule – a cow seen standing in the AM should be bred that night, and a cow seen standing at PM should be bred the following AM (Foote, 1979) – is still applied today. Nevertheless unsatisfactory estrus detection efficiency likely is the primary reason for introducing synchronization protocols on most farms (Tenhagen et al., 2004). Whether they are effective appears to vary in part based on management practices, for example, one study reported that conception rates based on first timed-AI were lower than those of AI after estrus detection (Tenhagen et al., 2004).

Synchronization protocols for dairy cattle breeding, also referred to as fertility treatments, have been developed to increase reproductive efficiency (Colazo and Mapletoft, 2014). More specifically they are used to induce a synchronized estrous episode in a group of cows, to induce a timed ovulation and act as a treatment for hormone responsive fertility problems (e.g. ovarian cysts) (Drost and Thatcher, 1992; Pursley et al., 1997). Artificially inducing estrous and ovulation allows for breeding without the need for estrus detection and enhances pregnancy rates (Pursley et al., 1995). Reportedly, the number of cows subjected to hormone protocols for first service, likely increasing the number of AI's at this time, has increased to ~35% (Miller et al., 2007);

providing some evidence that there is an increased reliance on hormonal manipulation. It appears that some farm managers initially make use of hormone protocols to aid in the induction of the first estrous cycle after parturition but then resort to estrus detection for the following estrous cycle (Caraviello et al., 2006). The question that warrants further investigation is whether using a longer VWP (together with live observation of estrus) would allow for the elimination of the hormonal protocols.

Comparison of the costs related to breeding using natural service by a bull, timed AI after estrus detection and timed AI after an hormonal synchronizing program have shown that the financial advantage of a hormonal protocol is farm dependent and marginal (Tenhagen et al., 2004; Lima et al., 2010). The next step in this type of analysis should be a comparison between the costs of breeding based on an estrus detection system verses a synchronization protocol but this is beyond the scope of this review.

A number of less invasive methods for early detection of animals that might develop fertility problems postpartum have been suggested. For example monitoring BCS throughout the transition period is essential for good reproductive management (Crowe et al., 2014). Additionally, the implementation of estrus detection devices might improve detection rates of animals that would normally be undetected by visual observation. On that note automatic monitoring of physical activity for the purpose of automatic estrus detection has been discussed for almost 40 years (Kiddy, 1977; Lewis and Newman, 1984), has developed greatly over the past decades (Maatje et al., 1997; Roelofs et al., 2005b) and continues to be investigated (Madureira et al., 2015a).

Given the potential questions surrounding the use of hormones in food animal production systems, including dairy cattle (von Keyserlingk et al., 2013), I encourage researchers to explore

the efficacy of using live estrus detection as the primary breeding method (especially considering the diverse technological detection aids nowadays available) and to limit the use of hormone protocols for cows with reproductive problems rather than adopting this procedure as standard practice applied to all cows. This approach is of growing interest especially considering the work of Tenhagen et al. (2003) who demonstrated that the financial benefit of an OvSynch-protocol was more pronounced in farms with low estrus detection efficiency. It might also be beneficial to redirect the money spent on hormones to the purchase and installation of an automatic monitoring system.

1.3.1.2 Hormones and hormonal protocols currently in practice

Despite the growing number of questions arising from the use of hormone protocols they are still widely used by dairy farms across North America. The use of hormone protocols allows for the synchronisation of follicle growth, CL regression and, in the case of timed AI, ovulation (Colazo and Mapletoft, 2014). The first protocols were based exclusively on the application of PGF (Lauderdale et al., 1974). This allowed for the synchronisation of estrous of multiple cows simultaneously and allowed for a narrow window for estrus detection. However, PGF administration can take up to 5 d to induce the cow to come into heat, thus estrus detection is still needed. To avoid missing heats, the use of GnRH and timed AI were introduced (Pursley et al., 1995).

Currently, two injections of PGF (two weeks apart) are used as a treatment for postpartum uterine infection as well as a presynchronisation protocol, in combination with either estrus detection or a timed AI protocol (Tenhagen and Heuwieser, 1999; Melendez et al., 2006). Three of the most common hormonal protocols for timed AI used within the dairy industry are

OvSynch, PreSynch and HeatSynch (see Caraviello et al., 2006). However, other modifications of the OvSynch protocol (such as adding an controlled internal drug release device or adding an injection of progesterone) have been described (Melendez et al., 2006). Another OvSynch modification that has been reported to increase pregnancy rates involves the addition of an injection of estradiol cypionate at 24 h after the PGF presynchronization protocol (Cerri et al., 2004).

The main hormones used in these injection protocols are PGF, GnRH, estradiol and progesterone. PGF is used to cause luteolysis of an existing corpus luteum, treatment for a luteal cyst, and to start a new follicular wave. The effect of GnRH is to induce LH release and ovulation followed by the emergence of a new follicular wave. Estradiol, normally administered 24 h after PGF, induces and synchronizes the LH surge and subsequent ovulation. Progesterone is used in controlled internal drug release devices that are placed into the vaginal cavity to keep circulating progesterone concentrations high enough to suppress GnRH release, but low enough to avoid natural PGF release from the uterus. This allows for normal follicular growth but prevents ovulation and causes a return to estrous once the device is removed (Colazo and Mapletoft, 2014).

The GnRH – PGF – GnRH treatment has been termed Ovsynch because the combination of follicular synchronization, estrus synchronization, and ovulation control are effective in more precisely timing ovulation of a fertile ovum (Pursley et al., 1995; Beal, 1998). This protocol can be started at any day throughout the cycle, however research has shown that fertility was improved, when started near mid-cycle (Vasconcelos et al., 1999). The first GnRH (administered at d 0 of the OvSynch protocol) is used to synchronize a new follicular wave and to ensure the presence of a CL during enrolment to OvSynch. The following injection of PGF on d 7 causes

luteolysis and growth of a new dominant follicle whose ovulation is aided by the administration of GnRH on d 9. This allows for timed breeding 8 to 24 h following GnRH administration (Figure 1.2).

Two modifications of the OvSynch protocol, which are widely used, are PreSynch (Lucy, 2007) and HeatSynch (Pancarci et al., 2002). PreSynch, also referred to as Presynch-OvSynch protocol, starts with a presynchronization consisting of 2 injections of PGF 14 d apart and is followed by an OvSynch protocol 14 d later, thus adding an extra 28 d to the treatment duration. The presynchronization ensures that cows are at the most responsive stage of the estrous cycle when the OvSynch is started. HeatSynch is similar to PreSynch, the difference being the administration of estradiol cypionate instead of GnRH at the end of the OvSynch, and breeding either after detected estrus or using timed AI 48 h later (Figure 1.2).

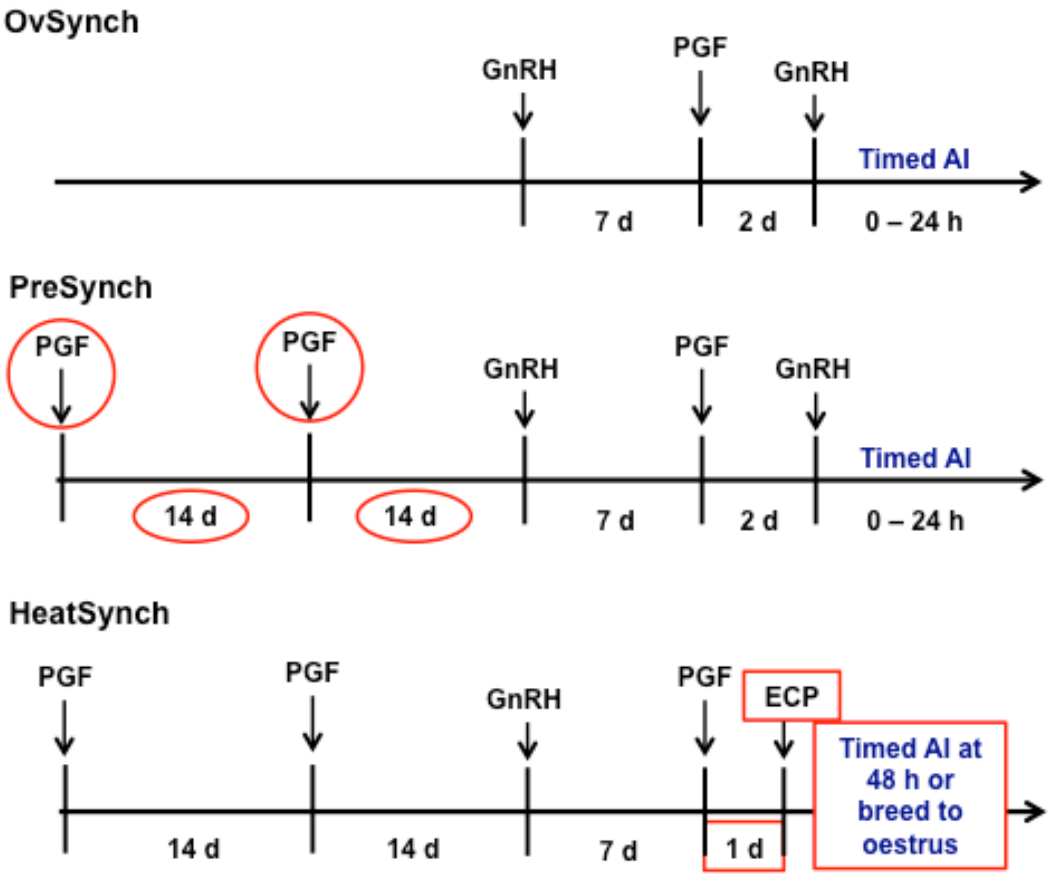

Figure 1.2: Timeline of the OvSynch, PreSynch and HeatSynch protocol.
Circles highlight differences between OvSynch and PreSynch and squares highlight differences between PreSynch and HeatSynch. (PGF = prostaglandin $F_{2\alpha}$; GnRH = gonadotropin releasing hormone; ECP = estradiol cypionate) (adapted from Caraviello et al., 2006).

1.3.1.3 Behavioural differences between spontaneous and induced estrus

Research on estrous behaviours expressed by cows enrolled in a synchronization protocol compared with cows exhibiting spontaneous estrus is limited. Walker et al. (1996) compared estrous behaviour of cows synchronized with PGF (presynchronization protocol) with untreated controls and reported that estrous behaviour between those 2 groups was similar, although, there was tremendous variation between cows within a treatment. These authors did make use of an

automatic mount detector and classified estrous behaviour solely as standing estrous (Walker et al., 1996) which may have influenced the results.

Differences in behavioural estrous expression may also be due to the hormones administered. Beal (1998) for example has shown that after receiving PGF and GnRH more cows were detected in estrous than after twice receiving a PGF application. Interestingly it was also illustrated that heifers responded differently than mature cows (Beal, 1998). It has also been reported that administration of estradiol cypionate caused estrous behaviour in anovulatory cows (Cerri et al., 2004) which would consequently lead to breeding of an animal that is not going to conceive.

Overall consideration must be given to both the type of hormone being used but also the timing relative to stage of estrous cycle as these both play a role in the animals' behavioural response.

1.3.2 Effect of management and housing on behavioural estrous

Over the past decades, we have seen a decrease in the number of commercial farms and a concomitant increase of numbers of cows on each farm (Capper et al., 2009). Housing and environmental conditions have long been established in contributing to reproductive efficiency of a dairy herd due to the influence on estrous expression (Gwazdauskas et al., 1981).

Research has shown that cows housed indoors expressed fewer standing events than cows kept on pasture (Palmer et al., 2010). This is likely due to the animals having altered standing behaviour on the indoor floorings surfaces which in turn affects standing heat; standing heat was observed only in 37% of cows in heat on slatted floors (Van Vliet and Van Eerdenburg, 1996). Furthermore a case study investigating concrete verses rubber flooring showed that the rubber

flooring increases estrous behaviour and movement in general (Platz et al., 2008). Finally, the modern dairy cows still appear to express behavioural estrous more at night and during the early morning than during the day time as previously described 40 years ago (Hurnik et al., 1975; De Silva et al., 1981).

Intensity of estrous as well as behavioural expression are decreased during high temperatures associated with heat-stress (Gangwar et al., 1965; Pennington et al., 1985). Gangwar et al. (1965) used a climate controlled approach and observed heifers during different times of the year, with or without adding air conditioning and reported that under hot conditions without cooling aid the only estrous signs observed were vaginal discharge and nervousness. The same authors also described a higher incidence of anestrous animals during high temperature periods. Similarly, Pennington et al. (1985) reported that mounting behaviour was higher during the winter (cold period) when compared to the summer observations (warm period). Interestingly these authors also reported an increase in rubbing and licking behaviour during the summer when compared to the winter. It appears that cows likely adapt their behavioural expression of estrous depending on environmental temperatures, a fact that should be considered for barns without an installed cooling system.

1.4 Conclusion and aims for this thesis

A decline in postpartum estrous detection related to high levels of milk production, high incidence of postpartum diseases, existing housing conditions and heat stress has been reported. This decline has been addressed by implementing hormonal protocols for better and faster successful breeding. Research on hormonal breeding protocols has been introduced at the time when dairy farming became more intensified, has been evolving over decades and is still a strong

focus for current research. While the implementation of hormonal breeding protocols may be one way to overcome the reproductive problems of today's high producing dairy cows, understanding estrus expression and detection may be another. There is still a gap in knowledge regarding the impact of housing conditions such as overstocking on cow behaviour and on their ability to express natural estrus behaviours in particular.

The cow's health is an important welfare concern in its own right, and can also affect milk production and the cow's ability to cycle and conceive. The transition period has been identified as an especially sensitive time for the cow as she is more susceptible to health problems during this time when she is undergoing metabolic and physiologic changes. With increasing herd sizes it has become more difficult for dairy farmers to focus on individual animals, and this is one reason why more farmers are relying upon technological aids for both estrus detection and health monitoring.

The overall aim of my thesis was to investigate how dairy cattle behaviour and estrous expression are affected by disease and housing conditions. My specific objective were to: 1) describe rumination behaviour pre and post-partum and to determine if changes in rumination and other feeding behaviours can be used to identify dairy cows at risk for disease post-partum (Chapter 2); 2) determine how stocking density affects estrous expression and biomarkers of stress in lactating dairy cows (Chapter 3); and 3) determine how standing and lying time change in response to estrus and how these behaviours are affected by lameness and stocking density (Chapter 4).

Chapter 2: Rumination and feeding behaviours differ between healthy and sick dairy cows during the transition period

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2.1 Introduction

High milk production places increased demands on the metabolism of dairy cows and increases the risk of disease (LeBlanc et al., 2006). Moreover, increased herd size may make the detection of sick animals more challenging (Guterbock, 2004). For both reasons there has been increasing interest in the development of methods to automatically identify sick cows and cows at risk of becoming sick. Dairy cows are at greatest risk of production diseases (e.g. metritis and subclinical ketosis) during the transition period (typically defined as 3 weeks before to 3 weeks after calving; Mulligan and Doherty, 2008).

Over the past decade there has been growing interest in how to use behaviour for the early identification of disease (see reviews by Weary et al., 2009; von Keyserlingk and Weary, 2010). In this context feeding and rumination behaviour are of particular interest. Feeding behaviour describes the parameters involved with active feeding, such as time spent feeding, amount of feed intake and rate at which the feed is ingested. Rumination behaviour (needed for e.g. particle breakdown and rumen pH-balance) describes the time spent ruminating the previously ingested feed and consists of regurgitation, re-insalivation, re-mastication and re-swallowing of feed boluses. Feeding behaviour has been shown to be sensitive to management situations and health,

especially during the transition period (von Keyserlingk and Weary, 2010). For example, Huzzey et al. (2007) and Goldhawk et al. (2009) found that changes in DMI and time spent feeding in the days before calving identified cows that were diagnosed with metritis or SCK postpartum, respectively. Similarly, (Sahar et al., 2019) reported that cows that spend less time eating during the first 90 min after fresh feed delivery before calving were at increased risk of developing hyperketonemia and metritis in the postpartum period. Other work has shown that lame cows spend less time feeding, have fewer feeding bouts and spend less time ruminating than non-lame animals (Miguel-Pacheco et al., 2014).

Different authors have argued that decreased rumination activity may be an indicator of anxiety (Bristow and Holmes, 2007), distress (Schirmann et al., 2011), imminent calving (Schirmann et al., 2013; Büchel and Sundrum, 2014), disease (Fogsgaard et al., 2012), and metabolic disorders (Hansen et al., 2003; DeVries et al., 2009). Recent work has also shown some promise in the use of rumination as an indicator for disease during the transition period (Soriani et al., 2012; Calamari et al., 2014). However, neither of these two latter studies monitored feeding behaviour and in both studies cows were grouped according average time spent ruminating, either during the pre-calving period (Soriani et al., 2012) or the early postpartum period (Calamari et al., 2014), and not according to their health status. Interestingly Calamari et al. (2014), found that 90% of the cows in the low rumination group were sick during the first month after calving. However, despite these new insights there is still no information on how time spent ruminating during the transition period changes according to health disorders and how these changes are related to feeding behaviour.

The objectives of this study were to use rumination and feeding data captured using validated technologies to (a) describe time spent ruminating and feeding behaviour (consisting of

DMI, time spent feeding, feeding rate and visits to the feed bin) of freestall-housed dairy cows in the weeks before and after parturition and, (b) to determine the relationship between postpartum disease and rumination behaviour before and after calving. We hypothesized that rumination and feeding behaviour of cows with health problems in the postpartum period would differ from healthy animals. We also speculated that these differences would already be present in the pre-partum period.

2.2 Methods

The study was conducted at the UBC Dairy Education and Research Centre in Agassiz, British Columbia, Canada. Animals were cared for following the guidelines of the (Canadian Council on Animal Care (2009) and all procedures were approved by the UBC Animal Ethics Committee (Protocol A 05-0660).

2.2.1 Animals, Housing and Diet

A total of 80 multiparous Holstein cows (parity 3.3 ± 1.6; mean ± SD) were enrolled over an 8-mo period. Only multiparous cows were included in this study, as previous research has shown differences in behaviour between primiparous and multiparous animals (e.g. Hasegawa et al., 1997; Gonzalez et al., 2003). Cows were enrolled at 18 ± 7 d (mean ± SD) before their actual calving date. At calving cows had an average dry period of 59 ± 5 d (mean ± SD). The behaviour and health status of every cow were recorded for 5 wk (2 wk before to 3 wk after calving).

Throughout the study cows were kept in a free-stall barn; 1 pen housed 12 prepartum cows and 2 pens, each containing 12 cows, housed the postpartum animals. Cows were alternatingly assigned to the postpartum pens. All pens measured 10.2 x 13 m, and were equipped with 12 lying stalls fitted with a mattress (Pasture Mat, Promat Inc., Woodstock, Ontario,

Canada) covered with approximately 5 cm of sand bedding, 6 Insentec feed bins (Insentec BV, Marknesse, the Netherlands) and 1 Insentec water bin. All cows had access to all feed bins and the water bin. Cow to stall ratio was 1:1 throughout the entire study. Water bins were emptied and cleaned weekly, feed bins were emptied and cleaned daily before the afternoon feeding and stalls were raked twice daily during each milking. Group composition was dynamic with cows entering and leaving the prepartum and postpartum pens depending on expected and actual calving dates. Cows in the prepartum pen were checked multiple times daily for signs of imminent calving. Cows showing signs such as vaginal discharge, relaxation of the pelvic ligaments or milk letdown, were moved to one of the two individual maternity pens. The maternity pens were 4.5 x 3.3 m and deep bedded with sand covered with fresh straw. While in the maternity pen cows had access to an automatic water trough and were provided fresh feed from the prepartum (before calving) and postpartum (after calving) diet. The calf was removed as soon as possible after calving. Cows remained in the maternity pen until the time of first milking (at either 0700 or 1700 h) and were then moved to one of the 2 postpartum pens. To maintain stocking density, cows that had spent longest in the postpartum pen were removed first. Cows were milked twice daily at approximately 0700 h and 1700 h.

Pre- and postpartum groups were fed a TMR formulated according to the recommendations provided by NRC (2001). Prepartum cows were fed once a day at approximately 0800 h and postpartum cows were fed twice daily at approximately 0700 and 1600 h. Representative samples of the TMR were taken once a week, at the time of feed delivery, and immediately frozen. Samples were thawed and then dried at 60°C for 48 h to determine DM content. Samples were then ground, pooled monthly and sent to Cumberland Valley Analytical Services Inc. (Maugansville, MD) for analyses according to the standards of the Association of

Official Analytical Chemists (AOAC, 2005) to determine average CP, ADF, NDF, and NE_L content of the diets fed throughout the study. The prepartum TMR consisted of 43.9% corn silage, 34.7% alfalfa hay and 21.4% pre-lactation concentrate and mineral mix on a DM basis (DM: 44.5 ± 3.4%; CP: 15.4 ± 0.9% of DM*;* ADF: 32.4 ± 1.7% of DM; NDF: 44.1 ± 2.5% of DM; NE_L: 1.4 Mcal/kg). The postpartum TMR consisted of 39.7% mineral and concentrate mix, 32.9% grass silage, 19.2% corn silage and 8.2% alfalfa hay on a DM basis (DM: 51.4 ± 3.2%; CP: 18.1 ± 0.9% of DM; ADF: 21.1 ± 1.6% of DM; NDF: 33.4 ± 0.9% of DM; NE_L: 1.6 Mcal/kg). Particle size distribution of the TMR was determined using the Penn State Particle Separator (Kononoff et al., 2003) consisting of 3 sieves and the bottom pan. The pore sizes of the 3 sieves were 19 mm (upper sieve), 8 mm (middle sieve), and 1.18 mm (lower sieve). The prepartum TMR was composed of 28.3% of particles >19 mm, 37.1% of particles >8 mm, 24.4% of particles >1.18 mm, and 10.2% of particles <1.18 mm. The postpartum TMR was composed of 20.8% particles >19 mm, 36.1% of particles >8 mm, 30.3% of particles >1.18 mm, and 12.8% of particles <1.18 mm. Particle lengths are reported for descriptive purposes only.

2.2.2 Behavioural recordings

Feeding and social behaviour. The Insentec system (Insentec, Marknesse, The Netherlands), validated by Chapinal et al. (2007), was used to monitor time spent feeding, duration and amount of intake consumed during each visit to a feed bin. DMI and time spent feeding were used to calculate feeding rate. A previously validated replacement criterion (Huzzey et al., 2014) was used to automatically assess social behaviour in the form of replacements at the feed bunk using data collected from the Insentec system. A replacement is defined as one cow (the actor) replacing another cow (the reactor) at the same feed bin.

Rumination behaviour. All cows were fitted with rumination loggers (HR-Tag, SCR, Netanya, Israel) that allowed for the continuous recording of time spent ruminating, summarized in 2-h intervals (for full description and validation see Schirmann et al., 2009). Infrared identification units were used for data transmission from the logger to the computer. The identification units were installed above each water bin and at the entrance to the milking parlour.

2.2.3 Health checks

Prepartum cows. Rectal temperatures were taken for all cows every morning in the home pen. Cows were also checked for signs of calving, injuries and visible signs of illness, such as dehydration or lameness. Blood ß-hydroxybutyrate (BHBA) was measured weekly using the validated Precision Xtra (Abbott, Abbott Diabetes Care Ltd., Witney, Oxon, United Kingdom) (Iwersen et al., 2009). At the same time all cows were scored for body condition on a 5-point scale in 0.25 increments (adapted from Ferguson et al., 1994).

Postpartum cows. Health checks were performed every morning after milking. Daily health checks consisted of measuring rectal temperature, auscultation of the rumen in the left paralumbar fossa for rumen motility (healthy cattle have 1 or 2 primary rumen contractions per 1 minute; according to Divers and Peek, 2008, p. 10), auscultation and simultaneous percussion for displaced abomasum (listening for abdominal pings; according to Radostits et al., 2007, p. 375), dehydration (skin test, dry mucosal tissue, sunken eyes) and retained placenta (RP; when the placenta was still attached > 24 h following parturition). Blood BHBA was determined 3x/wk (Monday, Wednesday and Friday) using the same method as described above and cows were classified as having subclinical ketosis (SCK) when the BHBA concentration was ≥ 1.2 mmol/L during the first or second week. Cows with BHBA > 3.0 mmol/L at any time throughout the

study would have been classified as ketotic (Oetzel, 2004) but none in this study reached this criterion.

Metritis diagnosis was based on the vaginal discharge, scored twice per week using the Metricheck (Metricheck, Simpro, New Zealand). The Metricheck device is a 50-cm-long stainless steel rod with a 4-cm hemisphere of silicon and has been evaluated for this application (Pleticha et al., 2009). Briefly, the vulva was cleaned with a dry paper towel and the Metricheck devise inserted to obtain a sample of the vaginal discharge. The discharge was visually assessed and scored: 0 = clear or no mucus; 1 = bloody or cloudy mucus or with small flecks of pus, bloody mucus; 2 = clear or cloudy mucus with larger flecks of pus up to 50% of the discharge; 3 = clear or cloudy mucus with > 50% of pus; 4 = watery, red-brown, putrid smelling (adapted from Sheldon et al., 2006). Cows with scores 0, 1, or 2 throughout the first 21 d postpartum were classified as non-metritic while cows with at least one score of 3 or 4 were classified as having puerperal metritis. Given the diurnal variation in fever (Vickers et al., 2010), fever was not considered. The assessment of odour for the classification of vaginal discharge is highly subjective (Sannmann et al., 2013) and was not considered. All cows with retained placenta or metritis were treated for 3d with Penicillin, following the standard procedure for our farm.

Cows were checked for clinical mastitis at every milking by a trained milker immediately before the attachment of the milking unit. The udder was manually checked for swelling, heat, pain and oedema and the milk was visually inspected for color, viscosity and the presence of clots.

2.2.4 Statistical Analyses

Of the 80 cows enrolled, 7 did not finish the study; 2 of these cows were sold and the others were removed due to severe health issues (2 with severe milk fever, 2 with persistent mastitis, and 1 with split legs). Another 9 cows were removed from the final data set as they were diagnosed with only a single health problem that was not metritis or subclinical ketosis (milk fever only = 2 cows, an elevated temperature as a single symptom = 3 cows, mastitis only = 4 cows). For the data analysis of our study we grouped cows according to their health status. Cows were classified as healthy when they presented no symptoms of any metabolic disorders or other health problems (healthy, n = 20). Cows were classified and grouped as having metritis (n = 18), subclinical ketosis (SCK; n = 9), having both of these diseases (metritis+SCK, n = 9) or a combination of either metritis or SCK or both plus another unrelated disorder (e.g. mastitis and SCK or milk fever and metritis; MULT, n = 8). Cows with a combination of retained placenta (RP) and metritis were classified as metritis, given that a RP is a major risk factor for this ailment (Paisley et al., 1986).

A sample size analysis based on differences in mean daily rumination behaviour yielded in a group size of n = 8 and was performed using Medcalc Software (Ostend, Belgium). In detail the sample size calculation was based on detecting a 45 min difference in mean daily time spent ruminating between groups. An alpha level of 0.05 and a beta level of 0.2 were used and the deviation of time spent ruminating for healthy and sick cows was expected to be 20 and 40 respectively.

Data loss of 2-h intervals of rumination data led to the exclusion of the entire day for that animal and resulted in a total loss of 4% of the data collected during the time relevant for the present analysis.

Further statistical analyses were performed using SAS software (SAS version 9.3; SAS Institute, Inc., Cary, NC) with cow as the experimental unit. DMI, feeding time, rumination and social behaviour data were summarized by 2-h interval, cow and day to obtain individual daily values. Individual feeding rates were calculated by dividing the DMI by the daily time spent feeding. Days -1, 0, 1 and 2 relative to calving were excluded as previous research has shown that calving and regrouping affect rumination and feeding behaviour (Schirmann et al., 2011; Schirmann et al., 2013). Data were initially plotted to identify differences in behaviours between health categories over time and were then divided into 4 periods; one pre-calving (d -7 to d -2) and three postpartum periods: period 1 (d 3 to 8), period 2 (d 9 to 14) and period 3 (d 15 to 20). These periods essentially refer to the weeks relative to calving, however, given that we excluded days around calving we decided to name the time frame 'period' rather than 'week'. We used a mixed model (PROC MIXED) with cow as a repeated measure, health status was a fixed effect and the interaction day x health status was evaluated and was not significant. Day, however, was significant in Periods 1 and 2 and kept in the model. Our preliminary models included parity and showed no effect. Therefore, parity was not included in the final model. To reduce the number of tests specified contrast statements were used to test for differences between the least square means between the healthy animals and the 4 sick groups (metritis, SCK, metritis+SCK and MULT). Residuals were plotted and visually examined to assess normality and homogeneity of variances. All values are reported as least squares means and the SE. Significance was declared at $P \leq 0.05$ and tendency at $P \leq 0.1$.

2.3 Results

2.3.1 Descriptive Data

Of the 64 cows included in the analysis 20 were healthy. Of the remainder, 18 had metritis, 9 SCK, 9 metritis+SCK and 8 multiple health problems. Mean BCS across all sick cows at parturition was 3.29 ± 0.25 (mean ± SD). In comparison BCS for healthy cows was 3.33 ± 0.22.

2.3.2 Rumination behaviour

Time spent ruminating showed a diurnal pattern (see Figure 2.1). This pattern appeared to be similar for each of the 4 observational periods and each of the health conditions. Figure 2.1A illustrates that cows with SCK appear to spend less time ruminating throughout the day during the pre-calving period while this difference is no longer visible during Period 3 (Figure 2.1B).

We noted a reduction in time spent ruminating for cows with SCK during the pre-calving period (Figure 2.2A). At this time healthy cows spent on average 14% more time ruminating than cows with SCK. Postpartum there were no differences in times spent ruminating.

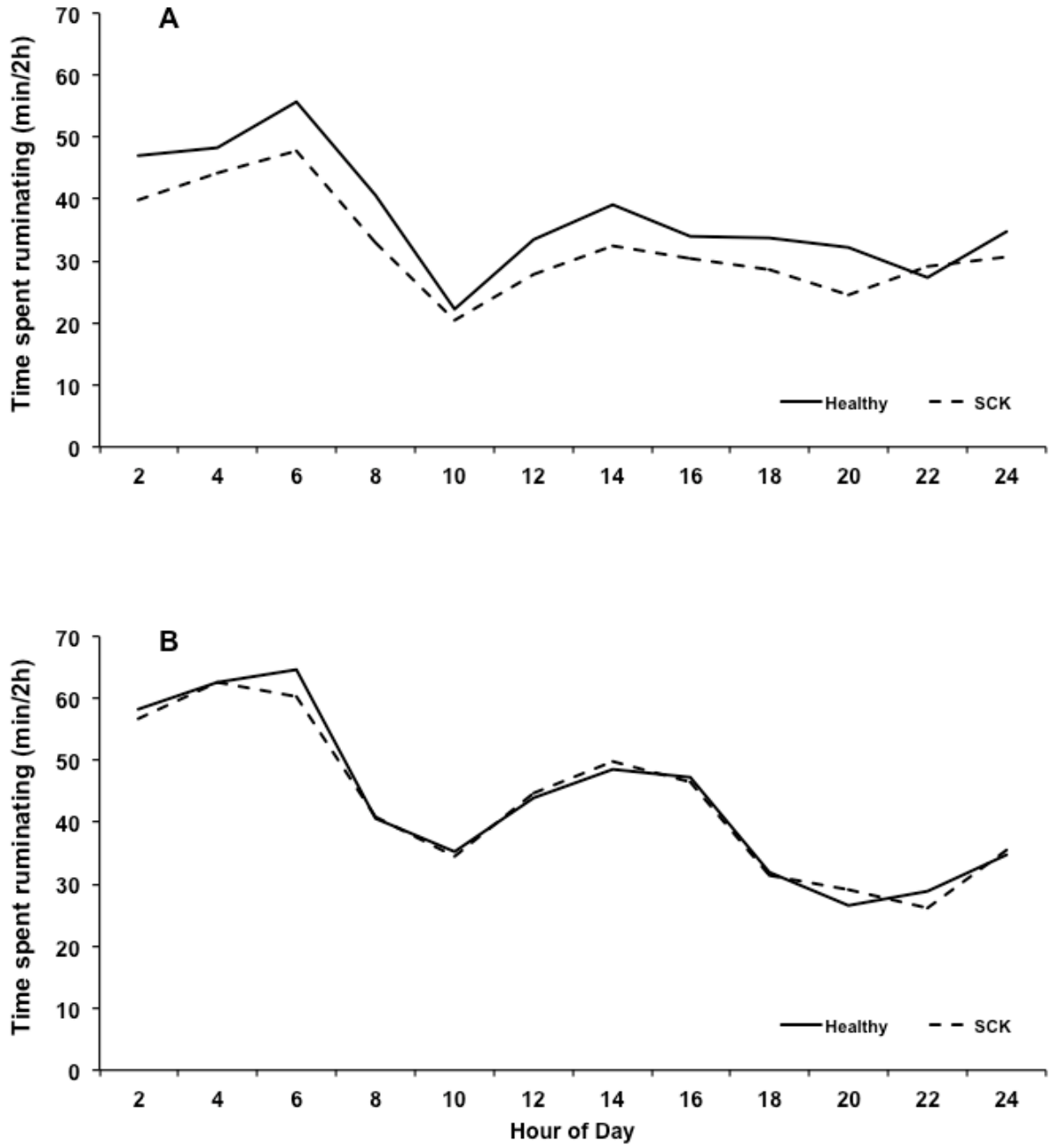


Figure 2.1: Diurnal pattern rumination.
Diurnal pattern for time spent ruminating for healthy cows and cows with SCK for the pre-calving period (A) and Period 3 relative to calving (B) illustrating the differences between healthy and SCK cows during the pre-calving period and the lack thereof during Period 3.

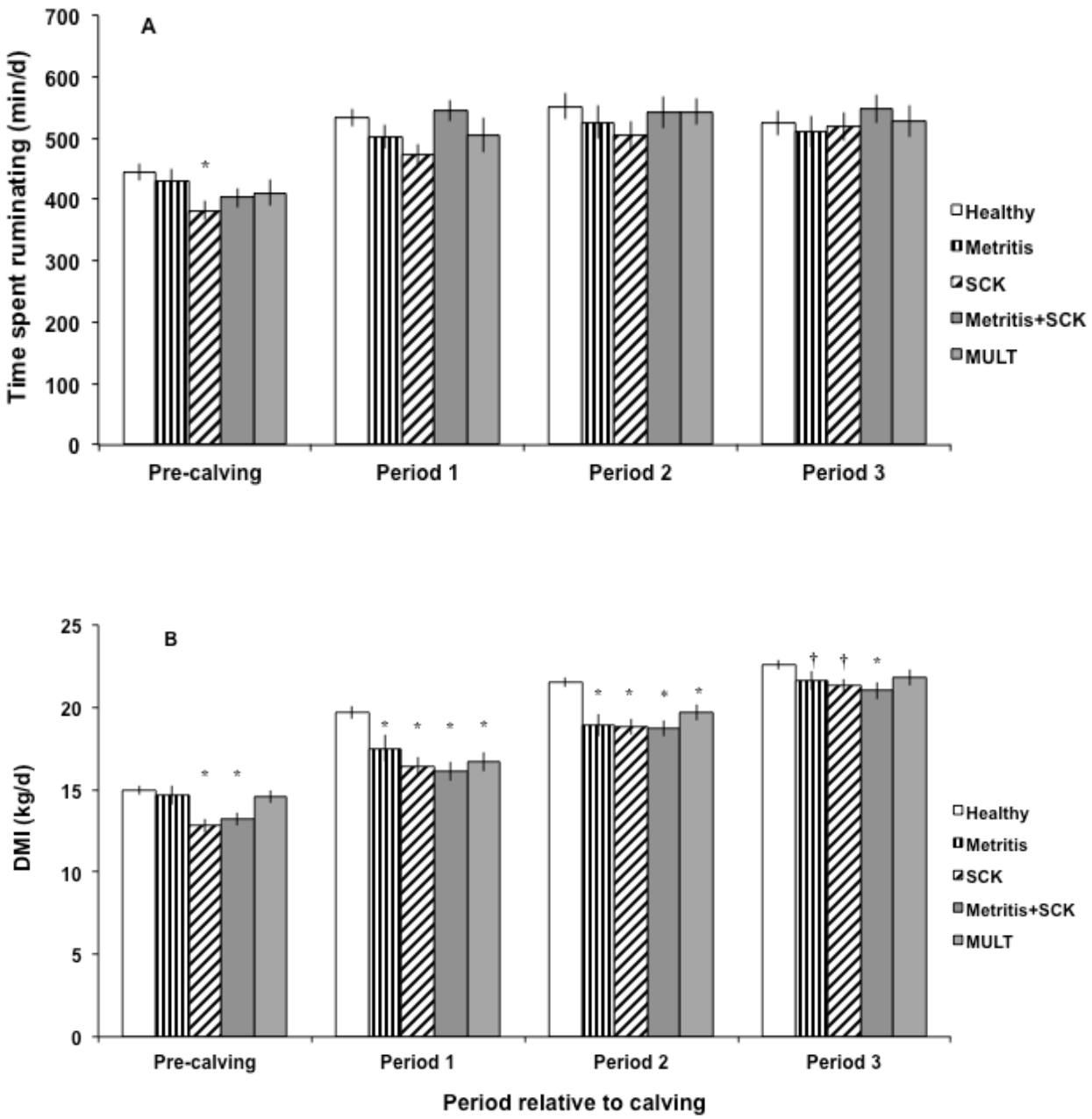


Figure 2.2: Average daily time spent ruminating and DMI.
Average daily time spent ruminating (LSM ± SE; A) and average daily DMI (LSM ± SE; B) for the 5 health categories (Healthy, n = 20; Metritis, n = 18; SCK, n = 9; Metritis+SCK, n = 9; and MULT, n = 8) within period relative to calving (pre-calving, Period 1, Period 2, and Period 3)[1].

[1]Significance level for differences between healthy cows and each of the other health categories within period relative to calving: $^{\dagger}P < 0.1$, $^{*}P < 0.05$.

2.3.3 Feeding behaviour

DMI. During the pre-calving period, DMI of healthy cows was 12 and 15% higher than that of metritis+SCK and SCK cows respectively (Figure 2.2B). Healthy cows consumed on average 15% more (range 11 to 18%) than the other health categories during Period 1 and 8 to 13% more during Period 2. During Period 3 healthy cows had a higher DMI than metritis+SCK cows while cows with SCK and cows with metritis only tended to differ.

Feeding time. During the pre-calving period healthy cows spent on average 18 to 29% more time feeding than cows with SCK, metritis+SCK and MULT (Table 2.1). In Period 1, feeding time for healthy cows was 13 to 25% higher than the feeding time of the other health categories. During Period 2 healthy cows spent up to 24% more time feeding than cows with metritis, SCK and MULT. In Period 3 only MULT cows differed from healthy cows and spent close to an hour less time feeding (22%).

Feeding rate. In the pre-calving period cows with SCK and MULT fed 25% and 34% faster than healthy cows. Post-calving only MULT cows differed from healthy cows and fed faster throughout all three periods (11, 21 and 24% for periods 1, 2 and 3 respectively).

Visits. Pre-calving healthy cows visited the feed bin 18, 24, and 40% more often than cows in the metritis+SCK, SCK and MULT groups respectively. Healthy cows visited the feed bins 14% more often than SCK cows in Period 1 and 36, 35 and 28% more frequently than MULT cows throughout Periods 1, 2 and 3 respectively. There were no differences in the number of visits between healthy cows and those with metritis at any time.

	Health category				
Parameter	Healthy	Metritis	SCK	Metritis+SCK	MULT
Feeding time (min/d)					
pre-calving	236 ± 5	221 ± 5	168 ± 5*	193 ± 7*	174 ± 8*
Period 1	197 ± 4	172 ± 4*	158 ± 6*	172 ± 6*	148 ± 7*
Period 2	231 ± 5	204 ± 7*	202 ± 7*	214 ± 6	175 ± 7*
Period 3	249 ± 5	236 ± 5	235 ± 7	232 ± 7	195 ± 8*
Feeding rate (g/min)					
pre-calving	65 ± 2	69 ± 2	81 ± 2*	70 ± 2	87 ± 3*
Period 1	103 ± 2	107 ± 2	105 ± 3	97 ± 3	115 ± 8*
Period 2	96 ± 2	98 ± 2	96 ± 3	89 ± 3	116 ± 3*
Period 3	93 ± 2	97 ± 2	96 ± 3	94 ± 3	115 ± 3*
Visits (no/d)					
pre-calving	62 ± 2	60 ± 2	47 ± 3*	51 ± 3*	37 ± 3*
Period 1	44 ± 2	43 ± 2	38 ± 2*	41 ± 2	28 ± 2*
Period 2	46 ± 2	46 ± 2	44 ± 2	45 ± 2	30 ± 2*
Period 3	47 ± 2	49 ± 2	48 ± 2	48 ± 2	34 ± 3*
Repl Actor (no/d) [2]					
pre-calving	13 ± 1	12 ± 1	10 ± 1	12 ± 1	9 ± 1*
Period 1	12 ± 1	12 ± 1	11 ± 1	14 ± 1	8 ± 1*
Period 2	14 ± 1	14 ± 1	15 ± 2	18 ± 1†	10 ± 2
Period 3	14 ± 1	15 ± 1	18 ± 2	19 ± 2*	13 ± 2
Repl Reactor (no/d) [3]					
pre-calving	12 ± 1	16 ± 1*	7 ± 1*	11 ± 1	6 ± 1*
Period 1	12 ± 1	13 ± 1	7 ± 1*	14 ± 1	7 ± 1*
Period 2	14 ± 1	16 ± 1	11 ± 2	19 ± 1*	8 ± 2*
Period 3	15 ± 1	16 ± 1	15 ± 2	22 ± 2*	10 ± 2*

Table 2.1 Feeding and social behaviour by health category and time period.
Least square means (± SE) for feeding and social behaviour for cows from each of 5 health categories (Healthy, n = 20; Metritis, n = 18; SCK, n = 9; Metritis+SCK, n = 9; MULT, n = 8) and for each of the 4 periods investigated (Pre-calving, Period 1, Period 2 and Period 3)[1].
[1]Significance of differences between healthy cows and each of the other health categories: †$P < 0.1$, * $P < 0.05$.
[2] Repl Actor = The Actor in a replacement is the cow actively displacing another cow from a feed bin and immediately thereafter taking her place at the same feed bin.
[3] Repl Reactor = The Reactor in a replacement is the cow that is being displaced from the feed bin.

2.3.4 Social behaviour

Pre-calving and during Period 1 healthy cows were 31 and 33% more successful at replacing another cow at the feed bin than MULT cows (Table 2.1). Compared with healthy cows, metritis+SCK cows were more often successful at replacing another cow during Period 3. During the pre-calving Period, metritis cows were 33% more often replaced than healthy cows. In Period 1 SCK and MULT cows were both replaced 42% less often than healthy cows. MULT cows were also replaced less often during Periods 2 and 3. In Periods 2 and 3 only, metritis+SCK cows were replaced at the feed bin 36 and 47% more often than healthy cows.

2.4 Discussion

According to our hypothesis this study shows differences in rumination and feeding behaviour between cows that stayed healthy and cows that experienced health problems in the weeks following parturition. Cows were grouped according to postpartum health and the mean daily rumination and feeding activity are described. We also describe differences in the diurnal pattern for rumination time during the pre-calving Period.

The percentage of cows with metritis reported in the current study (28%) is within the range of previous work (e.g. 12%: Machado et al., 2014; 40%: Patbandha et al., 2012). The prevalence of uterine infection varies considerably among studies, explained in part by differences in the diagnostic criteria used (Azawi, 2008). The percentage of cows with SCK reported here (14%) is also within the range reported in previous work. For example, McArt et al. (2012a) reported a prevalence of 43% among cows from 4 different farms, while Liboreiro et al. (2015) described a prevalence of 13%. In our study, excluding the metritis+SCK cows, 13% of animals experienced multiple unrelated health problems. Combined, the percentage of animals

with multiple health problems (27%) is somewhat higher than the 20% of animals with a combination of health problems as reported by Ospina et al. (2010).

Rumination is important for breaking down feed particles to facilitate passage through the reticulorumen to the omasum and promote saliva production (Beauchemin, 1991). The cessation of rumination is a clinical sign of disease in cattle (Radostits et al., 2007, p. 10). During the pre-calving period healthy animals in our study ruminated about 7.5 h/d; this value is similar to those reported elsewhere for pre-calving Holstein cows (Aikman et al., 2008; Calamari et al., 2014). The average time spent ruminating of approximately 9 h/d for healthy cows after calving is within the range previously reported for mid-lactation cows (Adin et al., 2009; Soriani et al., 2012). We observed no differences between healthy categories in time spent ruminating during Periods 1 to 3. However, the observed pre-calving difference in rumination activity between healthy animals and animals with SCK was in agreement with Kaufman et al. (2016). Interestingly, Kaufman et al. (2016) reported a tendency toward a decrease in rumination for cows with SCK during wk -1 and + 1 relative to calving. Rumination is known to decrease as calving approaches (Schirmann et al., 2013; Büchel and Sundrum, 2014; Pahl et al., 2014), therefore these times were excluded in the current study removing any effect of parturition per se. Furthermore rumination is known to vary greatly between animals (Byskov et al., 2015). Thus pre-calving differences in rumination between healthy animals and animals with SCK suggest that this behaviour may be useful for detecting cows at risk of metabolic problems postpartum. Also, Liboreiro et al. (2015) noted a reduction in daily rumination time (during the first 8 d postpartum) for cows with SCK. Furthermore, DeVries et al. (2009) found that when cows underwent an acidosis challenge they decreased time spent ruminating on d 1, followed by an increase over baseline on d 2 after the challenge, showing that metabolic changes can have a

rapid effect on rumination. These results indicate that changes in the metabolic status of a cow cause notable (and automatically detectable) changes in rumination behaviour.

We found no differences between health categories in time spent ruminating postpartum. This result differs from Calamari et al. (2014) who observed that the cows grouped as having low rumination behaviour plateaued in the amount of time spent ruminating at about 15 DIM. However, these authors' grouped cows as high and low according to the time spent ruminating at 3 to 6 DIM. These authors also investigated the relationship between inflammatory markers in the blood and rumination time, and noted that 90% of the animals in the low group showed evidence of clinical diseases in early lactation (versus only 40% of the cows in the high group). This information, combined with our finding that cows with SCK were already spending less time ruminating before calving, suggests that grouping animals according to rumination behaviour in the days immediately following parturition may result in grouping healthy versus sick cows.

A decrease in rumination time in combination with the onset of illness has previously been described for lameness (Miguel-Pacheco et al., 2014), mastitis (Siivonen et al., 2011; Fogsgaard et al., 2012) and metabolic changes (Hansen et al., 2003; DeVries et al., 2009). In agreement with these results, our work suggests that monitoring rumination pre-calving can be useful in the identification of cows likely to experience health problems, especially SCK, in the days after calving. Early detection of cows with SCK is worthy of further investigation given that untreated cows have a higher risk of displaced abomasum and are more likely to be culled within the first 30 DIM (McArt et al., 2012b). SCK also impairs reproduction, decreasing pregnancy rates at first insemination (Walsh et al., 2007). Measuring rumination after calving, ideally in addition to BHBA, may improve detection of metabolic disease. However, further research

focusing on identifying within cow cut-points for rumination is encouraged. Additionally, we suggest that future research focuses on the development of algorithms to identify sensitive and specific within-cow thresholds.

Average pre-calving and postpartum DMI, time spent feeding and number of visits to the feed bin for healthy animals in our study are in line with previous research (Huzzey et al., 2007; Goldhawk et al., 2009; Vickers et al., 2013). However, in contrast to Huzzey et al. (2007), we did not detect differences in feeding behaviour between metritic and healthy cows before calving. This difference between studies may be due to differences in days included in the analysis as well as scoring of vaginal discharge and inclusion criteria. We summarized our data to only one pre-calving period and excluded days -1 to 2 relative to calving from the analysis. Regarding metritis, Huzzey et al. (2007) differentiated between mild and severely metritic and also included smell and fever as diagnostic criteria; in our study smell and fever were excluded, as smell has been shown to be subjective (Sannmann et al., 2013) and fever has been shown to be highly variable (Vickers et al., 2010), so we used vaginal discharge only to distinguish between healthy and metritic cows. Differences in diet between studies may also have contributed to differences in feeding behaviour as dietary energy density has been found to influence behaviour at the feed bunk (Huzzey et al., 2013). The reduced DMI, lower feeding times and fewer visits to the feed bin before calving that we observed for cows with SCK may have been caused by malaise without diagnosable illness, and by reduced abdominal space because of the growing fetus. Goldhawk et al. (2009) also found reduced DMI in the week before calving in cows diagnosed with SCK. Given the within-cow correlation between DMI and rumination, it was not surprising that we noted reductions in both of these parameters. Reduced DMI prepartum may result in negative energy balance that is further exacerbated in the days following calving (given

the energy demands associated with milk production). To compensate for negative energy balance, fat is mobilized increasing the number of circulating ketone bodies and thus also increasing the risk of subclinical and clinical ketosis. Throughout the study MULT cows spent less time feeding, fed at a faster rate, and visited the feed bins less often than healthy cows. Interestingly, they also had lower DMI in Periods 1 and 2 despite the increase in feeding rate. The MULT cows were less often the receiver of a replacement in all 4 study periods, but the active involvement of MULT cows in a replacement was lower only during the pre-calving Period and Period 1. Other studies have shown that ill and lame cows will sometimes increase feeding rate, likely as a mechanism to maintain intake (González et al., 2008b; Norring et al., 2014). We suggest that when cows feel sick they are less able to compete successfully for access to the feed bunk, and thus use higher feeding rates when they do have access. Previous research has shown that socially subordinate cows, less able to aggressively compete for access to feed, eat at higher rates when they do have access to feed (Proudfoot et al., 2010). In our study cows in the MULT group not only increased their feeding rate, but also showed an added peak of feed intake in the early afternoon, a time of day when there is otherwise little activity at the feed bunk, providing another way for sick cows to avoid social interactions and competition at the feed bunk. We therefore suggest that cows in the MULT group adopted different coping mechanisms to maintain DMI, including increasing their feeding rate and feeding at less favourable times.

Some forms of illness likely reduce appetite, especially at the onset of disease. For example, Fogsgaard et al. (2012) reported that feeding behaviour decreased after *Escherichia. coli* mastitis was induced. The differences in postpartum feeding behaviour in our study were similar to those observed by Huzzey et al. (2007) and Goldhawk et al. (2009); these studies found lower DMI and feeding times during the first weeks postpartum for cows diagnosed with

metritis and SCK, respectively. One might argue that the findings in feeding behaviour, in part, could be due to overcrowding the cows at the feed bins. The cow-to-bin ratio in our study was 2:1 for the prepartum and the postpartum periods respectively. Using the same ratio of 2:1 and a ratio of 1:1 (Proudfoot et al., 2009) reported a tendency for lower DMI during the last week prepartum and lower feeding times during the first week postpartum for cows that were overstocked. Interestingly, Hosseinkhani et al. (2008), using the same two ratios as Proudfoot et al. (2009), found no differences for cows during weeks -3 and -2 relative to calving. Overall, our results suggest that the ill cows in our study were attempting to avoid competition, by increasing feeding rate or feeding at a less preferred time, but they were still motivated to feed.

The differences in feeding and social behaviour for MULT cows suggest the need for further research on behavioural changes for cows with more than one health problem. Furthermore, our results demonstrate the importance of distinguishing between animals with a single health problem and such with multiple health problems.

In summary, in comparison to healthy cows, cows with subclinical ketosis postpartum spent on average less time ruminating during the prepartum period, despite the large variation even among healthy animals. Regarding the feeding behaviour prepartum we noted reductions in feeding time and fewer visits to the feed bin for the sick groups except for cows with metritis. Feeding rate was increased for SCK and MULT cows. Postpartum there were no differences in rumination behaviour. All sick groups had a lower DMI and spend less time feeding during Period 1.

2.5 Conclusions

Our results indicate that monitoring rumination during the transition period is helpful in the detection of cows with metabolic problems. Furthermore, the findings from our study support

earlier work describing the applicability of monitoring feeding behaviour to detect health problems in the transition period. Based on our results, we conclude that automatic monitoring of individual rumination and feeding behaviour in transition dairy cows shows promise for the detection of health problems. Moreover, a combination of both behaviours may result in higher detection rates. Monitoring individual rumination behaviour is likely more feasible for commercial farms than is monitoring individual feed intake and thus especially warrants further development.

Chapter 3: Effect of stocking density on estrous expression and biomarkers of stress in dairy cows

3.1 Introduction

Decreases in reproductive performance and overall poor fertility have been noted for decades (Lucy, 2001) and, among other factors, have been linked to decreased estrous detection (Dobson and Smith, 2000; Dobson et al., 2007) as well as inappropriate management such as restricted access to feed and resting areas (LeBlanc, 2010). Behaviours typically associated with estrus, and used for estrus detection, include the primary sign standing to be mounted and secondary signs such as mounting other cows, increased activity (restlessness), sniffing and licking other cows (Hurnik et al., 1975; Peter et al., 2009a). Based on the knowledge that these behaviours change in relation to estrus, devices that automate behavioural monitoring have been developed with the aim of facilitating estrus detection. Many of these systems set out to detect deviations from a baseline, most commonly changes in activity, e.g. as number of steps walked, or changes in movement acceleration (as reviewed by Firk et al., 2002). These technologies have gained considerable traction, and tools such as automated activity monitors (**AAM**) are now integrated into many reproductive programs, either in combination with or instead of a hormonal protocol (Neves et al., 2012; Valenza et al., 2012; Neves and LeBlanc, 2015).

Evidence suggests that the management of farm animals can have a profound impact on fertility (Schefers et al., 2010; Ferguson and Skidmore, 2013). Previous studies demonstrated that chronic stress, induced by lameness, reduces intensity of estrous expression as well as progesterone concentration before estrus (Walker et al., 2008; Morris et al., 2011) that likely affects estrous behaviour. Schefers et al. (2010), using data collected from 108 freestall farms,

reported that a high-stocking density in the breeding pen was detrimental to pregnancy rate. This result, combined with the knowledge of increasing herd-sizes (USDA, 2008) and variations in stocking density within and between farms, raise the importance of investigating the effect of stocking density on dairy cattle reproduction and reproductive behaviour.

To our knowledge no literature has investigated the effect of stocking density on detailed measurements of estrous expression, ovarian dynamics and concomitant association with biomarkers of stress in healthy lactating Holstein dairy cows around 65 DIM. Thus, the objectives of the current study were to determine the effects of simultaneously over or under stocking at the lying stall and feed bunk on a) estrous expression parameters based on physical activity (intensity and duration), b) ovarian dynamics as measured by the pre-ovulatory follicle diameter and plasma estradiol and progesterone concentrations at estrus, and c) biomarkers of stress in plasma and feces during the experimental period. Our hypothesis was that overstocking would negatively affect estrous expression measurements, result in smaller pre-ovulatory follicles and negatively impact estradiol synthesis. Additionally, we expected overstocked cows to have higher concentrations of haptoglobin and fecal cortisol metabolites.

3.2 Methods

This study was conducted at the University of British Columbia's Dairy Education and Research Centre in Agassiz, BC, Canada. Animals were cared for according to the guidelines set by the Canadian Council on Animal Care (2009). All procedures were approved by the UBC Animal Ethics Committee (A 10-0290).

3.2.1 Animals, Housing, Diet and Milk Production

A total of 147 lactating Holstein dairy cows (parity 2.5 ± 1.3; mean ± SD) were enrolled in this study. Cows were housed in a freestall barn and study animals were assigned to one of

two stocking densities: 67% (understocked; n = 73) or 133% (overstocked; n = 74). In total, four study pens of 14.4 m length and 13.5 m width each (Figure 3.1) were used simultaneously, with 2 pens assigned to each of the stocking density treatments, balanced for parity. Pens were located on both sides of the barn with both stocking densities on either side. Each pen was equipped with 24 deep sand bedded lying stalls and 2 self-filling water troughs, one on either side of the pen. There were 16 cows in the 67% and 32 cows in the 133% stocking density pens resulting in a cow to stall ratio of 2:3 for understocked and of 4:3 for overstocked pens. The feed bunk was accessible via a post-and-rail-barrier and available feed bunk space/cow was 0.9 m for understocked and 0.45 m for overstocked pens. Available walking area was 7.5 m^2 and 3.25 m^2 for under- and overstocked cows, respectively. Group size was static while group composition was dynamic with simultaneous introduction of new animals and removal of others once a week.

Cows were milked and fed twice a day at approx. 0630 h and 1630 h. The TMR consisted of corn silage, grass silage, mineral and concentrate mix and alfalfa hay. Cows were milked twice daily in a double 12 side-by-side milking parlour (BouMatic Dairy Equipment Co., Madison, WI). Milk production was automatically recorded at every milking and AM and PM recordings were summarized for daily milk production.

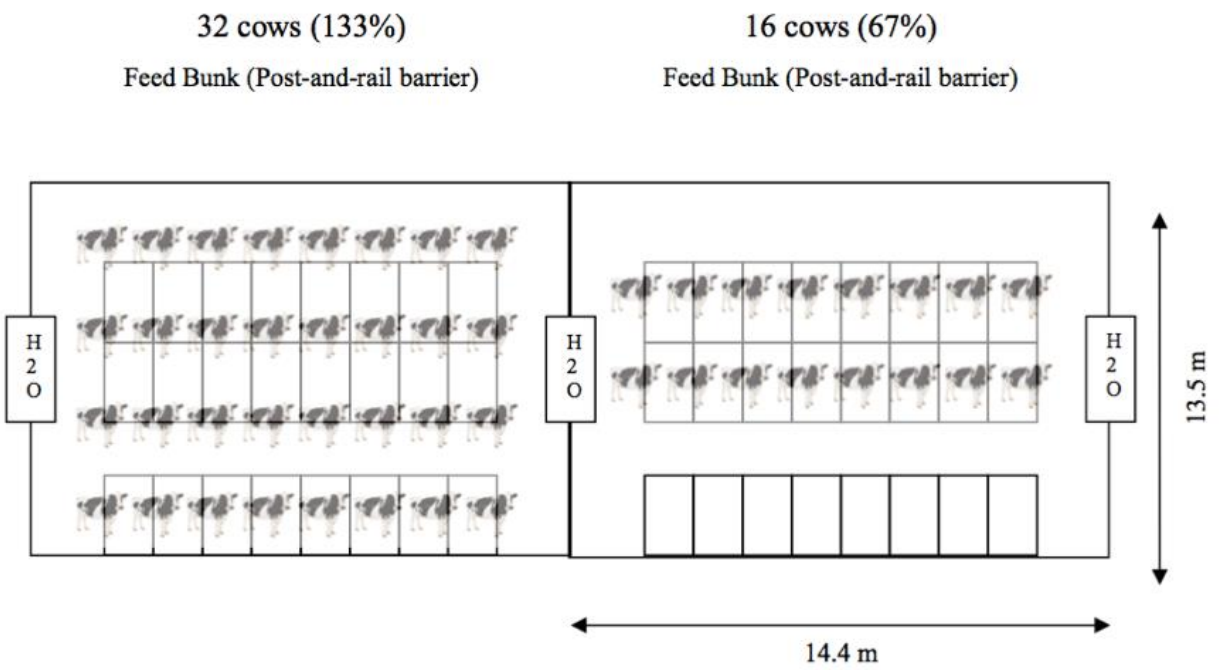


Figure 3.1: Illustration of the two stocking densities.
Illustration of pen design and dimensions of overstocked and understocked treatments.

3.2.2 Experimental design and estrus synchronization protocol

Animals were enrolled and m oved into the study pens at 40 ± 3 DIM and monitored for 34 d, therefore leaving the pen the earliest at 74 ± 3 DIM. Once a week new cows were enrolled and moved into the study pens with the simultaneous removal of an equal number of cows that had the highest DIM to maintain stocking density. Approximately half of the cows in each study pen were lactating filler cows that were either confirmed pregnant or had already been bred.

Before being enrolled into the study all cows underwent a basic health check, including gait scoring, by a veterinarian to ensure that the animals were healthy and sound. All cows received a synchronization protocol consisting of two i.m. injections of $PGF_{2\alpha}$ (**PGF**; Estrumate, Cloprostenol sodium, Intervet Canada Corp., Kirkland, QC, Canada) 14 d apart. The injections were given on study d 8 and d 22 (47 ± 3 DIM, **PGF1;** and at 61 ± 3 DIM, **PGF2**) regardless of

the presence or absence of a corpus luteum (**CL**) on the day of injection. Cows were examined, palpated and blood and fecal samples were taken on the day of enrolment (study d 1) and on study d 8, 22, 24, 25, 26, 27, and 34. The exception being animals that were bred after PGF2, these were palpated before breeding but not on the days immediately following to avoid interference with breeding and conception.

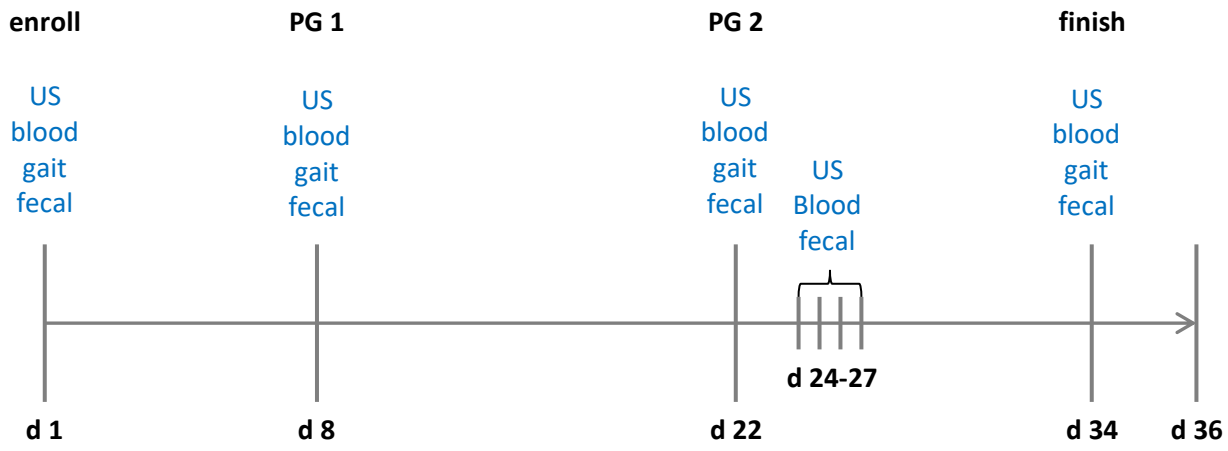


Figure 3.2: Study timeline

3.2.3 Ovarian ultrasonography

To determine cyclicity, stage of cycle and estrus, all cows were subject to *per rectum* palpation and examination by ultrasound (Ibex pro, E.I. Medical Imaging, Loveland, CO, USA) using a 7.5 MHz rectal linear array transducer. Cows were examined on study d 1 as well as on d 8 and 22 (PGF1 and PGF2). Cows were again examined on d 2 to 5 and d 12 following the second PGF injection, after which estrus was expected. The presence and diameter of the largest follicle and the CL were recorded to determine changes in follicle diameter, diameter of the pre-ovulatory follicle and occurrence of ovulation.

3.2.4 Blood and fecal sample collection

Blood and fecal samples were collected before ultrasound examination. Blood samples were collected by punctuation of the median coccygeal blood vessels utilizing K_2 EDTA coated vacutainer tubes (Becton & Dickinson Vacutainer systems, Rutherford, NJ), and cooled immediately. Blood samples were centrifuged at 2,700 rpm for 15 min to enable plasma harvesting. Plasma was divided into triplicates, stored at -80°C and later evaluated for progesterone (ELISA; Cerri et al., 2004), estradiol (RIA; Kirby et al., 1997) and haptoglobin concentration. The haptoglobin essay was performed using the commercially available PHASE Haptoglobin kit (Tridelta Development Ltd., TP801, Maynooth, CK, Ireland). Plasma was analysed for haptoglobin concentration using samples from study days 8, 22 and 34 (PGF1, PGF2, PGF2+12). Analysis for estradiol and progesterone concentration was performed on samples taken closest to estrous.

Fecal samples were collected either from spontaneous defecation or manually removed from the rectum. A new examination glove was used for each sample to avoid cross contamination. Each sample was sealed in a plastic container, cooled immediately and stored in a -20°C freezer for later analysis. Samples were analyzed for fecal cortisol metabolite 11, 17-dioxoandrostanes (**FCM**) concentrations using a competitive enzyme immunoassay developed by Palme and Mostl (1997) and validated for cattle (Palme et al., 1999).

3.2.5 Automated Activity Monitor (AAM)

For automatic recording of individual cows' activity and changes therein an activity monitoring system (Heatime, SCR Engineers Ltd., Israel) was used. An activity logger was mounted on the animals neck-collar and a wireless antenna that scans the barn in 2-h intervals automatically transferred the data from the collar to the computer. This AAM collects activity

data in a 2-min resolution and stores it in 2-h cells. An internal algorithm is used to detect animals in heat by comparing the current activity to a previously recorded baseline and using the standard deviation (**SD**) to calculate an activity index. The threshold for the activity index, used to create an automatic alert for high activity, indicating a cow in heat was 35 with a maximum index of 100. The Heatime batch tool was used to export the raw data to Excel files (Excel; Microsoft Corporation, Redmond, WA). A previously validated Excel macro (Madureira et al., 2015a) was then used to calculate percent increase in activity, highest peak activity, highest index and duration of high activity (duration of estrus; total amount of time the increased activity cluster was above the threshold) based on the dates when cows were defined as being in estrus by the AAM (index ≥ 35). Additionally baseline data from the 2 wks following the heat episode were used to assess the post-estrus baseline activity.

3.2.6 Standing behaviour

To assess standing time and bouts each cow was fitted with a data logger (Hobo Pendant G, Onset, Cape Cod, Massachusetts, USA) that has previously been validated for this purpose (Ledgerwood et al., 2010). The logger was attached to one of the animal's rear legs and programmed to record position of the cow once a minute. These measurements were used to calculate hourly and daily standing and lying times as well as frequency of daily standing bouts, average duration of standing and lying bouts and duration of the longest daily standing and lying bout. The analysis and discussion of this data are in Chapter 4.

3.2.7 Gait scoring

To evaluate the walking ability and for the detection of lame animals, all cows were gait scored by a veterinarian according to the scheme developed by Flower and Weary (2006) (scores 1 - 5; 1 = sound and 5 = severely lame) on study d 1, 8, 22 and 34.

3.2.8 Statistical analyses

A total of 147 cows were enrolled in the study. Cows that did not have a CL on one of the ovaries throughout the study period (n = 7; 3 in the overstocked and 4 in the understocked treatment) were retrospectively classified as non-cycling and excluded from the analysis. Another six animals were excluded due to severe lameness (at least one gait score of ≥ 4; 3 cows in each treatment). Out of the remaining 134 cows, 101 qualified for inclusion for the analysis. Inclusion criteria were as follows 1) the animals ovulated as confirmed by ultrasonography with either disappearance of the largest follicle until d 5 after PGF2, the presence of a CL on d 12 after PGF2 or a combination of both, and 2) the animals were able to walk freely with no gait score ≥ 4 throughout the study period. The 33 excluded cows all failed to ovulate during the monitored period (13 in the overstocked and 20 in the understocked treatment). Out of the 101 cows 60 were also detected by the AAM (29 of 55 cows in overstocked and 31 of 46 cows in understocked), resulting in an animal n = 60 for the analysis regarding the AAM. The sample size was calculated using a single mean sampling test (MedCalc 11, MedCalc Software, Belgium). The power analysis was based on a value of alpha = 0.05 and beta = 0.20. A conservative estimate was based on a previous study measuring peak intensity and duration (Madureira et al., 2015a). A mean value of 75 index (20 SD) with a null hypothesis value of 65 index yielded a minimum sample size of 32. All analyses for this experiment were performed using SAS (version 9.4; SAS Inst. Inc., Cary, NC) using cow nested within pen as the experimental unit as cows (experimental as well as these to fill up the pen to the desired stocking density) were moved in and out weekly, thus changing the group of animals within the pen. Prior to all analyses, data were checked for normality using Proc UNIVARIATE and probability distribution plots. Normality was visually assessed and confirmed by the Kolmogorov-Smirnov

method. Differences in terms of number of animals detected as being in estrus between the two methods, ultrasound and AAM, and the two treatments, overstocked and understocked, were analyzed using a chi-square test with testing for differences in proportions. Continuous variables were analyzed using the Proc MIXED, with cow nested within pen as the random error. An autoregressive covariance structure and repeated measures were used when multiple experimental days were included. estrus expression parameters, ovarian dynamics, haptoglobin and FCM were analyzed using Proc NPAR1WAY specified wilcoxon (duration, index, %-increase, progesterone) and Proc TTEST (peak activity, estradiol, pre-ovulatory follicle diameter). Correlations within and between automatic activity measurements (peak activity, index, %-increase of activity and duration) and physiological measurements (pre-ovulatory follicle diameter and plasma progesterone, estradiol, haptoglobin and FCM concentration) were determined by Spearman's correlation using the Proc CORR. Differences with $P \leq 0.05$ were considered significant and differences from $0.05 > P < 0.10$ were designated as tendency.

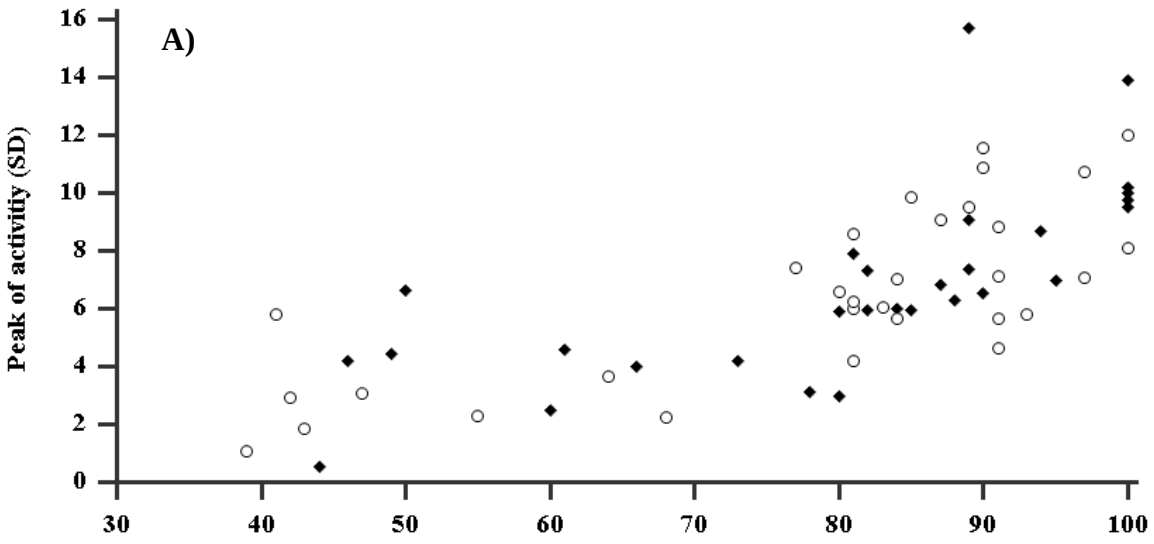
3.3 Results

3.3.1 Estrous expression parameters from AAM

Using the measures collected from the AAM we found no differences between treatments regarding the duration of estrus (12.1 h vs. 11.4 h for over and under stocked cows respectively), index (80.1 vs. 78.2), %-increase in activity (282.5 vs. 262.7) and the peak of activity (97.1 vs. 96.4). The index has no measurement unit as it translates the raw data from the system into a scale from 35 to 100 (Madureira et al., 2015a). Across treatments as well as within treatments we noted positive correlations for the index with duration of estrus, %-increase in activity and peak of activity (Figure 3.2).

Across treatments, %-increase in activity and pre-ovulatory follicle diameter tended to be positively correlated (r = 0.23, $P = 0.08$). When analyzed by treatment we noted that this was true for the overstocked cows (r = 0.49, $P < 0.01$) but not for the understocked cows (r = -0.02, $P = 0.9$). Only in overstocked cows did we observe positive correlations for pre-ovulatory follicle diameter with index (r = 0.58, $P < 0.01$) and pre-ovulatory follicle diameter with duration (r = 0.41, $P < 0.05$).

Overstocked cows tended to have a lower baseline activity post estrus than understocked cows (32.0 ± 0.9 vs. 34.8 ± 1.1 for overstocked and understocked cows respectively, $P = 0.06$).



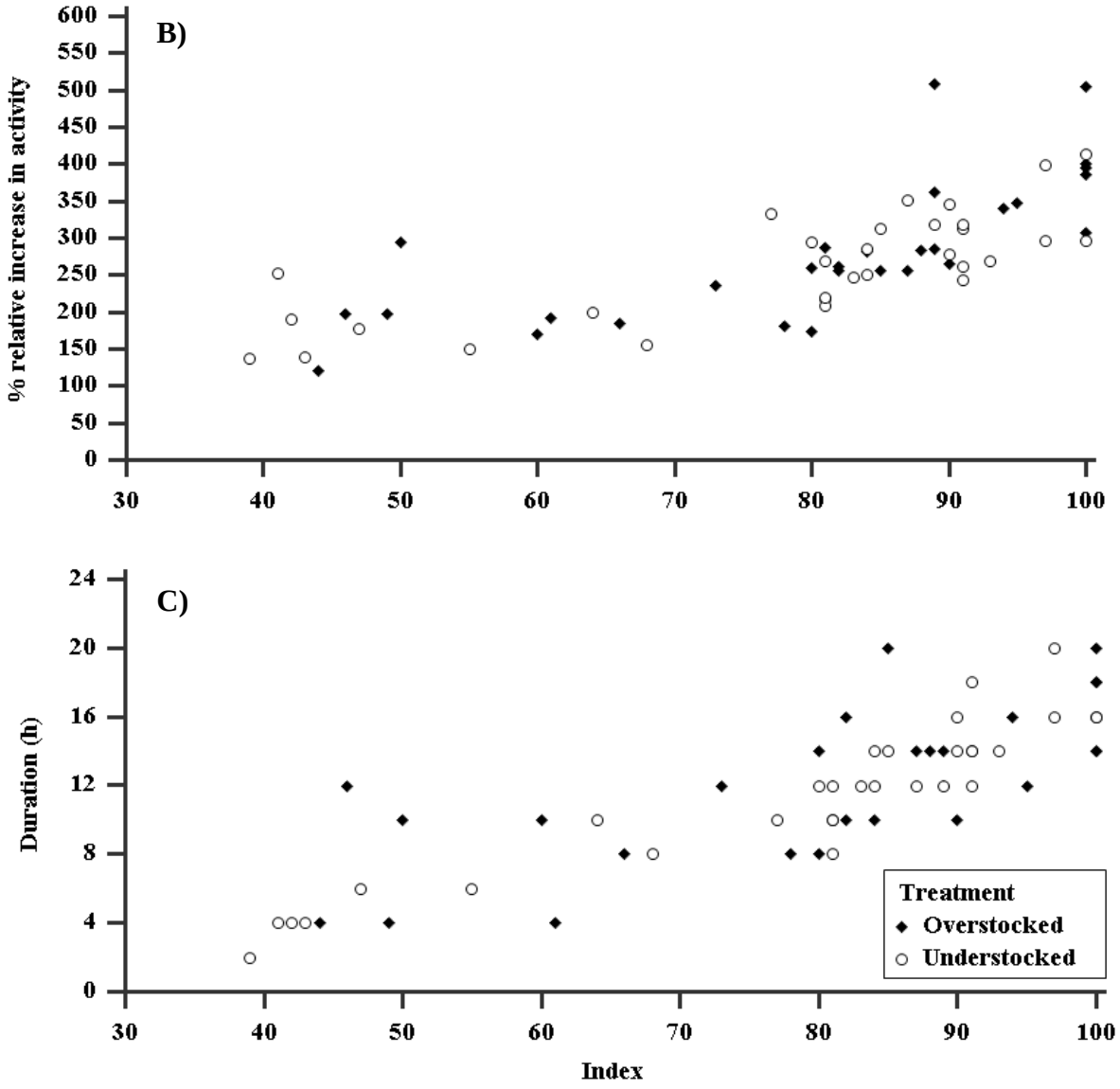


Figure 3.3: Across treatment correlations of the AAM activity measures.

Across treatment correlations between the index value of peak activity (measured by the AAM; min = 35, max = 100) and A) estrous activity in standard deviations (calculated using the raw peak activity value and baseline values of the previous 14 d from AAM data; r = 0.77, $P < 0.01$); B) the % relative increase in activity (calculated using the raw activity from AAM data; r = 0.79, $P < 0.01$); and C) the duration (h) of activity above the threshold (index of ≥ 35) measured by AAM (r = 0.83, $P < 0.01$).

3.3.2 Ovarian dynamics

We observed no difference between treatments regarding the pre-ovulatory follicle diameter (22.3 ± 0.8 mm vs. 21.3 ± 0.6 mm for over and under stocked cows respectively; P = 0.3), estradiol concentration (8.5 ± 0.4 pg/ml vs. 7.4 ± 0.6 pg/ml; P = 0.1) and progesterone concentration (0.8 ± 0.05 ng/ml vs. 0.8 ± 0.06 ng/ml; P = 0.9) at estrus. We noted a positive correlation for pre-ovulatory follicle diameter and estradiol concentration when analyzed across treatments (r = 0.3; P = 0.05).

3.3.3 Biomarkers of stress

Overstocked cows tended to have a higher haptoglobin concentration on PGF1 (study d 8; 1.9 ± 0.3 mg/ml vs. 1.4 ± 0.3 mg/ml for over- and understocked cows respectively; P = 0.7). We observed no differences between treatments later in the study (PGF2: 1.3 ± 0.2 mg/ml vs. 1.1 ± 0.1 mg/ml, PGF2+12: 1.2 ± 0.1 mg/ml vs. 1.2 ± 0.1 mg/ml for over- and understocked cows respectively). When haptoglobin concentration was compared across treatments over time we noted that across treatments haptoglobin concentration was greater on the day of PGF1 (study d 8) than on the day of PGF2 (study d 22) or at PGF2+12d (study d 34; Figure 3.3). When this was analyzed by treatment we noted that this was driven by the overstocked treatment (Figure 3.3).

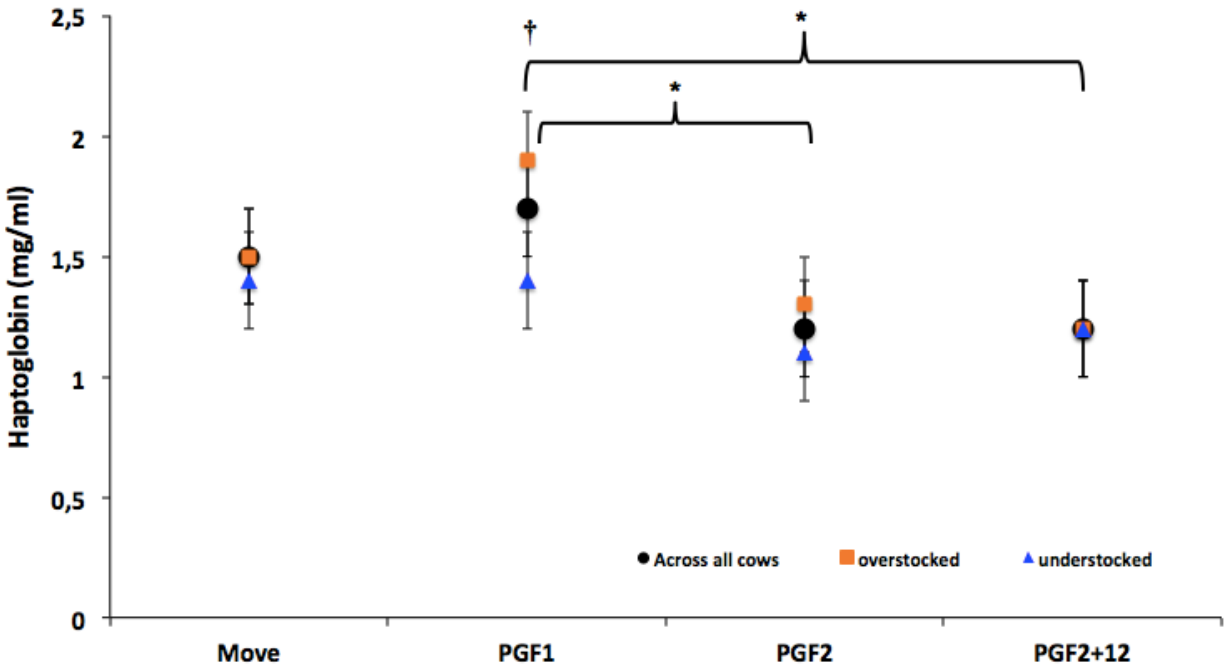


Figure 3.4: Mean Haptoglobin concentration across treatments and by stocking density. Mean haptoglobin concentration (LSM ± SE) across treatments (●), for over (■) and under (▲) stocked cows across time points (Move, PGF1, PGF2 and PGF2+12); * indicates a difference between treatment days across treatments and for overstocked cows with $P \leq 0.05$; † indicates a difference of $P \leq 0.1$ between treatments (over vs. under stocked cows).

There were no differences in fecal cortisol metabolites (FCM) between treatments and sampling days (Move: 32.4 ± 5.2 ng/g vs. 24.6 ± 3.3 ng/g, PGF1: 26.3 ± 3.8 ng/g vs. 29.1 ± 4.5 ng/g, PGF2: 24.1 ± 3.7 ng/g vs. 21.9 ± 3.0 ng/g, PGF2+12: 26.9 ± 5.7 ng/g vs. 26.9 ± 6.5 ng/g for over and under stocked cows respectively). Levels of FCM at PGF2 were positively correlated with those at PGF2+12d when correlated across treatments (r = 0.63; $P < 0.001$) as well as within both treatments separately (r = 0.69, $P < 0.01$ and r = 0.50, $P = 0.07$ for overstocked and understocked cows, respectively).

3.3.4 Milk production

On average primiparous cows had lower milk production than multiparous cows (34.1 ± 0.7 kg/d vs. 44.7 ± 0.6 kg/d for primiparous and multiparous, respectively; $P < 0.001$). Primiparous cows in overstocked conditions tended to produce less milk than those in the understocked treatment (32.9 ± 0.8 kg/d vs. 34.8 ± 0.7 kg/d for over and under stocked primiparous cows, respectively; $P < 0.1$). We observed no effect of treatment on milk production for multiparous cows.

3.4 Discussion

With this study we set out to investigate the effect of stocking density on automatic collected estrus activity data, ovarian parameters and biomarkers of stress. We hypothesised that overstocking would have a negative effect on ovarian parameters and increase biomarkers of stress. The AAM used in this study detected 60% of the 101 animals that were confirmed as having ovulated; however, treatment did not explain this discrepancy with cows not identified as having ovulated being balanced across both treatments. We speculate that the failure to detect animals in heat in both treatment groups was likely due to silent ovulations or ovulations with a reduced expression of behavioural estrus, a phenomenon that has increased over the past decades (Lucy, 2001). As the cows in this study were all just approaching the end of the voluntary waiting period it is also possible that some of the animals had only just returned to cyclicity and therefore a sub-optimal exposure to progesterone may have contributed to silent estrus events. However, several factors, such as milk production (Lopez et al., 2004), lameness (Morris et al., 2011) and mastitis (Huszenicza et al., 2005; Morris et al., 2013) have been identified as factors causing declines in behavioural estrus expression indicating that the influences on depression of behavioural expression are manifold.

A correlation of AAM data (duration of estrus, index, %-increase in activity and peak of activity) within and across stocking densities was not unexpected given that by design we only included cows that were detected by the AAM system. Madureira et al. (2015) have previously demonstrated a strong correlation between these activity parameters. However, we caution readers as to the interpretation of these types of results given that the activity measure of the specific logger used in this study is not fully understood. Future research should investigate, for example, the highest recorded index for cows not identified by the system as being in estrus, as a way to validate the pre-assigned AAM index threshold recommended by the supplier, which can be adjusted for each farm individually.

Contrary to our hypothesis we did not observe treatment differences for estradiol, progesterone or pre-ovulatory follicle size. Possible explanations that may explain this finding are: first, the high and low stocking densities were too short-lived and did not result in any physiological alterations; 2) the use of a pre-synchronization program overrode or masked any potential differences; or 3) dairy cattle are in general very resilient and are able to cope well under the influence of a single stressor. We did, however, observe positive correlations for pre-ovulatory follicle diameter with estradiol concentrations at estrus, %-increase in activity and with duration of high activity for overstocked cows. This finding is consistent with the observed linear relationship of follicle size and estradiol concentration in previous studies (Cerri et al., 2004; Perry et al., 2014b).

Haptoglobin, an acute phase protein, is typically used as a marker for inflammation, trauma and disease (Petersen et al., 2004). Its potential use as a biomarker of stress has previously been evaluated for beef steers after weaning and transportation (Arthington et al., 2008), for beef cattle at lairage at the slaughterhouse (Giannetto et al., 2011) and for dairy cattle

after exposure to complex stress (Lomborg et al., 2008). As reviewed by Petersen et al. (2004) the acute phase response is seen shortly after exposure to a triggering event and serum levels of acute phase proteins return to baseline levels when the triggering factor is no longer present. Thus we hypothesized that overstocked cows would have higher haptoglobin concentrations when compared to understocked cows. However, in our study we observed only a tendency for higher haptoglobin levels in overstocked cows on PGF1 (study d 8) and no further differences between treatments. Lomborg et al. (2008) detected an increase in haptoglobin after exposing the animals to a complex stressor composed of transport, solitude and slippery floors. Thus, given that our stressor was limited to a single factor (stocking density) it follows that our results are in agreement with Silva et al. (2016) and (Tarantola et al. (2016) who also found no differences in haptoglobin concentration when comparing 80% stocking density with 100% or moving cows from tie-stalls to free-stall barns. Arguably, 100% stocking density at the feed bunk is not high enough to cause an acute phase response, or that the change from tie-stall to free-stall was a positive change. However, it is also possible, that the animals adapted to the new situation and were therefore not in an acute situation of stress. This falls in line with the detected within treatment decrease in haptoglobin concentration for overstocked cows.

The measurement of cortisol as an indicator of stress is well established. After exposure to a stressor glucocorticoids (e.g. cortisol) are released almost immediately into the blood and are quickly metabolized and excreted via urine and feces (Palme, 2012). The hormone levels in fecal samples represent the cumulative secretion of hormones and are less affected by short episodic fluctuations, thus making them more useful for the detection of chronic stress (Palme, 2012). As previously described by Munksgaard and Simonsen (1996) lying deprivation can cause a change in blood cortisol concentration. Thus, we hypothesized that, in comparison to understocked

cows, overstocked cows would have higher levels of FCM as we expected them to be chronically stressed. Similar to the effect on Haptoglobin we observe no effect of stocking density on fecal cortisol metabolites. Most of the research on overstocking dairy cows has been conducted using dry dairy cows. Huzzey et al. (2012) analysed FCM from dry cows that were subjected to a 'normal' housing condition (stall to cow ratio = 1:1 and 0.67 m feed bunk space/ cow) and then to an overstocked housing situation (stall to cow ratio = 1:2 and 0.34 m feed bunk space/ cow) and observed only a tendency for elevated FCM during the overstocked period. More recently, and slightly different from Huzzey et al. (2012), Fujiwara et al., (2019) reported no effect of stocking density on FCM for cows in the dry period. Similar to our findings Krawczel et al. (2012) noted no effect of stocking density on FCM when simultaneously overcrowding for lying and feed bunk space during a 14-d-period. In summary it can be speculated, that cows are good at adapting or that FCM may not be ideal for the detection of housing related stressors in dairy cows. The implementation of a good coping strategy by the cows is supported by our observed positive correlation between FCM at PGF2 (study d 22) and FCM at PGF2+12d (study d 34).

The influence of parity on milk production has long been established (Waltner et al., 1993) therefore the fact that primiparous cows had lower milk production than multiparous animals was not unexpected. Neave et al. (2017) also reported dramatic differences in behaviour between primi- and multiparous cows. We did note that there was a tendency for overstocked primiparous cows to have reduced milk yield than under stocked primiparous cows. Similar to our findings Krawczel et al. (2012) reported that first-parity cows were more affected by overcrowding when housed together with older cows. This is in line with the reported benefits of primiparous cows with regard to space availability (Naess et al., 2011). Unfortunately, the reports on the effect overstocking on milk production from multiparous cows are inconsistent;

Bach et al. (2008) reported a decline in production when cows were housed in overcrowded conditions; whereas, (Bewley et al. (2001) failed to show any differences in milk production between stocking densities based on survey results. Overall the effect of stocking density on milk production is likely dependent in part on parity but also in part on the extent and duration of overstocking.

Our aim was to investigate the effect of overstocking lactating dairy cows on automatic estrus detection, follicle size, hormone concentrations (estradiol, progesterone), biomarkers of stress (haptoglobin, fecal cortisol metabolites) and milk production. However, our results showed no difference for most of the investigated parameters. In summary the results from this study show only mild effects of short-term exposure of lactating dairy cows to a high stocking density (133%) and highlight how robust dairy cows are to short-term sub-optimal housing conditions.

Chapter 4: Lying behaviour and behavioural expression of estrus in dairy cows vary with stocking densities

4.1 Introduction

Over the past decades the total number of dairy farms has been declining while the number of animals per farm has increased (USDA 2008), bringing changes in the housing and management as well as production and fertility of dairy cows (Ferguson and Skidmore, 2013; Barkema et al., 2015). The observed increase in production of individual animals has been associated with a decline in fertility (Butler, 1998; Walsh et al., 2011). It is thus not surprising that research on dairy cattle estrous behaviour, including automated methods of assessing this, have been the focus of research efforts (Kiddy, 1977; Nebel et al., 2000). Also, over the past two decades, welfare has also become more of a focus in dairy cattle research (von Keyserlingk and Weary, 2017), and has been identified as a top priority issue by stakeholders in the dairy industry (Bauman et al., 2016). Measures of standing and lying behaviour can be relevant to cow comfort, one component of cow animal welfare; these measures are now widely used in dairy research (Tucker et al., in press). For example, measures of lying behaviour have been used in studies on a variety of research questions, including lameness (Ito et al., 2010) and more general illness (Siivonen et al., 2011).

The Canadian Dairy Code of Practice (National Farm Animal Care Council, 2009) recommends a cow-to-stall ratio of 1:1 and 0.6 m linear feed bunk space per cow for dairy cows housed in freestall systems. Research has shown that a higher stocking rates for free stalls can cause reductions in lying behaviour (Fregonesi et al., 2007; Krawczel et al., 2012). Likewise, research on feeding behaviour has shown that providing less than 0.6 m of linear feed bunk space

per cow increases competition, causing profound changes in feeding and social behaviour (Huzzey et al., 2006; Proudfoot et al., 2009b), particularly after fresh feed delivery (DeVries et al., 2003). The differing effects of stocking density on dairy cow behaviour in relation to key resources (feedbunk; Huzzey et al., 2006; lying stalls; González et al., 2008a; lying stalls; Winckler et al., 2015) have been investigated. Collectively these authors concluded that overstocking can compromise welfare. For example, overstocking lying stalls reduces the time that cows spend lying down, and shifts lying to less favourable times when lying space becomes available (Fregonesi et al., 2007). The majority of research to date has focused on dry cows; little to no work has been done on the effects of overstocking on lactating cows. These animals are perhaps especially important to consider as these animals may be reproductively active, and any changes in standing behaviour may also affect estrus expression.

Estrus includes primary and secondary behaviours. The primary behaviour is 'standing to be mounted' and refers to a cow in estrus allowing herself to be mounted by others. There are also numerous secondary behaviours, including restlessness and mounting of other cows. The tracking of these secondary behaviours can provide an early and reliable identification of when dairy cows are approaching estrus (Roelofs et al., 2010). Although historically visual detection of behavioural estrus was used, it has become increasingly difficult (Roelofs et al., 2010) due, in part, to the primary behaviour - standing to be mounted - being the least frequent behaviour (Reith and Hoy, 2018). Thus there is growing interest in tracking secondary behaviours, in particular changes in 'restlessness' (Reith and Hoy, 2018); it is these changes in activity that are assessed by automated activity monitoring technologies (see review by Roelofs and van Erp-van der Kooij, 2015).

To our knowledge no research has directly investigated the effect of stocking density on general changes in standing behaviour including standing related to estrous. The objectives of the current study were to determine the effects of over or under stocking at the lying stall and at the feed bunk on standing and lying behaviour and on estrous expression as characterised by changes in standing behaviour. We expected that overstocking would increase standing times and negatively affect standing behaviour around estrus.

4.2 Methods

This study used data generated by the study presented in Chapter 3. Housing, management, study design and procedures are briefly described here and methods pertaining specifically to this study are added where appropriate.

4.2.1 Animals, Management and Housing

A total of 147 lactating Holstein dairy cows (parity 2.5 ± 1.3; mean ± SD) were enrolled in this study. Cows were housed in a freestall barn and study animals were assigned to one of two stocking densities: 67% (understocked; n = 73) or 133% (overstocked; n = 74). In total, four study pens of 14.4 m length and 13.5 m width each were used simultaneously, with 2 pens assigned to each of the stocking density treatments, balanced for parity. Each pen was equipped with 24 deep sand bedded lying stalls and 2 self-filling water troughs, one on either end of the pen. Lying stalls were set up in three rows and cleaned twice daily, at the time of milking. Each pen had two alleys (front and back) and two crossovers from the front to the back to allow for cows to get away from other animals and to avoid being cornered (Figure 4.1). Alleys were cleaned with automatic scrapers 6 times a day and crossovers were manually cleaned twice daily while cows were outside of the pen being milked.

There were 16 cows and 32 cows in the 67% and 133% stocking density pens, respectively. This equated to a cow to stall ratio of 2:3 in the understocked pens and 4:3 in the overstocked pens, resulting in an available walking area of 7.5 m^2 and 3.25 m^2 for under- and overstocked cows, respectively. The feed bunk was accessible via a post-and-rail-barrier (see Huzzey et al., 2006 for description) and available feed bunk space/cow was 0.9 m and 0.45 m for the understocked and overstocked pens, respectively. Group size was static while group composition was dynamic with simultaneous introduction of new animals and removal of others into the pens taking place once a week.

Cows were milked and fed twice a day at approx. 0630 h and 1600 h. Cows were fed a TMR consisting of corn silage, grass silage, mineral and concentrate mix and alfalfa hay. Cows were milked in a double 12 side-by-side milking parlour (BouMatic Dairy Equipment Co., Madison, WI). Milk production was automatically recorded at every milking and AM and PM recordings were summarized for daily milk production.

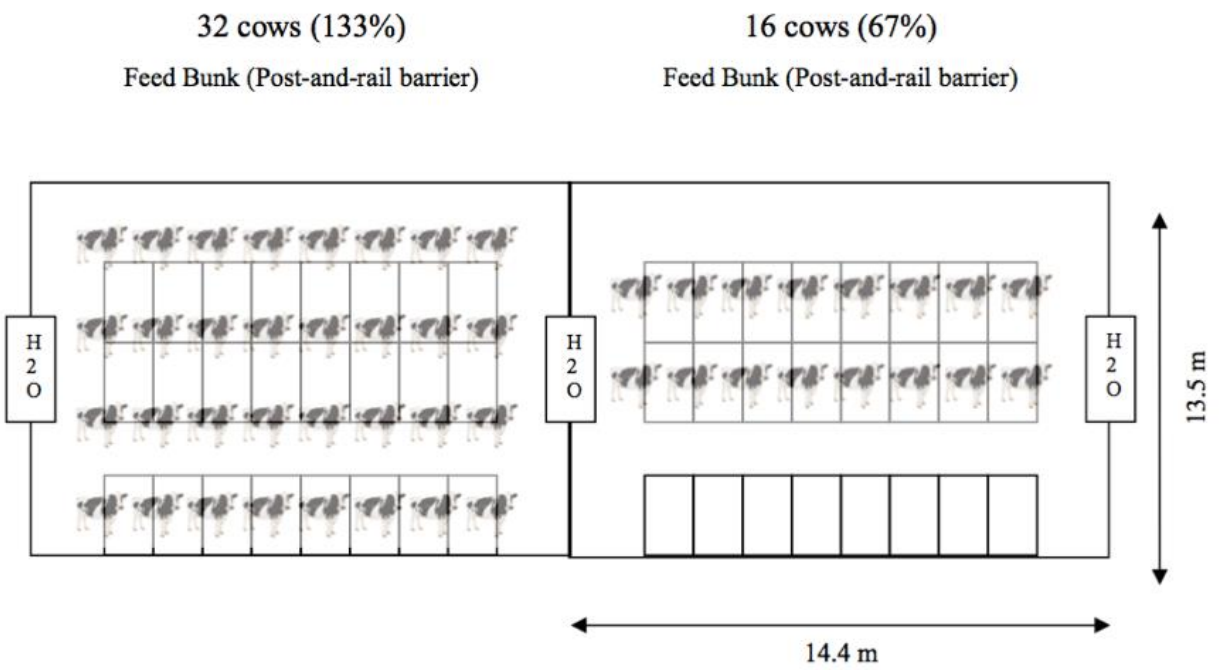


Figure 4.1: Illustration of the two stocking densities.
Illustration of pen design and dimensions of overstocked and understocked treatments.

4.2.2 Experimental design and estrus synchronisation protocol

Animals were enrolled and moved into the study pens at 40 ± 3 DIM and monitored for 34 d; thus, leaving the pen no earlier than 74 ± 3 DIM. Once a week new cows were enrolled and moved into the study pens with the simultaneous removal of an equal number of cows that had the highest DIM; thereby, maintaining stocking density. Approximately half of the cows in each study pen were lactating 'filler' cows (i.e. used to maintain density but not monitored); these cows were either confirmed pregnant or had already been bred.

Before being enrolled into the study all cows underwent a basic health check by a veterinarian, including locomotion scoring using a 1 (sound) to 5 (severely lame) scale (see Flower and Weary 2006); to ensure that the animals were both healthy and sound. All cows received a synchronization protocol consisting of two i.m. injections of $PGF_{2\alpha}$ (**PGF**; Estrumate,

Cloprostenol sodium, Intervet Canada Corp., Kirkland, QC, Canada) 14 d apart. The injections were given on study d 8 and d 22 (47 ± 3 DIM, **PGF1;** and at 61 ± 3 DIM, **PGF2**).

4.2.3 Ultrasonography

To determine cyclicity, stage of cycle and estrus, all cows were subject to *per rectum* palpation and examination by ultrasound (Ibex pro, E.I. Medical Imaging, Loveland, CO, USA) using a 7.5 MHz rectal linear array transducer. Cows were examined on study d 1, d 8 (PGF1), d 22 (PGF2) and 24 to 27 and 34 (d 2 to 5 and d 12 following the second PGF injection).

4.2.4 Standing and lying behaviour

To assess individual standing and lying times each cow was fitted with a data logger (Hobo Pendant G, Onset, Cape Cod, Massachusetts, USA) that has previously been validated for this purpose (Ledgerwood et al., 2010). The logger was attached to one of the animal's rear legs and programmed to record position of the cow once a minute. These measurements were used to calculate hourly and daily standing and lying times as well as average duration of standing and lying bouts, frequency of daily standing bouts and duration of the longest daily standing and lying bout.

4.2.5 Automated Activity Monitor (AAM)

An activity monitoring system (AAM; Heatime, SCR Engineers Ltd., Israel) was used to monitor individual cow activity. The logger was mounted on a neck-collar and a wireless antenna in the barn ensured automatic data transfer in 2-h intervals from the collar to the computer. The AAM collected activity data at a 2-min resolution and stored it in 2-h cells. The loggers' internal algorithm was used to detect animals in heat by comparing individual current activity with a previously recorded baseline and using the standard deviation (**SD**) to calculate an

activity index. The threshold for the activity index, used to create an automatic alert for high activity indicating a cow in heat, was pre=set to 35 by the manufacturer as reported previously (e.g. Madureira et al., 2015b; Burnett et al., 2017).

4.2.6 Gait Scoring

All cows were gait scored by a veterinarian on d 1, 8, 22 and 34, using a score from 1 to 5 (where 1 = sound and 5 = severely lame; Flower and Weary, 2006).

4.2.7 Statistical analyses

A total of 147 cows were enrolled in the study. Due to severe lameness (at least one gait score of ≥ 4), 6 cows were excluded from the analysis (3 from each treatment). Data from all other cows were used to assess general differences in standing and lying behaviour between treatments. For analysis of treatment differences in standing and lying behaviour as estrous expression, only cows with confirmed ovulation (via ultrasonography) that were also detected by an AAM were included in the analysis. All analyses for this experiment were performed using SAS (version 9.4; SAS Inst. Inc., Cary, NC) using cow nested within pen as the experimental unit. Before analyses data were checked for normality using the univariate procedure and probability distribution plots. Normality was visually assessed and confirmed by the Kolmogorov-Smirnov method. Continuous variables were analyzed using the mixed procedure of SAS, with cow nested within pen as the random error. An autoregressive covariance structure and repeated measures were used when multiple experimental days were included. Standing behaviour was first assessed over a period of 20 d to detect differences between stocking densities and then further analyzed within stocking density by testing data from the days around estrus (d -2, -1, 0, 1, and 2 relative to estrus) against a baseline value (consisting of an average

over 5 d) using contrast statements. Differences $P \leq 0.05$ were considered significant and differences from $0.05 > P < 0.10$ were designated as tendency.

4.3 Results

4.3.1 General effect of stocking density on lying behaviour

Over the 20-d duration of this study the overstocked cows spent on average 1.2 h (10%) more time standing per day and had nearly 13% fewer standing bouts per day than the understocked cows. Overstocked cows increased their average standing bout duration by approximately 20 min (i.e. by about 33%) and the longest standing bout of the day by 17 min (8.5%), compared to understocked cows (Figure 4.2). The diurnal pattern for standing time shown in Figure 4.3 helps to visualize differences in standing times between the two stocking densities.

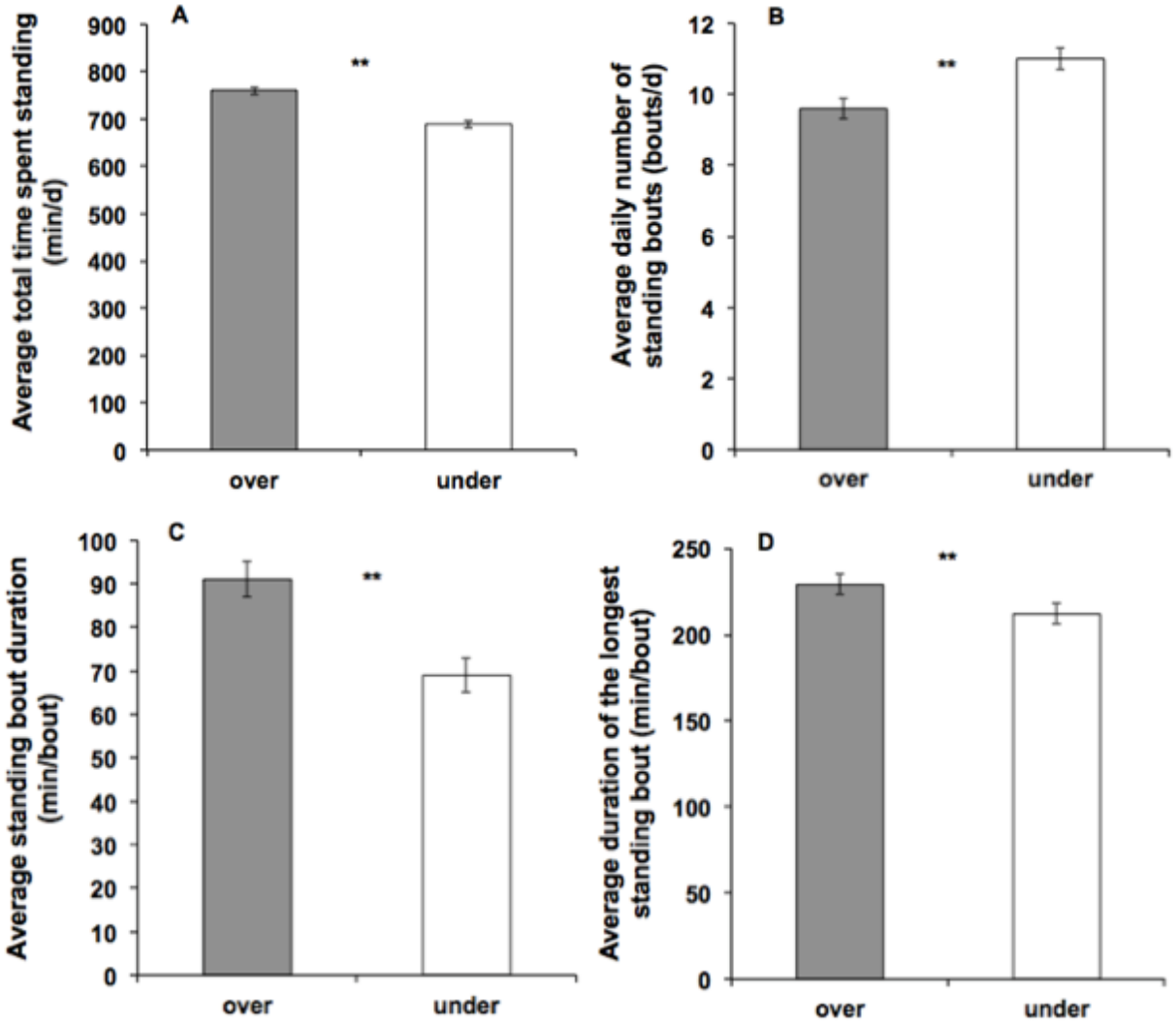


Figure 4.2: Standing behaviour by stocking density.

Average (LSM ± SE) daily standing time (A), number of standing bouts (B), standing bout duration (C) and duration of the longest daily standing bout (D) for over- and under-stocked dairy cows.

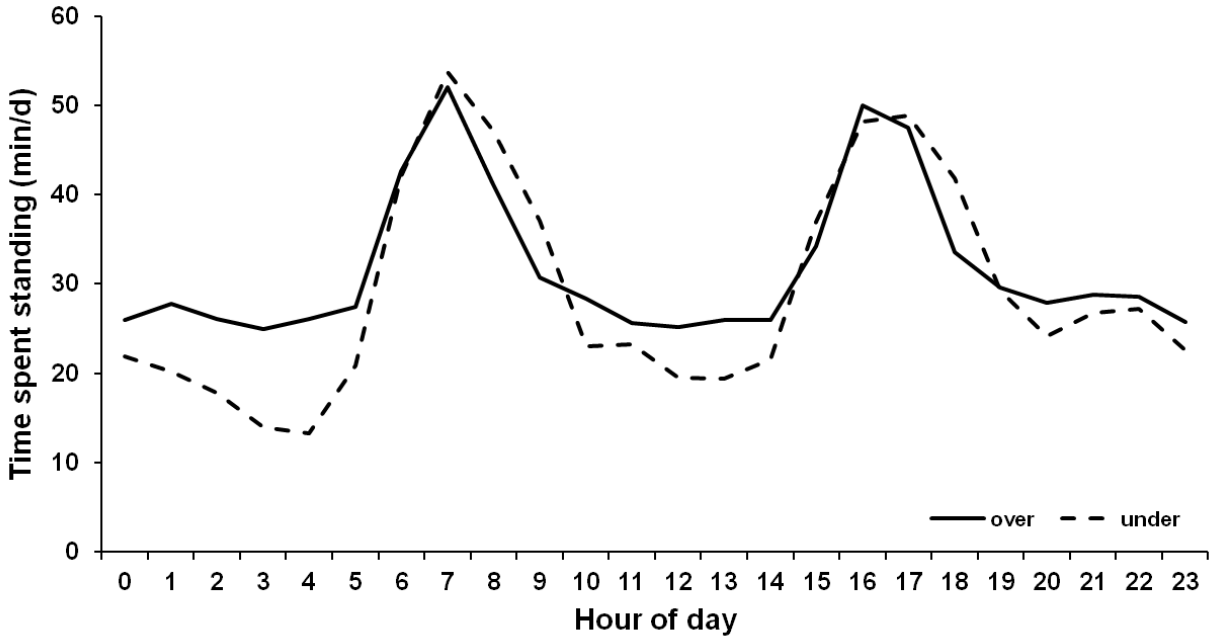


Figure 4.3: Diurnal pattern of time spent standing for over- and under-stocked dairy cows. Values are means calculated over the 20-d study period. Cows were fed and milked at approximately 0630 h and 1600 h.

4.3.2 Estrus related changes in lying behaviour and the effect of stocking density

Overstocked cows. Within treatment, when compared to a baseline value, overstocked cows spent on average 2.5 h more time standing on the day of estrus ($P < 0.001$; Figure 4.4). Average daily standing bout duration and duration of the longest standing bout of the day were higher on the day of estrus then during baseline or the day after estrus (average bout duration: baseline = 86 ± 1 min/bout, estrus = 121 ± 11 min/bout, d 1 after estrus = 85 ± 1 min/bout; longest bout of the day: baseline = 209 ± 21 min/longest standing bout, estrus = 351 ± 22 min/longest standing bout, d 1 after estrus = 230 ± 22 min/longest standing bout; $P < 0.01$ in all cases). Overstocked cows increased their longest lying bout of the day on the day after estrus when compared to the day of estrus (estrus = 139 ± 9 and d 1 after estrus = 153 ± 9 min/longest lying bout; $P < 0.05$), but we noted no difference when compared to the baseline.

Understocked cows. Compared to their baseline, understocked cows increased standing time on the day before estrus by 1.4 h ($P < 0.01$) and by another 1 h (to a total of 2.4 h) on the day of estrus ($P < 0.001$; Figure 4.4). Understocked cows had fewer standing bouts on the day of estrus (8.8 ± 0.7 bouts/d) than during baseline (11.0 ± 0.7 bouts/d; $P = 0.01$) or the day after estrus (10.0 ± 0.7; $P = 0.05$). Average standing bout duration was higher on the day of estrus (104 ± 6 min/bout) than during baseline (68 ± 5 min/bout; $P < 0.001$) or on the day after estrus (68 ± 1 min/bout; $P < 0.001$). Compared to baseline (208 ± 16 min/longest standing bout) understocked cows tended to increase their longest standing bout on the day before estrus (250 ± 18 min/longest standing bout; $P = 0.08$) and increased the longest standing bout on the day of estrus (343 ± 18 min/longest standing bout; $P < 0.001$). The duration of the longest daily standing bout on the day of estrus was higher than on the day before estrus ($P < 0.001$) and the day after estrus (212 ± 18 min/longest standing bout; $P < 0.001$).

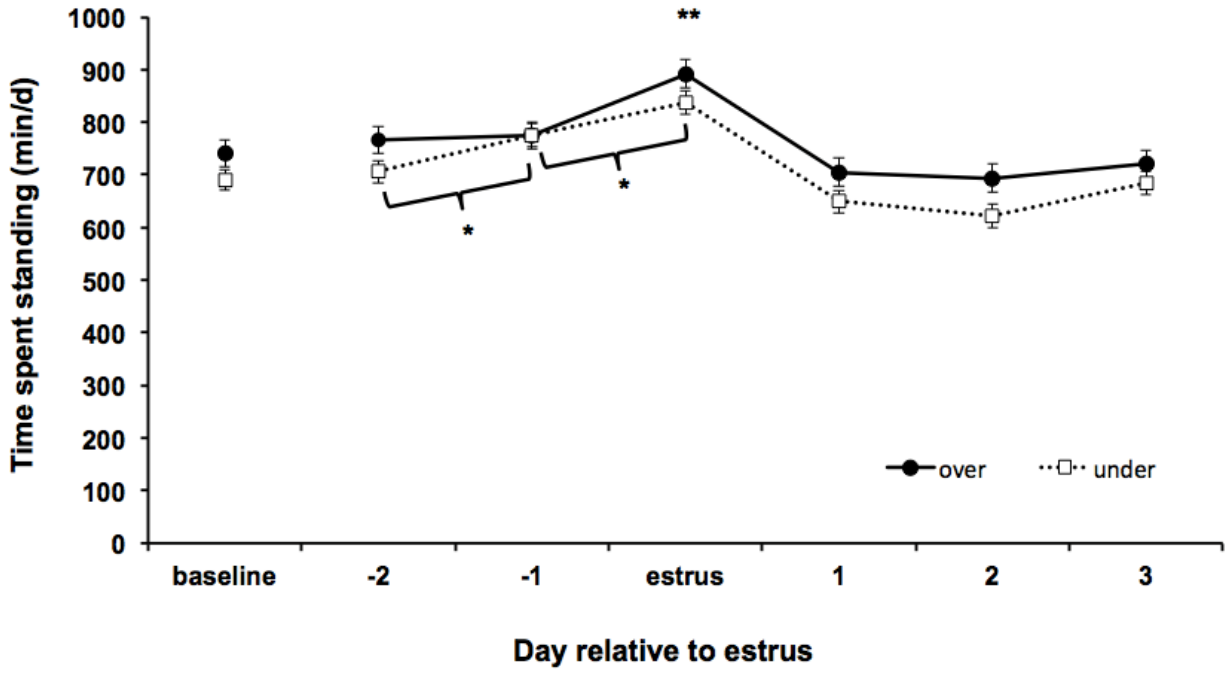


Figure 4.4: Time spent standing relative to estrus and stocking density.
Average (LSM ± SE) daily time spent standing relative to estrus for over- and under-stocked dairy cows.
** Within treatment differences for all days, except d-1 for understocked cows, compared to estrus $P \leq 0.01$; *****indicates a difference of $P \leq 0.05$ for understocked cows between d-2 and d-1 as well as d-1 and estrus.

4.4 Discussion

Standing and lying behaviour of dairy cattle are widely used to assess the effect of housing conditions and illness (e.g. Hill et al., 2009; Neave et al., 2018; Solano et al., 2016; Tucker et al, in press). In this study, we used standing behaviour to investigate the effect of stocking density on standing time and detected an average increase of more than 1h in daily standing time for overstocked cows, which confirmed our hypothesis that overstocking would increase standing times. Furthermore, we showed that this effect of stocking density was fairly uniform across different times of day. However, the diurnal lying behaviour pattern does indicate

that overstocked cows appeared to be much faster to seek and secure a lying stall after milking and fresh feed delivery than cows housed in pens with enough available lying stalls. These finding are consistent to those of Fregonesi et al. (2007) who reported that cows housed at 150% (in relation to lying stalls) laid down about 13 min sooner after milking than cows housed at 100%, with some cows foregoing the opportunity to feed in order to secure a lying space. Similarly, Cooper et al. (2007) found that after being deprived of lying, cows would spend less time feeding in order to lie down. Collings et al. (2011) investigated the effect of overcrowding at the feed bunk while maintaining a 1:1 cow:stall ratio and found no effect of on daily lying time. We thus conclude that the increased standing times in the present study were due primarily to the limited availability for lying stalls in the 133% treatment and not to overstocking at the feedbunk. Taken together with the reduced latency to lie down after milking, it is apparent that at least some of the overstocked cows in our study preferred to lie down rather than feed.

Our findings on standing time are also in agreement with other work on the effects of overstocking, all of which report reductions in standing time with overstocking (Krawczel et al., 2012; Telezhenko et al., 2012; Winckler et al., 2015). However, in contrast to our study, Krawczel et al. (2012) and Telezhenko et al. (2012) found no differences in numbers of lying bouts. This difference may be due to the types of overstocking treatments investigated or duration of treatment. For example, although Telezhenko et al. (2012) looked at a range of stocking densities, the highest was 100%, (such that every cow had access to a lying stall). Krawczel et al. (2012) considered stocking densities similar to those used in the current study, but so for a shorter duration; cows were overstocked for 14 d and standing times were recorded over a 5-d period. In contrast, we applied stocking density treatments for 34 d; allowing for a 14 d habituation period followed by a 20-d recording period. Furthermore, in contrast to our

dynamic group structure, Krawczel et al. (2012) monitored a stable group of cows. Also, they blocked lying stalls and feed bunk space to increase the stocking densities, whereas we put more cows into the overstocked pens thereby decreasing space per cow (mimicing what happens on commercial farms). The number of times cows are milked can also affect behaviour (Hart et al., 2013); in the Krawczel et al. (2012) study cows were milked three times per day whereas the cows in our study were milked twice. It is possible that cows in the overstocked treatment, that otherwise faced difficulties in accessing a lying stall, lay down immediately upon return from milking; thus an increase in daily milking frequency would increase the chance of cows gaining access to a lying stall and likely also affect the standing bout duration.

Previous research has shown that reduced feeding space results in increased time spent standing idle while waiting for access to feed, thereby increasing daily standing time (Olofsson, 1999; Huzzey et al., 2006). Increasing standing times can be detrimental for claw and leg health and put cows at risk for developing lameness (Cook and Nordlund, 2009; Proudfoot et al., 2010). We therefore speculate that permanent overstocking could lead to a higher risk of lameness.

As expected and in agreement with previous work (Dolecheck et al., 2015; Silper et al., 2015), cows increased average daily standing time on the day of estrus most likely reflecting increased restlessness' as cows approached estrus (Roelofs et al., 2010). Interestingly, cows in the understocked treatment had an initial increase in their daily standing time on the day before estrus but then showed a further increase in standing time on the day of estrus; this finding was not observed in the overstocked cows. This notable increase in standing time on the day before and the day of estrus in the understocked pens is worthy of future research, as this more pronounced behavioural response may facilitate the detection of estrous.

Brehme et al. (2008) reported that cows with a silent estrus episode still showed an increase in standing time, despite a lack of increase in activity. In our case the difference in onset of increased standing time can be interpreted as an indication that, when given the opportunity, cows start to show interest in their surroundings before the onset of estrus.

Our finding that the duration of the average lying bout length was longest the day after estrus may reflect the cows need to spent more time resting after the increased activity on the day of estrus. This need to rest the day after estrus is perhaps another indication that resting is of high priority for cows.

In summary, the results from this study indicate that even a short-term increase in stocking density to 133% (cow to stall ratio of 4:3 can have detrimental effects on the standing behaviour of healthy lactating dairy cattle. Our results also show that individual standing times can be an indicator for the onset of estrus, particularly in understocked cows.

Chapter 5: General discussion

5.1 Findings, implications and future research

The research summarized in my thesis focuses on the interactions between reproduction, health and farm management, with a special focus on the common practice of over stocking. More specifically, I set out to investigate dairy cows' behaviour and estrus expression in relation to illness and management decisions. Where possible, I included the use of precision technologies to monitor the behaviour of dairy cows. Thus, in addition to contributing to the disciplines of dairy cow health, reproduction, ethology and farm management, this thesis also contributes to the growing body of work on precision farming technologies. This latter contribution specifically set out to improve our understanding of how the use of automated measures of behavioural changes can potentially facilitate the early detection of disease (see Foris et al., 2020) and estrus (i.e. Burnett et al.,2017) .

In Chapter 1, I reviewed the literature to convey to the reader an understanding of the natural estrus cycle of the dairy cow, including hormonal and behavioural changes and reasons for anestrus. Given my training as a veterinarian, I was particularly interested in improving our knowledge on the topic of identifying cows at risk for illness and to include work on understanding how management impacts lameness. My review of the literature identified the negative effects of postpartum illness on the resumption of the estrus cycle and quality of the follicles. It is clear that timely detection and intervention – with the goal of either circumventing the illness event all together or shortening the number of days a cow is sick - is the key to minimizing the negative health and welfare consequences that affect the individual cow. Additionally, in this first chapter I presented an overview of the effect of direct and indirect human intervention on identifying cows in estrus. While reviewing this body of literature I noted

that the research focus is largely in the area of what type of hormonal breeding protocols can mitigate the fertility issues, and how implementation of these pre-planned schedules allows for the elimination of the need to visually observe estrus behaviour. Although my research described in this thesis (Chapters 3 and 4) has included use of these timed artificial insemination protocols, I was also interested in how the use of precision technologies could minimize reliance on these timed artificial insemination protocols given the indications that they may not be accepted by the public in the future (Pieper et al., 2016).

In Chapter 2, my first aim was to describe how time spent ruminating and other measures of feeding behaviour (DMI, time spent feeding, feeding rate and visits to the feed bin) collected using validated technologies (Chapinal et al., 2007; Schirmann et al., 2009) change in cows in the weeks before and after parturition. My second aim was to determine if there was a relationship between postpartum disease and rumination behaviour before and after calving. Specifically, I set out to investigate if there are behavioural differences between cows that got sick during the postpartum period and those that stayed healthy during the postpartum period. This work is important given that approximately 35% of dairy cows succumb to illness around the time of calving (LeBlanc, 2010). For the analyses I focused on data collected beginning one week before calving until 21 d postpartum and then retrospectively assigned cows by health status (n=64) as either healthy (n = 20), metritic (n = 18), subclinically ketotic (SCK; n = 9), having both of these diseases (metritis+SCK, n = 9) or a combination of either metritis or SCK or both plus another unrelated disorder (e.g. mastitis and SCK or milk fever and metritis; MULT, n = 8). Despite my predictions, only changes in rumination behaviour proved to be sensitive for identifying cows that succumbed to SCK; cows that developed SCK postpartum spent less time ruminating during the week prior to calving. Most interesting was that the diurnal pattern of

when rumination peaked did not change; cows simply spent less time ruminating. My findings are not consistent with some earlier work (Calamari et al., 2014; Liboreiro et al., 2015), but are in agreement with the work of Kaufman et al. (2016). Reasons for these differences among studies may have do to with how animals were grouped in different studies; for example, in my study cows were grouped by health status while Calamari et al. (2014) grouped by high and low rumination times on specific days. The discrepancies between the studies suggest that topic is complex and that further work is required to elucidate factors that affect feeding behaviour, including rumination. My work does contribute to the body of literature indicating that there is potential to detect cows at risk for SCK using rumination pre-partum. However, before this approach can be implemented into everyday management practices, future research must address what thresholds to implement into automatic alert systems for farmers. Whether these thresholds should be based on an individual animals' change from one day to the next, or summarized using a rolling average based on multiple days or weeks will also need to be elucidated.

The findings that arose out of this research with regard to feeding behaviour were in line with other reported research, showing differences in DMI between healthy and sick cows in the periods before and after calving (i.e. Huzzey et al., 2007; Goldhawk et al., 2009). For the pre-partum period, the difference was only detected for SCK and SCK+Met cows, but post-partum all sick groups had a lower DMI than did their healthy counterparts. While these results for feeding behaviour were as expected, the technology that allows the monitoring of individual feeding behaviour (including DMI) is still not readily available for commercial farms, so from a practical on-farm perspective the use of individual rumination behaviour appears to be far more practical and promising.

The objective of Chapter 3 was to investigate the effect of stocking density on detailed measurements of estrous expression (physical activity), ovarian dynamics (pre-ovulatory follicle diameter and concentrations of plasma estradiol and progesterone at estrus) and concomitant association with biomarkers of stress (fecal cortisol and plasma haptoglobin concentration). We hypothesized that overstocking would negatively affect estrous expression, result in smaller pre-ovulatory follicles and negatively impact estradiol synthesis. Additionally, we expected overstocked cows to have higher concentrations of haptoglobin and fecal cortisol metabolites. To create the desired stocking densities, we used identical pens with 24 lying stalls with either 16 cows (i.e. understocked with a cow to stall ratio of 2:3 or 67%) or 32 cows (i.e. overstocked with a cow to stall ratio of 4:3 or 133%); this treatment also resulted in differences in feed bunk space availability per cow (0.9 m vs. 0.45 m) and available pen space per cow ($7.5m^2$ vs. 3.25 m^2). This approach to overstocking pens mirrors the approach on commercial farms (see von Keyserlingk et al., 2012). Although the current Code of Practice on the Care and Handling of Dairy Cattle (NFACC 2009) recommends a maximum of 120% stall stocking density, this is not always followed; Charlton et al. (2014) reported that stocking ranges from 52% to 160% on Canadian farms. Thus the findings of my work are relevant to Canadian dairy farms. That said, in some previous studies overstocked situations were created by blocking off available resources (e.g. lying stalls or feed bunk space; Huzzey et al., 2006; Krawczel et al., 2012), and keeping the number of cows constant; this design avoids the obvious confound of group size, but my design has the advantage of more closely relating to real practices on farms.

Chapters 3 and 4 are based on the same study but address different objectives. My aim in Chapter 3 was to investigate the effect of overstocking on automatic estrus detection, follicle size, hormone concentrations (estradiol, progesterone), biomarkers of stress (haptoglobin, fecal

cortisol metabolites) and milk production. Although we did observe positive correlations for pre-ovulatory follicle diameter with the following parameters - estradiol concentrations at estrus, increase in activity (%) and duration of high activity for overstocked cows - we were only able to show a tendency for increased haptoglobin but surprisingly found no difference in fecal cortisol. I also hypothesized that, in comparison to understocked cows, overstocked cows would have higher levels of fat corrected milk but again found only a tendency was observed. Indeed, when taken together these results suggest that dairy cows are reasonably resilient to short-term sub-optimal housing conditions, which may explain the lack of noticeable effects on the feeding behaviour measures and milk production, and explain why many farms continue to overstock.

Chapter 4 is, to my knowledge, the first study to investigate the effects of stocking density on standing behaviour in relation to estrous. My prediction was that overstocking would increase standing times and negatively affect standing behaviour around estrus. The results indicate that even a short-term increase in stocking density to 133% can have detrimental effects on the standing behaviour, and that individual standing times can be an indicator for the onset of estrus, particularly in understocked cows. Indeed to my knowledge this is the first study to show these effects of stocking density on standing behaviour in relation to estrus behaviour. Although my study was not powered to investigate effects on lameness of increased standing times, other research has shown that increased standing times can put cows at risk, especially given that for the most part cows did not have a dry place to stand (other than in the stall). More research that looks at the interactions between standing time, lameness, reproductive behaviour and cow factors such as parity and previous lameness cases is needed. This type of work may be facilitated by the move towards precision dairy farming that allows for better monitoring of cows at the individual level.

Additional limitations to this thesis:

There are a number of limitations to this thesis. One limitation would be the small number of sick cows in Chapter 2. Although I did a sample size analyses based on a difference of 45 minutes in time spent ruminating (determined from reference values in the literature), when combined with today's knowledge on within-cow between days and between cow differences (Beauchemin, 2018), it is now clear that I underestimated this difference and underpowered the study. Also, the use of the synchronisation protocols on the cows in the work on stocking density (Chapters 3 and 4) was likely not ideal given that one of my research questions were very focused on behaviour (Chapter 4)– especially when considering the very recent research that these protocols potentially alter the behavioural estrus expression (Schweinzer et al., 2020). In hindsight, while the use of the synchronization protocols allowed us to increase the probability of an estrus episode within a certain time frame, they may also have lowered the behavioural response. Lastly, it should not be overlooked that all of the empirical research described in this thesis took place at the UBC Dairy Education and Research Centre. This university farm is designed and managed in such a way as to facilitate interventions (such as over and understocking) and measures (such as those on rumination and feeding behaviour) that would be difficult or impossible on most commercial farms; therefore, readers should be cautious in generalizing the results to other farms in other regions.

5.2 Final thoughts through the lens of my first degree – veterinary medicine

My thesis contributes to knowledge on dairy cow behaviour, and especially on how certain behaviours are altered by illness, reproductive status and housing conditions. That dairy cows continue to suffer from high rates of illness (e.g. postpartum disease), and that management practices (e.g. overstocking) potentially harmful to cows are still common, concerns me in my

role as a veterinarian. I had hoped during the course of my dissertation to provide greater insights into how to identify cows at risk of disease and to improve our understanding of the effects of overstocking on both reproduction and behaviour, my results allow only limited inferences. The results of Chapter 3 especially indicate that dairy cows are reasonably resilient to at least the short-term effects of overstocking. That said the findings described in Chapter 4 are the first to show that standing and lying behaviours are affected, including in ways that may impact the detection of when cows are in heat.

As a veterinarian, I was trained to place value on biological functioning and health, one of the three commonly recognized components of animal welfare (see Fraser et al., 1997). During the journey of doing the research included in this dissertation, which included moving to Canada to do a Ph.D. in conjunction with the UBC reproduction team and the UBC Animal Welfare Program, I have come to realize that providing a dairy cow a good life must also consider the other components – including the ability to engage in motivated natural behaviours like estrus expression, and to avoid unpleasant affective states like the pain associated with lameness and other ailments. I have also become interested in the profound individual differences between cows, including why some cows adapt to change and stay healthy while others fall ill; understanding these individual differences is an important area for future research. I hope my work may motivate other veterinarians to consider research in cattle welfare, and to consider some of the topics that have emerged from the research I have described in this thesis, by combining their expertise in the assessment of biological functioning and health with other measures related to cow welfare.

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