CHARACTERISTICS OF ESTROUS BEHAVIOUR IN HEIFERS AND LACTATING COWS AND ITS ASSOCIATIONS WITH FERTILITY

by

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Abstract

Detection of estrus in dairy cows is challenging, partly because of poor behavioural expression. Automated activity monitors allow quantification of estrus expression based on restlessness. The main goals of this thesis were to use automated measurements and visual observation of behaviour to increase understanding of estrus characteristics, variation among animals, risk factors for poor expression, and its association with fertility. In the first study, the behaviour of heifers was video-recorded and activity peaks were identified from accelerometer data; estrus was validated by ovarian ultrasonography. Chin rest, sniff, back mount, crossover, and follow had the largest increase in frequency during estrus. Estrus relative increase in walking activity (290 ± 160%) and duration (14 ± 4 h) varied greatly and were affected by estrus order, season and time of the day. The second study investigated how estrus affected automated measurements of lying and standing behaviour, a less explored aspect of estrus. At estrus, bout frequency was lower, daily standing time was greater, and heifers stood uninterruptedly for twice longer than at baseline. Relative changes in standing behaviour at estrus were smaller for estrus starting between 1200 h and 0300 h. The third experiment investigated the agreement between estrus characteristics in heifers fitted with two accelerometers. Both systems were precise (PPV = 84.7% [Heatime] and 98.7% [IceTag]) and provided similar characterization and timing. Plasma estradiol was not correlated with follicle diameter, duration, intensity, or presence of estrus signs. Finally, estrus lying behaviour of lactating cows and its associations with fertility were studied. Daily lying time and bout frequency were reduced at estrus (65 ± 21% and 65 ± 24% of baseline). Ovulation and pregnancy at d 32 after AI were 4.9 and 1.6 times more likely if estrus lying time was < 75% of baseline. Collectively, results suggest potential application of lying behaviour towards fertility prediction. We have also highlighted features such as variability, risk factors and basal activity that can contribute to assessment of methods and practices for increased expression. Additional future directions include investigation of physiological bases of estrus-fertility association and real-time applications of characterization data.

Preface

The research presented in this thesis was performed at the University of British Columbia's Dairy Education and Research Centre (Agassiz, BC, Canada) and at a commercial dairy farm located in Araras (SP, Brazil). The research done at the University of British Columbia (Chapters 2 and 3) was approved by the local Institutional Animal Care Committee (A14-0019) and followed the requirements of the Canadian Animal Care Council (CCAC, 2009). Research done at the commercial dairy farm in Brazil (Chapter 4) followed the requirements and practices of the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999). Publication details are presented below; similar information has been included at the start of Sections 2.1 and 2.2, and Chapters 3 and 4. Throughout this thesis, I have employed the first person singular to express my personal opinions and assumptions. Alternatively, I have used the first person plural when referring to co-authored research.

The content of Chapter 2 resulted in two publications:

- *B.F. Silper, I. Robles, A.M.L. Madureira, T.A. Burnett, M.M. Reis, A.M. de Passillé, J. Rushen, R.L.A. Cerri. 2015. Automated and visual measurements of estrous behavior and their sources of variation in Holstein heifers. I: Walking activity and behavior frequency. Theriogenology. 84:312-320.* Ideas for this manuscript were developed by Silper, de Passillé, Rushen, and Cerri. Writing was done by Silper under supervision of Rushen, de Passillé and Cerri. Experimental procedures were executed by Silper, Robles, Madureira, Burnett, and Reis. Data compilation and interpretation were aided by Robles and Reis. Co-authors helped with manuscript editing process.
- *B.F. Silper, L.B. Polsky, J. Luu, T.A. Burnett, A.M. de Passillé, J. Rushen, R.L.A. Cerri. 2015. Automated and visual measurements of estrous behavior and their sources of variation in Holstein heifers. II: Standing and lying patterns. Theriogenology. 84:333-341.* Ideas for this manuscript were developed Silper, de Passillé, Rushen, and Cerri. Writing was done by Silper under supervision of Rushen, de Passillé and Cerri. Experimental procedures were executed by Silper, Luu, and Burnett. Data compilation

and interpretation were aided by Polsky and Luu. Co-authors helped with manuscript editing process. The main ideas for both publications were researched by Silper.

A version of Chapter 3 has been published: *B.F. Silper, A.M.L. Madureira, M. Kaur, T.A. Burnett, R.L.A. Cerri. 2015. Short communication: Comparison of estrus characteristics in Holstein heifers by 2 activity monitoring systems. J. Dairy Sci. 98:3158-3165.* Ideas for this manuscript were developed by Silper, Madureira and Cerri. The manuscript was written by Silper and Kaur, who were supervised by Cerri. Experimental routine was performed by Silper, Madureira and Burnett. Silper and Kaur processed and analyzed data. Co-authors helped with manuscript editing and reviewing process. The main ideas for this publication were researched by Silper.

A version of Chapter 4 has been submitted for publication: *B.F. Silper, A.M.L. Madureira, L.B. Polsky, S. Soriano, A.F. Sica, J.L.M. Vasconcelos, R.L.A. Cerri. Daily lying behavior of lactating Holstein cows during an estrus synchronization protocol and its associations with fertility.* Experimental routine and data collection were done by Madureira, Polsky, Soriano and Sica. The idea was developed by Silper, Madureira, Vasconcelos and Cerri, and analyzed by Silper and Madureira. The manuscript was written by Silper under supervision and contributions of Cerri. Co-authors participated in final writing process. The main ideas for this manuscript were researched by Silper.

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List of Abbreviations

AAM = Automated Activity Monitor

AI = Artificial Insemination

ANOVA = Analysis of Variance

BCS = Body Condition Score

CL = Corpus Luteum

CV = Coefficient of Variation

DIM = Days in Milk

DMI = Dry Matter Intake

E2/P4 = Estradiol and progesterone-based synchronization protocol

FN = False Negative

FP = False Positive

FSH = Follicle-Stimulating Hormone

GnRH = Gonadotropin-Releasing Hormone

IGF-I = Insulin-like Growth Factor I

IGFBP = IGF-Binding Proteins

LH = Luteinizing Hormone

LSM = Least-Squares Mean

NEB = Negative Energy Balance

OR = Odds Ratio

P/AI = Pregnancy per Artificial Insemination

PPV = Positive Predictive Value

Q1 = 25^{th} percentile

Q3 = 75th percentile

SD = Standard Deviation

SEM = Standard Error of the Mean

TAI = Timed Artificial Insemination

TMR = Total Mixed Ration

TP = True Positive

Acknowledgements

In February 2012, when coming to Canada for the first time, I could not have guessed where I'd be and who I'd be today. Every accomplishment and learning would not have been possible without all the special people that have been by my side.

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Bruna

Para Edir e Bela

Chapter 1: Introduction

Estrus is defined as the period of sexual receptivity and fertility in cows, which is characterized by distinctive behavioural display and physiological changes. Estrous is the adjective for description of events related to this phase of the cycle. In this thesis, I use the word 'estrus' to refer to the phase of the estrous cycle, comprising its behavioural and physiological aspects. When specifically referring to behavioural determination of estrus, the terms 'behavioural estrus' or 'estrous behaviour' are used. 'True estrus' is used to describe events that had its physiological and behavioural components assessed.

Dairy herds depend on regular calving intervals to maintain optimal average herd milk yield, but fertility of lactating dairy cows has decreased over the last decades (Lucy, 2001; Garnsworthy et al., 2008). Rolling herd average milk production has increased 40% since the 1970's, period during which the calving interval and the number of services per conception also increased (Lucy, 2001). Others have reported intervals from calving to conception (i.e. days open) to be 46 d longer, and number of artificial inseminations (AI) per conception to have increased from 2 to 3 between 1976 and 1999 (Washburn et al., 2002). From 1985 to 1999, estrus detection rate reduced 20% (Washburn et al., 2002). The apparent association between increased milk production and reduced fertility is, however, the product of a complex relationship, given the wide range of factors affecting reproductive performance (Santos et al., 2009). Therefore, it is important to ask: are cows indeed less fertile, or are management practices not adequate for modern dairy cows? (LeBlanc, 2010).

Lactation induces metabolic and physiological adaptations that can affect the reproductive function. Compared to nulliparous heifers, lactating cows have lower circulating estradiol and progesterone, despite larger preovulatory follicles and corpus luteum (CL; Sartori et al., 2004). This lower circulating steroid concentration has been associated with greater metabolic rate in high producing cows (Sangsritavong et al., 2002; Vasconcelos et al., 2003). Sartori et al. (2004) hypothesized that issues such as delayed ovulation (relative to time of luteolysis), ovulation failure, double-ovulation, and poor estrus expression could be explained by suboptimal estradiol and progesterone concentrations.

Herd reproductive performance can be evaluated via days open, rate of submission to AI, or pregnancy and conception rates, for example. Pregnancy rate is the product of conception and AI submission rates. In Canada, a recent survey reported average conception risk, 21-d insemination rate, and 21-d pregnancy rate of 40.5%, 44.1% and 17.6%, respectively (Denis-Robichaud et al., 2016a). Early pregnancy losses (up to 42 d after AI) are apparently the main reason for low conception rates observed in lactating dairy cows. In spite of 76% fertilization rate at d 2 after AI, conception rate is approximately 40% at d 28 and 35% at 42 d (Santos et al., 2004). Rate of submission to AI is dependent on detection of estrus by herd personnel and can be insufficient because of poor observation routine or poor expression of estrus.

Behavioural estrus is traditionally detected by acceptance of mounts from other cows (i.e. standing to be mounted). This behaviour has been reported to occur infrequently and during a short time interval, especially in high yield cows (Lopez et al., 2004); thus, estrus is likely to be missed when observation takes place twice or thrice a day. This has been partially overcome with use of hormonal synchronization protocols (timed artificial insemination; TAI) because this practice does not require estrus detection for submission to AI. However, higher fertility has been reported when cows expressed estrus at TAI (Cerri et al., 2004; Souza et al., 2007; Pereira et al., 2014). This raises questions about the importance of behaviour as a phenotypical marker for fertility and the underlying associations between behaviour and reproductive physiology. It is now easier to detect estrus and measure its expression using automated activity monitors (AAM), which monitor behaviour continuously and provide estrus measurements that could be used in basic and applied reproduction science. In addition, recent research has suggested potential for fertility-oriented genetic selection based on AAM measurements, as it was observed that interval from calving to first high activity event (i.e. first post-partum estrus) is heritable and genetically correlated with estrus characteristics and days to first post-partum AI (Ismael et al., 2015).

This chapter starts with a review of dairy cow reproductive physiology and management, concerning aspects relevant for the study of estrus expression and detection. Factors related to poor reproductive performance are presented and the use of AAM is discussed as a potential, although partial, solution. Sensors that monitor animal behaviour, including AAM, are under increasing availability and diversification (Rutten et al., 2013), although the idea of automating measurements of cow activity is not new (Kiddy, 1977). This literature review is followed by a summary of gaps where research would contribute to improve dairy cow reproductive

performance from the estrous behaviour and detection approach. Lastly, I present the general and specific research questions that were addressed in the experiments that compose this thesis (Chapters 2, 3, and 4).

1.1 Physiology of the Estrous Cycle

Estrous cycles are divided into proestrus and estrus (follicular phase), and metestrus and diestrus (luteal phase). These phases are determined according to ovarian structures and dominance of estradiol or progesterone, the major ovarian hormones. Estrous cycles start and end at ovulation, usually defined as d 1 of the cycle. Luteolysis marks the transition between diestrus and proestrus, bringing the luteal phase to an end and providing the endocrine milieu for final follicle development and ovulation (Forde et al., 2011). Estrous cycles in cattle are expected to last 21 d, ranging between 18 and 24 d (Forde et al., 2011). Sartori et al. (2004) reported mean cycle duration (interovulatory interval) of 22.0 ± 0.4 d for heifers and 22.9 ± 0.7 d for lactating cows.

Follicular growth occurs in waves, each wave comprehending the interval from emergence to atresia - or ovulation - of the dominant follicle (Lucy, 2007). In general, cows have two or three waves of follicular growth per estrus cycle. The last wave of a cycle ends with ovulation of the dominant follicle; dominant follicles of other waves loose dominance and undergo atresia (Ireland et al., 2000). Sartori et al. (2004) observed a predominance of two-wave cycles in heifers and lactating cows (56% and 79% of cycles, respectively). Follicular waves start with the emergence of a follicle which is 4 to 5 mm in diameter. This follicle, which was selected among a recruited cohort of primordial follicles, continues to grow, eventually establishing its dominance over the subordinate follicles. The process through which the dominant follicle achieves greater growth rate and becomes larger than the remaining follicles is termed deviation (Ireland et al., 2000).

Growth of the recruited cohort of primordial follicles is induced by a surge of FSH, which occurs at the time of and shortly after the preovulatory LH surge (Lucy, 2007). The decrease in FSH concentration after its surge marks the end of the selection process, i.e. continued growth of only one follicle, which will become dominant. All other follicles (the subordinate follicles) undergo atresia at this point. Although gonadotropins are not necessary for recruitment, this

process benefits from gonadotropin presence (Ireland et al., 2000). Considering ovulation as d 1, granulosa cells of d 3 follicles express FSH receptors, marking the start of gonadotropin-dependent follicular growth. Growth and proliferation of granulosa cells are stimulated by FSH. Initiation of aromatase activity (conversion of androgens to estrogens) in granulosa cells also occurs at this stage (Forde et al., 2011). Expression of mRNA for P450 aromatase and P450 side chain cleavage in granulosa, and P450 alpha-hydroxylase in thecal cells of follicles > 4 mm in diameter result in estradiol synthesis (Lucy, 2007). Follicles with diameter > 1 mm present steroidogenic acute regulatory proteins, which regulate steroidogenesis together with P450 aromatase (Braw-Tal and Roth, 2005).

Approximately at the end of selection, one follicle deviates from the remaining subordinate follicles due to achievement of greater growth rate (Ireland et al., 2000). In this faster-growing follicle, FSH induces increased protease activity of IGFBP-4 and 5, resulting in greater concentration of free IGF-I in the follicular fluid. IGF-I stimulates gonadotropin action, cellular growth, and estradiol synthesis (Ireland et al., 2000; Rivera and Fortune, 2003; Lucy, 2007). The dominant follicle thus benefits from differential IGFBP metabolism that influences its steroidogenic capacity (Canty et al., 2006). This differential regulation results in greater IGF-I to IGFBP ratio in the follicular fluid of dominant follicles than in the subordinate follicles, modulating follicular growth and atresia (Ireland et al., 2000). IGF-I and FSH act synergistically in this follicle to stimulate estradiol synthesis (Lucy, 2007). This results in more than twice the amount of estradiol and 10 times less IGFBP-4 in the follicular fluid of the follicle that will become dominant (d 3 follicles; 7.6 ± 0.4 mm in diameter), in comparison with subordinate follicles (5 to 8.5 mm in diameter; Mihm et al., 2000).

Estradiol, synthesized by follicular cells (two-cell/two-gonadotropin model) has a central role in induction of estrous behaviour and regulation of oocyte maturation and ovulation (hypothalamic-pituitary axis feedback pathways). The two cell/two gonadotropin model starts with LH binding to LH receptors in the theca cell membrane to induce synthesis of androgens from cholesterol. Androgens are then aromatized to form estradiol within granulosa cells (Forde et al., 2011). Other molecules present in the follicular fluid, such as inhibin and activin, act in paracrine or autocrine manner. Inhibin down-regulates FSH secretion, whereas activin supports estradiol synthesis via positive feedback on FSH (Ireland et al., 2000; Forde et al., 2011).

Estradiol and inhibin down-regulate FSH secretion via negative feedback, inducing atresia of subordinate follicles (Ginther et al., 2000). Only the dominant follicle will continue to develop in this low FSH environment, because of its acquired expression of granulosa cell LH receptors (Xu et al., 1995; Beg et al., 2001; Forde et al., 2011). Frequency and amplitude of LH pulses regulate the follicle's steroidogenic capacity and maintenance of dominance (Ireland et al., 2000; Forde et al., 2011).

In addition to increased expression of LH receptors, dominance and continued growth after reduction of FSH secretion are also related with increased presence of steroidogenic enzymes (Bao et al., 1997). Bao et al. (1997) reported follicular expression of P450 aromatase and P450 side chain cleavage mRNA 12 h after follicular emergence, while LH receptors were expressed in the granulosa only 24 h later. Estradiol-active dominant follicles have more granulosa cells, greater estradiol, and greater estradiol to progesterone plus androgen ratio in the follicular fluid, besides the greater expression of LH receptors in granulosa and theca cells (Ireland et al., 2000). The period during which a follicle exerts dominance over others, termed follicular dominance, lasts approximately 5 to 8 d. The dominant follicle's main functions are oocyte nourishment and hormonal synthesis (Lucy, 2007). Inhibin and estradiol exert GnRH-independent negative feedback on FSH secretion, preventing follicular emergence while a dominant follicle is active (Haughian et al., 2013).

The dominant follicle of the first wave, and possibly that of the second wave, does not ovulate because of progesterone's negative feedback on LH pulsatility. Under progesterone influence, LH pulses have low frequency and amplitude, characteristics that do not support prolonged periods of follicular dominance (Ireland et al., 2000; Forde et al., 2011). There are approximately 20 to 30 LH pulses/24 h in the early luteal phase, whereas in the late luteal phase pulses occur 6 to 8 times/24 h. Progesterone reduces to basal concentration within 24 h of luteolysis induced by $PGF_{2\alpha}$ injection (Chenault et al., 1976). Endometrial $PGF_{2\alpha}$ release occurs in response to oxytocin binding to its receptor, which has its functioning coordinated by ovarian steroids (Mann et al., 2001; Mann and Lamming, 2006). When basal progesterone concentration is achieved and the negative feedback on LH is removed, the increasing estradiol concentration is able to induce a GnRH-dependent LH preovulatory surge that results in ovulation. Estradiol coordinates this positive feedback to hypothalamus and anterior pituitary, as well as the final

follicular maturation, ovulation, and behavioural estrus (Forde et al., 2011). Mechanisms leading to ovulation of the dominant follicle and to behavioural expression of estrus will be discussed in the next section.

1.2 Neuroendocrine Regulation of Estrus and Ovulation

The hypothalamus is the control center for neural, endocrine and sensory integration that regulates estrous behaviour (Frandson et al., 2006). It also coordinates gonadotropin secretion via GnRH, a neurohormone secreted by neurons from the tonic centre (basal GnRH secretion) and surge centre (preovulatory GnRH surge). Each of these centres is composed of hypothalamic nuclei, which are groups of neurons with similar functions. Those involved in regulation of female sexual behaviour and GnRH secretion are the Ventromedial Nucleus and Arcuate Nucleus, located in the medial basal area of the hypothalamus and forming the tonic centre, and the Sexually Dimorphic Nucleus and Anteroventral Periventricular nuclei, which are part of the Medial Preoptic Area in the surge centre (Simerly, 1998; Carlson, 2013).

Neurons in the Ventromedial Nucleus and Medial Preoptic Area integrate behavioural and hormonal information by responding to afferent chemosensory stimuli (e.g. pheromones, sensory information from the genitals and flanks) and to estradiol. The efferent neurons leaving those areas, by establishing synapses within the central nervous system, deliver the signal to motor neurons in the spinal cord, eliciting responses such as lordosis in female rats (Carlson, 2013). Neurons in the Anteroventral Periventricular region have synapses with GnRH neurons, thus influencing GnRH and gonadotropin secretion (Simerly, 1998). GnRH neurons have been reported to express estradiol receptor-β, evidencing direct control of GnRH secretion by estradiol (Petersen et al., 2003). The responsiveness of neurons in the Ventromedial Nucleus and Medial Preoptic Area to estradiol and their connections with GnRH neurons indicates indirect control of GnRH secretion by estradiol (Petersen et al., 2003).

In addition to estradiol receptor-β expressed in GnRH neurons, estradiol receptors-α have been observed in Ventromedial Nucleus and Medial Preoptic Area during the luteal phase and in the arcuate nucleus during estrus and metestrus (van Eerdenburg et al., 2000). In the uterus, expression of estradiol receptors in the endometrial glandular epithelium was greater during proestrus (d 17 to 20) and estrus-metestrus (d 0 to 6) (Kimmins and Maclaren, 2001). Expression

of estradiol receptors in the luminal epithelial was increased only at d 14 and 16 of the cycle (Kimmins and Maclaren, 2001). Estradiol and its receptors are key factors in synchronization of physiological and behavioural reproductive events (van Eerdenburg et al., 2000).

In cattle, induction of estrous behaviour and ovulation is dependent on estradiol 17-β and conditional to low progesterone concentration (Allrich, 1994). Studies with ovariectomized and intact cows reported that injection of various dosages of estradiol induced similar behavioural expression (Katz et al., 1980; Allrich, 1994). This led to a postulation that estradiol promotes estrous behaviour in an "all or none" fashion: estrous behaviour would be induced once estradiol reaches a determined concentration and the behavioural expression would not be affected by further increases of estradiol concentration. More recently, Reames et al. (2011) observed a linear increase in duration of standing estrus (first to last acceptance of mount) with increasing estradiol supplementation, but estradiol supplementation cannot be directly compared among these experiments. Reames et al. (2011), testing 4 estradiol dosages, reported between-cow differences in the estradiol concentration required for induction of estrous behaviour. Individual behaviours, metabolic rate, and expression of estradiol receptors are some of the potential factors that could interfere with the estrus expression response.

In addition to inhibition of estrous behaviour (Allrich, 1994), progesterone at AI above basal concentration (> 0.4 ng/mL) has been associated with poor fertility (Wiltbank et al., 2014). Accordingly, Kimmins and Maclaren (2001) concluded that there is self and paracrine regulation between sex steroids and their receptors in the endometrium. The role of progesterone in priming the bovine brain for estradiol (regulating expression of estradiol receptors) is evidenced by the absence or low expression of estrus associated with the first post-partum ovulation or the pubertal estrus, which are not preceded by a luteal phase. Greater expression of estrus at time of AI as part of synchronization protocols with progesterone supplementation provide further evidence (Rhodes et al., 2002).

Although regulation of estrus expression likely involves additional factors, estradiol is at least required as a trigger (Roelofs et al., 2010). It is important to consider that if greater concentration of estradiol does not benefit behavioural expression of estrus, it might still improve aspects of reproductive tract function such as sperm transport and uterine protein secretion for embryo nourishment (Allrich, 1994). Positive associations between estrus expression and

conception rate (Garcia et al., 2011; Gilmore et al., 2011; Madureira et al., 2015) suggest that both processes share common regulators such as estradiol. Investigations of neuroendocrine regulation of estrus expression could increase the understanding on how estrous behaviour and physiological mechanisms relate to fertility.

Ovulation occurs after the end of behavioural estrus, and processes leading to ovulation are also controlled by estradiol (Bloch et al., 2006; Forde et al., 2011). When progesterone concentration decreases after luteolysis, its negative feedback on LH is removed and amplitude and frequency of LH pulses increase. The LH surge is GnRH-dependent and is induced by the increasing estradiol concentration resulting from follicular steroidogenic activity. The LH surge then induces final follicular maturation and ovulation (Forde et al., 2011).

The intervals among events occurring between luteolysis and ovulation are timely regulated (Figure 1.1). Variation in these intervals, especially in relation to time of estrus onset, could contribute to the low conception rates observed in Holstein cows (Saumande and Humblot, 2005; Valenza et al., 2012). Assuming an interval of 28 h from estrus onset to ovulation and 25 h from LH surge to ovulation (Bloch et al., 2006), estradiol peak, GnRH surge, and LH surge occur in a time frame of only 3 h. By the end of behavioural estrus, ovulation is the only event left to complete the cycle. Onset of estrus measured by AAM occurred 29 ± 8 h before ovulation (Valenza et al., 2012). A farm using AAM would nowadays breed cows 7 to 12 h after the onset of estrus determined by high physical activity (Neves and LeBlanc, 2015), resulting in an interval of approximately 10 h between AI and ovulation. Timing between estrus onset and ovulation has been reported to be similar when onset of estrus was determined by standing to be mounted or by high physical activity (26.4 ± 0.7 h vs. 24.6 ± 0.7 h, respectively; Stevenson et al., 2014).

Greater peak estradiol at estrus, obtained with induction of luteolysis earlier in the cycle (d 6 to 9 vs. d 14 to 15), resulted in a 10 h shorter interval from luteolysis to onset of standing estrus (Stevenson et al., 1998). This shorter interval is in agreement with observations that greater estradiol concentration results in LH peak and ovulation closer to onset of estrus (Saumande and Humblot; 2005; Bloch et al., 2006). This could favour fertility because of ovulation of a high quality oocyte (not prematurely activated) and better synchrony between AI and ovulation. Accordingly, Bloch et al. (2006) reported that very long intervals between estrus and ovulation

(35 to 50 h) coincided with lower peak estradiol, lower progesterone in the previous cycle, and smaller amplitude of LH surge. Indications that the hypothalamic centres for GnRH surge are more sensitive to estradiol than the behavioural centres have been reported. In addition, it has been suggested that an association between a minimum estradiol concentration needed for LH surge and intensity of behavioural estrus is not likely to exist (Reames et al., 2011).

Besides inducing ovulation, the LH surge stops aromatase activity and terminates estradiol synthesis (Forde et al., 2011). Accordingly, a reduction of estradiol concentration to 50% of peak concentration by 5 h post-LH surge has been reported (Chenault et al., 1975). By 14 h post-LH surge, estradiol is already at basal level (2 pg/mL; Chenault et al., 1975), or is less than 2 SD above basal level (Aungier et al., 2015). Relatively to behavioural estrus, peak estradiol concentration has been detected at time of maximal behavioural expression according to a scoring system by van Eerdenburg et al. (1996). Estradiol has also been reported to reduce to 60% of peak concentration 6 h after maximal behavioural expression (Lyimo et al., 2000). It could be inferred from Figure 1.1 that estradiol synthesis ends approximately 3 to 9 h after estrus onset, thus towards mid to end of estrus. It can be extrapolated that the last hours of behavioural estrus occur under lowering concentrations of estradiol, so that behaviour at that time results from stimulation by other sources or by continued estradiol-induced neuroendocrine pathways. Because high producing cows have greater metabolic clearance of estradiol (Sangsritavong et al., 2002), reduction in circulating estradiol after the LH surge is likely faster in these cows and could result in shorter and less intense estrus expression (Lopez et al., 2004).

Suboptimal concentrations of estradiol or progesterone can negatively impact physiological mechanisms and reproductive performance (Sartori et al., 2004). In a comprehensive study of ovarian function and steroid concentration in heifers (10 to 16 mo old) and lactating cows (56 ± 4 days in milk; DIM), Sartori et al. (2004) observed that cows had ovulatory follicles of greater diameter (16.8 vs. 14.9 mm) and greater volume of luteal tissue (11,120 vs. 7,303 mm^3). Although structures were larger, lactating cows had lower maximum serum estradiol (7.9 vs. 11.3 pg/mL) and progesterone concentration (5.6 vs. 14.9 ng/mL) than heifers.

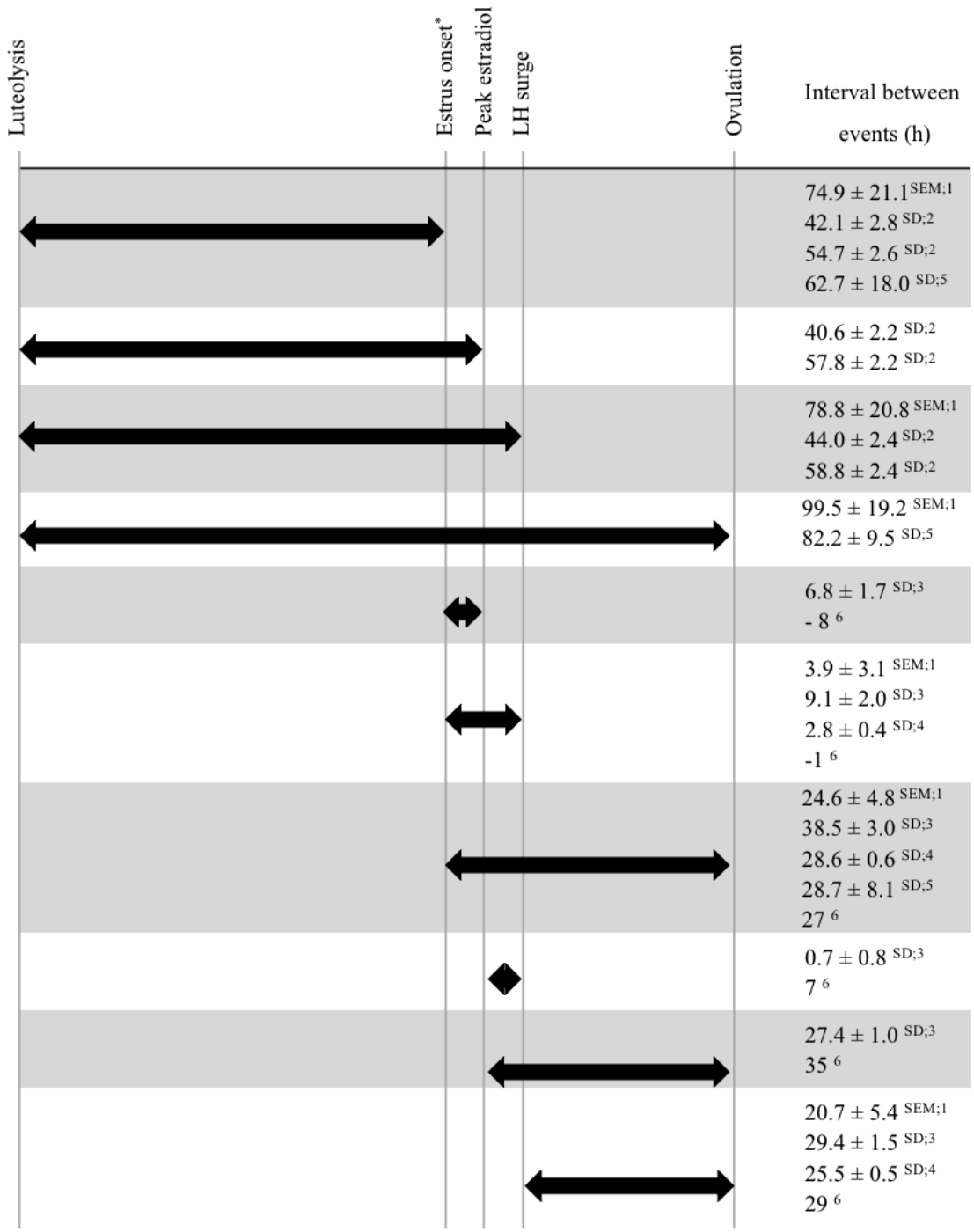

Figure 1.1 Time interval between events occurring from luteolysis to ovulation

Time intervals (in hours) are means ± SEM or SD (indicated in the superscripts). *Estrus onset determined by: visual observation of standing estrus ([1]Chenault et al., 1975; [3]Saumande and Humblot, 2005; [4]Bloch et al., 2006), electronic mount detectors ([2]Stevenson et al., 1998; luteolysis at d 6 to 9 or d 14 to 15), or increased physical activity ([5]Valenza et al., 2012; [6]Aungier et al., 2015).

Low progesterone and estradiol have been linked to increased metabolic rate and clearance of steroid hormones that occur in response to increased blood flow to gut and liver post-feeding (Parr et al., 1993; Sangsritavong et al., 2002; Vasconcelos et al., 2003). Due to metabolism and feed intake, lactating cows have greater liver blood flow (1,183 L/h) than dry cows (757 L/h) (Sangsritavong et al., 2002). Estradiol metabolic clearance rate remains elevated for 4.5 h post-feeding in lactating cows, resulting in reduction of circulating estradiol to half of the pre-feeding concentration at least during that 4.5 h-period (Sangsritavong et al., 2002). Vasconcelos et al. (2003) reported reduction of circulating progesterone by approximately 0.5 to 1.5 ng/mL during 8 to 9 h post-feeding if cows were fed 50 or 100% of their daily DMI requirement.

The length of follicular waves, and thus the number of waves in a cycle, is under control of progesterone and estradiol (Sartori et al., 2004). Low progesterone concentration delays follicle turnover and prolongs follicular dominance (Cerri et al., 2009) due to reduced inhibition of LH pulses by progesterone. A profile of high progesterone concentration during the preovulatory follicle growth phase, conversely, might further inhibit LH pulse frequency, avoid premature oocyte activation and prevent delayed ovulation or ovulation failure, thus improving reproductive outcomes. Follicles that grew under high progesterone concentrations also had greater follicular fluid IGF-I concentration (Cerri et al., 2011a), which contributes to follicular development and estradiol synthesis. Embryos from follicles that grew under progesterone concentration greater than 1.4 ng/mL (high progesterone; ovulation of second vs. first follicular wave dominant follicle) had better quality (Rivera et al., 2011). Early pregnancy losses can originate from compromised oocytes (poor quality embryos) or from inadequate uterine environment (Santos et al., 2004), as well as from untimely inseminations (Dalton et al., 2001). Finally, reduced luteal phase progesterone concentration results in premature expression of estradiol receptors and increased concentration of PGF-metabolite after oxytocin challenge, leading to premature luteolysis (Cerri et al., 2011a).

Control of luteolytic mechanisms is also impaired by reduced estradiol during the preovulatory period. Suboptimal estradiol concentration does not fully inhibit oxytocin receptors, resulting in premature luteolysis (Mann and Lamming, 2000). This mechanism was proposed to explain the short cycles following the first post-partum ovulation and supports the effect of low peak estradiol concentration on conception rates and luteolysis before maternal recognition of pregnancy (d 16 after AI), leading to early pregnancy losses (Santos et al., 2004). Estradiol also

influences the length of the luteolysis-to-ovulation interval by altering the timing of GnRH and LH surges (Mann and Lamming, 2000). This implies a longer period of follicular growth under lowering progesterone concentrations. In addition to untimely AI, this delay results in ovulation of a larger and older follicle that likely holds a poor quality oocyte. A reduction of 1.5 d in the length of follicular dominance improved embryo quality, although fertilization rate was not affected (Cerri et al., 2009).

At the moment of follicular emergence, lower estradiol concentration results in lesser negative feedback on FSH, which by remaining at high concentration allows for emergence of more than one follicle from a single cohort. This model has been proposed to explain the greater incidence of double ovulations and twinning in association with high milk yield (Wiltbank et al., 2000). Double-ovulation rates of 64% have been reported in lactating cows in the summer, while heifers had a rate of 1.3%, and lactating and dry cows of 17% in winter conditions (Sartori et al., 2002).

Low circulating steroid concentration can also impair estrus expression. The first estrus post-partum and the pubertal estrus are examples of events not preceded by periods of high progesterone where estrus can be silent and followed by a short luteal phase. The first estrus post-partum, characterized by less intense behavioural manifestation, is shorter and has smaller increase in physical activity (Aungier et al., 2012). High milk yield has been reported to affect duration of estrus and rate of multiple ovulations, mediated by elevated steroid metabolism and low plasma estradiol concentration (Wiltbank et al., 2006). Lopez et al. (2004) observed larger follicles (18.6 ± 0.3 vs. 17.4 ± 0.2 mm) and lower circulating estradiol (6.8 ± 0.5 vs. 8.6 ± 0.5 pg/mL) in cows producing averages of 46.8 and 32.3 kg/d, respectively. Moreover, higher yield cows had shorter standing estrus (7.0 ± 1.1 vs. 11.9 ± 1.4 h).

Overall, a greater incidence of reproductive abnormalities (e.g. ovulation failure, multiple ovulations, ovarian cysts) as well as poor estrus expression are likely to originate from lower circulating estradiol in the preovulatory period (Sartori et al., 2004). Milk production, a factor that is frequently linked to poor reproductive performance, induces metabolic changes such as negative energy balance (NEB) at early lactation and is associated with high metabolic rate, partly due to large feed intake and increased metabolic clearance of steroids. These factors can

result in impaired ovarian and uterine function and might contribute to the historical decline in reproductive performance (Lucy, 2001; Wiltbank et al., 2006).

Comparisons between lactating cows and nulliparous heifers demonstrate an effect of milk production on reproduction (Sartori et al., 2002; Sartori et al., 2004). Two other factors should be considered: level of individual milk production per day and parity. Some have reported negative effects of milk production on reproductive function (Lucy, 2001), while others have not (López-Gatius et al., 2006; Madureira et al., 2015). The ability of individual cows to cope with high milk yield and current management practices are important in determining negative effects of lactation on fertility. These are complex associations because cows with low milk production might be affected by diseases that also impact reproductive function, while high producing cows can be among the healthiest in a given herd (Santos et al., 2009). Primiparous have extra demands for growth, in spite of lower milk production. Analysis of milk production per kg of dry matter intake or per kg of body weight could reveal differences in metabolism and partitioning of nutrients in primiparous cows. Although primiparous are less likely to resume cyclicity before 65 DIM, they achieve greater conception rate than multiparous (Santos et al., 2009).

Primiparous and multiparous cows differ in post-partum tissue mobilization, resulting in different metabolic and endocrine profiles. In general, cows that are still growing have greater post-partum IGF-I concentration (Wathes et al., 2007b), which has been identified as the trait with greatest influence on interval to first service and conception in multiparous cows (Wathes et al., 2007a). Lower IGF-I is part of the altered metabolic and endocrine profile during periods of NEB. Low glucose and insulin, and reduction of LH pulse frequency have also been observed (Butler, 2003). IGF-I is essential for follicular growth, estradiol synthesis, and potentiation of gonadotropin actions in the follicle (Wathes et al., 2007b; Garnsworthy et al., 2008); in addition, it interacts with estradiol receptor-α in the modulation of sexual behaviour (Cardona-Gómez et al., 2002; Woelders et al., 2014). Insulin is similarly potent at stimulating steroidogenesis, follicular growth and hastening resumption of cyclicity (Wathes et al., 2007b; Garnsworthy et al., 2008), and both insulin and IGF-I also regulate GnRH secretion (Garnsworthy et al., 2008). Metabolites such as NEFA and BHBA can impair follicular growth and indirectly influence steroidogenesis (Wathes et al., 2007b). Because NEB lasts 10 to 12 wk (Butler, 2003) and follicular growth from early antral stage to ovulation takes approximately 40 d (Wathes et al.,

2007b), it is likely that the ovulatory follicle of the first cycles following the voluntary waiting period (around 60 DIM) develops under an altered endocrine profile.

1.3 Behavioural Estrus

Cows present a characteristic behavioural display when in estrus, where standing still when mounted by another female (i.e. standing to be mounted) is the primary sign (Roelofs et al., 2010). The mounting behaviour among female cattle has been explained as a strategy for communication of sexual receptivity to males, or as the result of an instinctive selection by herdsmen for cows displaying such behaviour when in estrus. This hypothesis originates from historical information that female *Bos Taurus* cattle have been herded apart from males for centuries; estrous behaviours was then used to determine when bulls should be brought to a herd (Baker and Seidel, 1984; Albright and Arave, 1997).

Research done using continuous visual observation of estrous behaviour in the 1970's and 1980's observed greater frequencies and longer periods of standing to mounted than observed in more recent research (Figure 1.2a and b). It is important to highlight the large variation in number of mounts received per estrus (0 to 91 mounts/estrus; Pennington et al., 1985; Pennington et al., 1986), as well as the percentage of cows detected in standing estrus. For example, Sveberg et al. (2011) reported standing estrus in 14 out of 20 cows, but 2 cows had a single standing event. Although it seems evident that frequency of standing to be mounted per estrus has declined, one might argue that such comparisons are not valid, given that behaviour can vary with number of cows in estrus, flooring surfaces and group sizes (Albright and Arave, 1997). The previously mentioned reports (Hurnik et al., 1975; Pennington et al., 1985; Pennington et al., 1986) refer to free-stall housed cattle, with permanent or partial access to a dry lot. Mounting and standing to be mounted have been reported to be approximately 2-fold greater on dirt than on concrete trial pens, which were accessed by free-stall housed cows for 30 min thrice/d (Britt et al., 1986). Accordingly, Pennington et al. (1985) observed that 80% of the mounting activity occurred in the dry lot area. Whether a 1980's cow would mount as infrequently as the modern cow if access to dry lots was denied is unknown. Palmer et al. (2010) observed numerically less standing to be mounted events and shorter duration of estrus in dairy cows housed in cubicles when compared to cows on pasture. The effect of access to dry lots or to

other sources of non-slippery flooring on estrus expression should be further studied. This question concerns not only reproductive performance, but also welfare, housing costs, feasibility, and incidence of mastitis and lameness, for example.

The rate at which standing to be mounted is observed might not be sufficient for satisfactory estrus detection in most dairy farms. Using the traditional scheme of 30 min of visual observation twice/d, acceptances of mount are likely to be missed, with only 37% of estrus events detected (Van Vliet and Van Eerdenburg, 1996). With 30 min of observation four times/d, Dolecheck et al. (2015) observed standing estrus in 18 of 32 cows enrolled in a synchronization protocol, while 29 had an ovulation confirmed by blood progesterone profile. Accordingly, Roelofs et al. (2005) reported standing estrus in 20% of estrus events if a single cow was in estrus at a given time; this increased to 79% when more than two cows were in estrus simultaneously. Although a detection rate of 79% would be seen as satisfactory, it was obtained with a demanding visual observation scheme (30 min rounds at 3 h intervals).

Occurrence of standing to be mounted depends, evidently, on the willingness of other cows to mount. In accordance with the trend of reduced frequency of standing to be mounted, there is indication of similar reduction in display of mounting behaviour over time (Figure 1.2c). Mounts are performed mostly by cows in proestrus and estrus (Helmer and Britt, 1985). Hurnik et al. (1975) reported an average of 34 mounts per estrus and median of 16 mounts, showing a positively skewed distribution where most cows displayed low frequency of mounts and few performed many mounts. Mounting can be classified as oriented (back mounts), disoriented (front and side mounts), attempt (the receiver cow did not stand), or successful mount (standing behaviour by the receiver). Previous reports (Britt et al., 1986; van Eerdenburg et al., 1996) have suggested that disoriented mounts are good discriminative behaviours for estrus detection as they only occur during or within 12 h of standing estrus. Another useful tool would be the ratio between accepted and rejected mounts, which could inform if a cow received mounting attempts and was not receptive, or if there were no mounting attempts. It can be suggested that behavioural manifestation of estrus has changed, most likely in response to resources we provide to or restrain from the cows. It is clear that modern dairy cows in the standard dairy farm setup do not stand to be mounted frequently enough for visual observation of estrus to yield adequate AI submission rates. Alternatives that could be considered are 1) provision of an environment that stimulates behavioural expression, 2) improvement of methods to detect standing to be

mounted, and 3) development of methods for detection of alternative behaviours. The last two alternatives have been addressed more extensively, respectively with use of tail chalk or electronic mount detectors, and with the development of AAM.

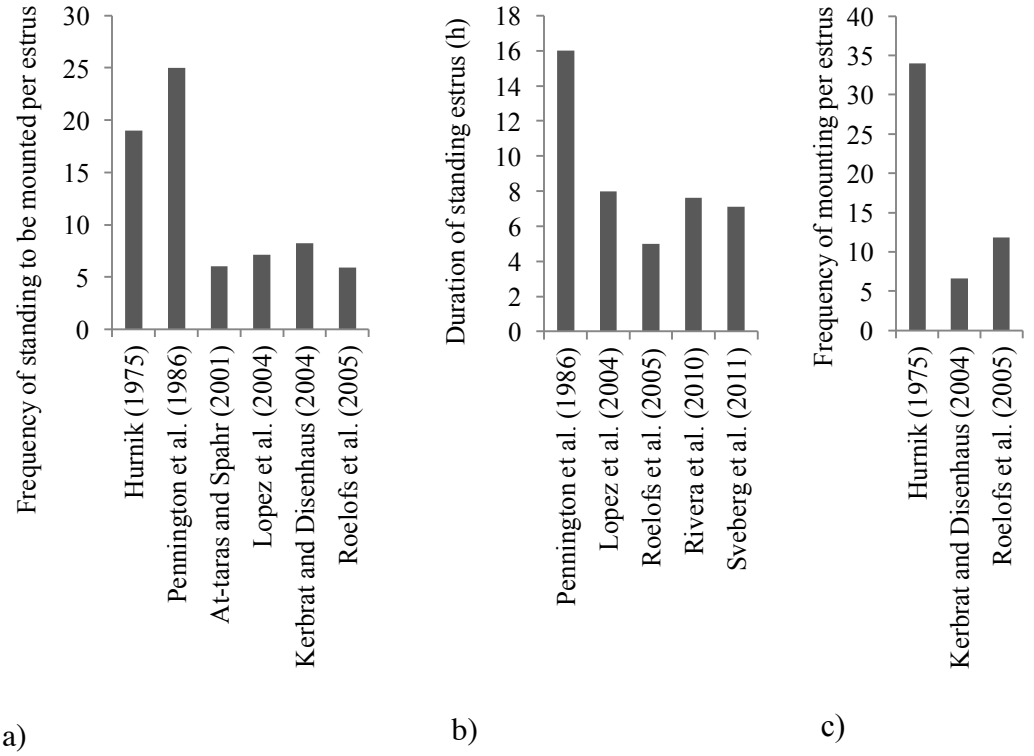

Figure 1.2 Characterization of standing estrus according to studies from 1975 to 2011

a) Frequency of standing to be mounted per estrus, b) Duration of standing estrus (h), and c) Frequency of mounts per estrus

Similar to mounting and standing to be mounted, secondary estrous behaviours are classified as active (initiated by an actor cow) or passive (received by a reactor cow). During standing estrus, active secondary signs are observed at greater frequency than passive secondary signs (Sveberg et al., 2011). Monitoring secondary estrous behaviours, such as active mounting, chin resting, sniffing the vulva, and restlessness, could improve the efficiency of estrus detection (van Vliet and van Eerdenburg, 1996). Even though secondary behaviours are not specific to estrus, they are infrequent and occur at random when cows are not in estrus (Sveberg et al., 2011). Initiation of a variety of secondary behaviours was reported to increase during the 6 h preceding standing estrus, while increased receptivity to secondary behaviours was simultaneous

to onset of standing estrus (Sveberg et al., 2011). A sequential behavioural expression has been proposed: at a first moment, sniffing and chin resting would occur, followed by mounting, and finally by standing to be mounted (Roelofs et al., 2005). Sveberg et al. (2011), conversely, pointed to secondary behaviours occurring in an active fashion before onset of standing estrus and in a receptive fashion during standing estrus (general receptive state).

Secondary signs might be useful especially in situations were standing to be mounted is not frequently observed. Detection focused on active behaviours is likely more efficient because display of such behaviours depends only on the cow in estrus. Chin resting is one of the secondary signs of estrus that apparently has great frequency of display. When in estrus, cows usually respond to a received chin rest with immobilization reflex, similarly to standing to be mounted (Albright and Arave, 1997). Chin resting could be interpreted as a receptivity test, usually followed by mount in case of positive immobilization reflex. Chin rests were initiated during 100% of estrus events, but also during non-estrus periods (Roelofs et al., 2005). Sveberg et al. (2011) reported increased frequency of chin rests received and initiated during estrus (3.2 and 3.5 counts per h, respectively, comparable to frequencies of zero during days with no cows in estrus). Another potentially important secondary estrous behaviour is to follow or be followed. Trailing (Diskin and Sreenan, 2000) and circling with partner (Phillips and Schofield, 1990) likely refer to the behaviour named here as ‘following’. This behaviour was described as one cow closely trailing the movements of another, and was reported to increase during estrus but at a lesser extent than chin resting and sniffing (Sveberg et al., 2011).

Observation of estrous behaviour by producers should not be restricted to standing to be mounted (Roelofs et al., 2010). Secondary behaviours are not specific, but are relevant due to their high frequency of expression. van Eerdenburg et al. (1996) created an estrus detection method based on a sum of scores attributed to primary and secondary signs of estrus. They identified a threshold that allows 100% specificity for estrus detection, even though scores were low and easily achieved if one judges their scale. Nonetheless, their results evidence that estrus detection methods based on frequent expression of secondary behaviours can be employed.

Research by Sveberg et al. (2011) described an increase in counts per h of chin rest, sniffing, following and other secondary behaviours during estrus. This is one of the recent publications addressing the importance of secondary behaviours for estrus detection, but research

was done with cows producing 6,016 kg of milk/lactation and housed in a soft-floored pen. Similar research performed with cows of greater yield and housed in free-stalls could bring insight into causes of low estrus expression. In addition, Sveberg et al. (2011) reported reduced duration of standing estrus and low frequency of mounts, despite the previously mentioned production and housing aspects.

Given the actor-reactor nature of most estrous behaviours, it is expected that cows in estrus will form sexually active groups. Increased mounting frequency has been described in the presence of five heifers (Helmer and Britt, 1985) or two cows (Sveberg et al., 2011) simultaneously in standing estrus. Sveberg et al. (2013) defined that cows in such groups should "…participate in a minimum of 1 estrous behavior per 5 min while staying within 3 m (2 cow lengths) of its partner(s) for a minimum of 5 min". Formation of sexually active groups certainly facilitates the identification of cows potentially in standing estrus, but it does not provide confirmation. Increased behavioural display resulting from participation in sexually active groups likely contributes to the restlessness that is characteristic of estrus. Restlessness, commonly cited as one of the main behavioural changes during estrus (van Eerdenburg et al., 1996; Roelofs et al., 2010), has been interpreted as a search for mating partners or as a tool for advertising sexual receptivity (Kerbrat and Disenhaus, 2004). Although this behaviour is subjective when visually evaluated (van Eerdenburg et al., 1996), it constitutes the basis for automated estrus detection. The display of secondary behaviours likely contributes to increased physical activity, which is the most common parameter measured by AAM. Restlessness can also affect lying behaviour. An increased number of lying bouts in tie-stall housed cattle (Walton and King, 1986; Kerbrat and Disenhaus, 2004) and a decrease in the total daily lying time during estrus have been suggested as alternatives for automated estrus detection (Kerbrat and Disenhaus, 2004, Dolecheck et al., 2015). Lying time and bout frequency reduced approximately 60% during a ± 6 h interval surrounding the first visually observed standing event (Dolecheck et al., 2015). Investigation of lying behaviour during periods of estrus up to this date has mostly been done with small number of observations or with low-yield cows (Kerbrat and Disenhaus, 2004, Livshin et al., 2004, Dolecheck et al., 2015).

1.4 Automated Detection of Estrus

Low rates of submission to AI can be linked to poor expression of estrus or to inefficient detection. Considering that the problem was detection rather than expression, Senger (1994) proposed three automation alternatives: pedometers, pressure-sensitive mount detectors, and monitors of impedance of vaginal mucus. Pedometers - and other sensors that measure physical activity - became the most promising technology. A survey of large dairies in North America (613 ± 46 cows) reported that, in addition to visual observation, the most used method for estrus detection was tail chalk, followed by pressure-activated mount detectors and pedometers (Caraviello et al., 2006). The tail-chalk technique consists in applying chalk to cows' tail-heads, with the objective of detecting estrus by removal of chalk as a consequence of standing to be mounted. This method has low cost, intermediate labour requirements, and has been applied with considerable success (Firk et al., 2002), but its detection principle is dependent on mounting activity. Automated activity monitors have been under development for a couple decades and are presented as a promising tool due to enhanced estrus detection via continuous surveillance of behaviours that are not directly dependent on standing to be mounted, as well as because of their simultaneous application towards other areas (e.g. health and welfare) and additional potential benefits to reproductive management and performance, which will be presented in this section.

Technology for automated estrus detection has been developing quickly, increasing the availability of sensors that are more precise, longer-lasting, and have greater capacity for data storage and transmission. Consequently, behavioural descriptions that had been previously done by visual observation (Hurnik et al., 1975; Pennington et al., 1985) are now being studied with aid of automated sensors such as mount detectors (At-Taras and Spahr, 2001; Rivera et al., 2010) and physical activity monitors (Løvendahl and Chagunda, 2010; Aungier et al., 2012; Valenza et al., 2012).

In one of the earliest studies using pedometers for estrus detection, Kiddy (1977) observed that step counts during estrus were 4-fold greater than during baseline periods. Others have reported maximum step counts 8 h after peak behavioural expression and estradiol concentration, and concluded that activity was an inefficient measurement due to timing relative to other events (Lyimo et al., 2000). Nowadays, sensors can generate real-time alerts at the onset of high activity and indicate optimal insemination timing based on time of estrus onset instead of time of peak

activity. The most important detection criteria should be those that can predict ovulation time with greater accuracy. Sensor development until the early 2000's had already set activity as the most reliable measurement for automated estrus detection, although error rates still limited its application (Firk et al., 2002).

Modern automated detection tools monitor behaviour continuously and generate alerts when deviations from baseline behaviour are detected for a given animal (e.g. when a cow is in estrus, lame or sick). Examples of AAM measurements are step counts, acceleration of leg or neck movements, rumination time, lying time and lying bouts. Automated activity monitoring systems are composed of sensors attached most frequently to a cow's neck or limb. These sensors store information until it is transferred to a central computer via antennas at the milking parlour entrance or automatically every 1 or 2 h. Activity indexes are calculated by algorithms, which also generate insemination alerts to be visualized using specific software. Although algorithms are proprietary to companies, their calculation is usually based on the relative change in activity of a given cow compared to that same cow's previous activity profile. In Canada, AAM are being used by 52% of free-stall dairy herds (Denis-Robichaud et al., 2016a).

Detection rates of 71 and 72% have been reported for cows housed in free-stall barns (Valenza et al., 2012) or on pasture (Aungier et al., 2012), respectively. Another experiment with collar-mounted sensors attached to grazing cows reported 62% sensitivity and 77% positive predictive value (PPV; Kamphuis et al., 2012). After presynchronization with 2 injections of $PGF_{2\alpha}$ 14 d apart, Fricke et al. (2014) observed 70% of cows in high activity until start of an Ovsynch protocol 12 d later. It should be highlighted that AAM can improve the detection rate if estrous behaviour is expressed. Alternatively, if the problem is lack of estrus expression, another focus is needed. Identification of factors impacting the degree of estrus expression could be used to diagnose poor behavioural estrus at herd and cow levels. Once these factors have been identified, actions concerning management, housing, nutrition, or even genetic selection can be taken in favour of improved estrus expression and overall herd performance.

Intensity (magnitude of behavioural change) and duration of estrus are measurable characteristics of estrus expression. While standing estrus lasts approximately 5.5 to 9.5 h (At-Taras and Spahr, 2001; Lopez et al., 2004; Rivera et al., 2010; Sveberg et al., 2011), duration of high physical activity (i.e. time above threshold) approximates 10 to 16 h (Løvendahl and

Chagunda, 2010; Aungier et al., 2012; Valenza et al., 2012). A longer duration of increased physical activity is likely associated with greater display of mounting or other secondary behaviours prior to standing estrus, although these measurements were not obtained within a single experiment for comparison purposes.

Quantification of activity increase (i.e. estrus intensity), is usually reported relatively to a baseline period and processed as standard deviations, percent change, fold change, or proprietary indexes. Research has reported estrus intensity in the order of 2 to 4-fold increase [334 ± 156%, Madureira et al. (2015); 377 ± 156%, López-Gatius et al. (2005b); 2.8-fold; Løvendahl and Chagunda, (2010)]. These results are comparable to the pioneer 4-fold increase obtained by Kiddy (1977). Descriptive estrus intensity data are frequently omitted in exchange of detection rate and sensor precision. Intensity and duration of AAM alerts could be applied towards identification of false positives, prediction of fertility, and genetic selection, and should therefore be thoroughly investigated. The development of a body of literature on estrus characteristics for cows of varied production levels and housing systems is essential for development of more accurate AAM and expansion of its secondary applications.

Other behaviours such as rumination, feeding and lying patterns can also be automatically recorded. Changes in feeding times, which are in accordance with the increased physical activity and restlessness characteristics of estrus, have been associated with decreased rumination and feeding time at d -1 and d 0 relative to AI (Pahl et al., 2015). Restlessness can be measured via increased physical activity, as well as by changes in lying behaviour or even in feeding behaviour. There has been little research on the use of lying and standing behaviour for estrus detection. Rutten et al. (2013) reviewed 48 papers but only two reported lying and standing patterns (Brehme et al., 2008; de Mol et al., 2009). The first evaluated six cows and one estrus; the second studied 10 cows and 40 estrus events. Sensors that measure lying time could be applied to estrus detection but there is a dearth of information on how lying and standing times change during estrus in free-stall housed dairy cattle. Given the increase in walking activity during estrus, it is logical to expect an increase in standing time. Recently, Dolecheck et al. (2015) reported a reduction in lying time and bout frequency during estrus in lactating cows and suggested that these measurements could be used for automated estrus detection. Combination of multiple measurements within a single detection system can reduce error rate and increase

accuracy of AAM (Firk et al., 2002; Dolecheck et al., 2015). Another practical benefit of AAM, in addition to increased detection rate, is the prediction of interval to ovulation. Data on time of estrus onset and knowledge of ovulation timing relative to estrus onset can be used to determine optimal AI timing (Stevenson et al., 2014), potentially increasing fertilization and conception rates.

One challenge with AAM is the accuracy of detection, i.e. occurrence of false negative events (missed preovulatory periods) and false positive alerts. The error rate ($FP / [TP + FP] * 100$; complementary to $PPV = TP / [TP + FP] * 100$) indicates the percentage of false alerts. The combined evaluation of detection rate ($sensitivity = TP / [TP + FN] * 100$) and error rate provides information about a system's performance (Firk et al., 2002). A large number of false positives has been reported (Aungier et al., 2012), but this should be carefully interpreted considering the criteria used to validate events. Estrus is usually confirmed by ovarian ultrasonography, concentration of progesterone in blood or milk, or visual observation of standing to be mounted. False positive alerts had reduced duration and intensity compared to true positives, which also had a clear positive association between intensity and duration (Aungier et al., 2012). Examination of the cow and use of herd-level data could also flag false estrus (Roelofs et al., 2010). As previously mentioned, the combination of measurements in a single sensor is another tool to enhance accuracy by increasing specificity (Firk et al., 2002). While false positives can be double checked, characterized, and potentially removed by algorithms, false negative events mean failure of estrus detection. This might lead to cows remaining nonpregnant for another estrous cycle, unless pharmacological interventions are applied. Valenza et al. (2012) highlighted the importance of establishing a comprehensive reproductive program that ensures breeding of cows that fail to be detected by AAM, or that fail to express estrus.

A second challenge with use of AAM is the large variation of estrus expression, which contributes to the difficulty of determining AI and ovulation timing (Roelofs et al., 2005). Variation can also be seen as potential for use of AAM measurements as selection criteria, as Cummins et al. (2012) observed greater estrus activity in cows with a "Fertility +" genotype. López-Gatius et al. (2005b) reported a range of estrus activity increase of 80 to 993%. It has also been reported that the majority of estrus events are characterized by long duration and low frequency of standing to be mounted, while events of long duration and high frequency of standing to be mounted are the least frequent combination (Dransfield et al., 1998; Lopez et al.,

2004; Figure 1.3). In addition, circadian rhythms might contribute to variation in behavioural expression. More cows were in estrus between 0600 h and 0800 h, although onset of standing estrus occurred at greater frequency from 1800 h to 0000 h (Hurnik et al., 1975). Peralta et al. (2005) observed 43% of estrus starting between 0100 h to 0600 h when detected by pedometers. This could be related to lower basal activity during the night and resulting greater relative change, what can be corrected with refined algorithms. A more modern AAM attached to cows on pasture did not evidence circadian effects on time of estrus onset (Aungier et al., 2012).

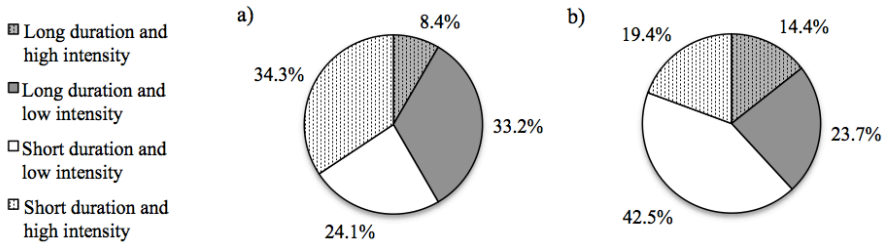

Figure 1.3 Frequency distribution of estrus characterized by intensity and duration

Intensity (frequency of standing to be mounted/h) and duration (time interval between first and last standing to be mounted) were measured based on standing estrus. Measurements were taken with radio-telemetric mount detectors. a) Dransfield et al. (1998): intensity [high if at least 1.5 standing to be mounted events/h]; duration [long if > 7 h]; b) Lopez et al. (2004): intensity [high if at least 2.7 standing to be mounted events/h; duration [long if > 8.7 h].

1.5 Timed Artificial Insemination

While reviewing the literature on estrous behaviour, it became evident that most research was published until the late 1980's, while in the last decade, there has been abundant research on automated estrus detection. During this time interval, most AAM developmental research was published (Firk et al., 2002). A reduced focus on estrous behaviour during that time could also be attributed to the advance of TAI (Pursley et al., 1995), which allows achievement of satisfactory pregnancy rates by submitting a greater number of cows for AI without the need for estrus detection.

There are two main types of TAI synchronization protocols. The first is based on GnRH supplementation to induce ovulation, formation of a new CL, and start of a new follicular wave

(e.g. Ovsynch; Pursley et al., 1995). The second type, estradiol-progesterone based (E2/P4) protocols, starts with estradiol benzoate to induce follicular atresia and emergence of a new follicular wave, followed by insertion of an intravaginal controlled drug-releasing device (CIDR) impregnated with progesterone (Pereira et al., 2015). In both cases $PGF_{2\alpha}$ is used to induce luteolysis. GnRH (Ovsynch) or estradiol cypionate (E2/P4 protocol) can be used to induce ovulation, and cows are inseminated at a fixed time according to the expected ovulation time.

Ovsynch successfully synchronized 87% of ovulations, with 6% of ovulations occurring before the day of TAI and 7% failing to ovulate until 48 h after GnRH injection (Vasconcelos et al., 1999). Cows in that study had conception rate between 32 and 42%, with the advantage of 100% insemination rate. Similarly, Pereira et al. (2015) reported conception rate of 30% with an E2/P4 based protocol. Ovulation rate was approximately 85%, and estrus expression rate was of 80 to 87%, season-dependent (Pereira et al., 2015). Because cows are given estradiol cypionate as the last injection of E2/P4 protocols, behavioural estrus is more frequently observed than in GnRH-based protocols, but all enrolled cows are inseminated in either case. Cerri et al. (2004) demonstrated that conception and pregnancy rates of high producing Holsteins are improved by adding exogenous estradiol to TAI protocols.

Although conception rates are usually similar between TAI and breeding after spontaneous estrus, TAI resulted in more cows pregnant at 60 and 100 DIM (Pursley et al., 1997), likely due to greater rate of submission to AI. The improved reproductive efficiency obtained with TAI comes with product costs, increased labour and animal handling. The use of TAI as a major tool for reproductive management of dairy cows has been challenged by increased consumer concern about food choices, particularly regarding administration of drugs to farm animals, animal welfare, and environmental impact of animal production. A search for sustainable solutions for dairy cow fertility moves away from TAI as a herd treatment in favour of individual fertility treatments. Increased estrus detection and potentially increased conception rates obtained with use of AAM present an alternative for reproductive management with reduced hormone use, considering that expression of estrus is satisfactory.

Comparisons between TAI and AAM as main reproductive management tools have reported similar conception rates (30% vs. 31%) and days to pregnancy (137 and 122 d) among cows bred by TAI or following automated estrus detection, respectively (Neves et al., 2012).

Recent studies that have experimented with different combinations of AAM and hormonal synchronization programs (Stevenson et al., 2014; Fricke et al., 2014; Burnett et al., 2017) indicate comparable pregnancy rates among those strategies. Similar observations were reported in a survey of Canadian dairy herds (Neves and LeBlanc, 2015).

It is worth mentioning that expression of estrous behaviour at the day of TAI has been associated with increased in conception rate (Cerri et al., 2004; Souza et al., 2007; Pereira et al., 2014). For example, Pereira et al. (2014) reported conception rates of 51 and 39% for cows expressing estrus and not expressing estrus at TAI, respectively; these authors also reported reduced pregnancy losses between 32 and 60 d when cows showed estrus at TAI. This raises questions about the relationships between estrus, its associated physiological mechanisms, and fertility. Automated activity monitors provide the necessary tools to measure estrus expression and investigate these hypotheses.

1.6 Summary of Gaps in the Literature

Behavioural expression of estrus has been studied for many decades, as it is essential in determining when and which cows to inseminate. This rate at which cows are inseminated (i.e. service rate) is seemingly persistently low. Helmer and Britt (1985) stated that "detection of estrus continues to be a problem on dairy farms". Denis-Robichaud et al. (2016a) reported an insemination rate of 44% in Canadian dairy herds. Currently, AAM allows increased detection rate and provides measurements of estrus beyond a dichotomous detection response. Although there is considerable amount of research investigating the efficiency of these sensors, little is known about estrus characteristics and the potential of transforming these data into valuable information for reproductive management. Focusing on the use of AAM-generated data, two main areas in need of further research were identified.

The first refers to knowledge about estrus characteristics across categories of cattle and for a same cow, and identification of factors affecting these characteristics. Current AAM are efficient in detecting estrus, but there is added potential in understanding how estrus measurements are influenced by cow and environment-related factors. Together with identification of new measurements, the refinement of technologies can lead to improved sensitivity and reduced error rates.

A second gap concerns the application of quantified estrus expression as a phenotypic measurement of fertility. This is a complex research area, given the multitude of factors influencing behaviour and fertility. An initial approach to this question would be to investigate the association between estrus characteristics (e.g. estrus duration and intensity) and pregnancy outcomes. Knowledge on variability of estrus expression could contribute to this area of research, which would also benefit from information on agreement between estrus characteristics provided by different systems or based on different criteria.

1.7 Thesis Research Questions

The general objective of this thesis was to improve understanding of automated measurements of estrous behaviour in heifers and lactating cows, where heifers represent a sample population without influence of high milk yield. This research aimed at supporting AAM data interpretation and application by studying sources of variation, patterns of estrus expression between contemporary pen-mates and among estrous cycles of a same individual, and by measuring agreement between different estrus characteristics and AAM systems. Secondary objectives were to investigate the potential of these systems and point to achievable future features. By studying the associations between estrus characteristics and fertility, we anticipated to assess the importance of estrus expression as a phenotypical marker of fertility and the potential of AAM to provide real-time information about individual and herd-level reproductive performance.

Using a variety of AAM to characterize estrous behaviour, the following specific questions were addressed:

- *Chapter 2:*
    - What is the magnitude of change in walking activity of heifers in estrus? Can we identify sources of variation and patterns of expression among and within heifers?
    - How is lying behaviour of heifers affected by estrus? Is it associated with walking activity?
- *Chapter 3:*
    - What is the level of association between automated measurements of estrus by two different AAM?

- *Chapter 4:*
    - Does lying behaviour of lactating cows change with estrus? Is this change associated with fertility?

Chapter 2: Automated and Visual Measurements of Estrous Behaviour and their Sources of Variation in Holstein Heifers

2.1 Walking Activity and Frequency of Behavioural Display

A version of Section 2.1 has been published: B.F. Silper, I. Robles, A.M.L. Madureira, T.A. Burnett, M.M. Reis, A.M. de Passillé, J. Rushen, and R.L.A. Cerri. 2015. Automated and visual measurements of estrous behavior and their sources of variation in Holstein heifers. I: Walking activity and behavior frequency. Theriogenology. 84:312-320.

2.1.1 Introduction

Estrus detection is an essential component of reproductive programs in dairy cattle, but concerns about low rates of estrus detection are not recent (e.g. Helmer and Britt, 1985). During the past 20 years, timed AI protocols have improved service and pregnancy rates with satisfactory results (Santos et al., 2004). Recent interest in reproductive programs with minimal pharmacological intervention presents a new opportunity for the use of automated estrus detection tools, thus the need for further research on behaviour, detailed measurements from automatic monitors of activity, and variability between and within cows.

Automated systems for estrus detection identify preovulatory follicular phases with variable rates of success depending on pre-determined thresholds (Aungier et al., 2012). There are relatively few studies of detailed automated estrus activity measurements that include sufficient number of observations to provide statistical power above 0.80. Furthermore, walking activity and behavioural measurements of estrus are subject to variation originating from lactation, social interactions, housing, age, genetics, and physiological aspects (Galina and Orihuela, 2007). Studies of estrous behaviour and walking activity of heifers provide assessment of individual and environmental sources of variation while controlling for some of the lactation-linked factors.

Further studies of automated and visually measured estrous behaviour, their relationship, and inherent variation are essential for improvement of currently available automated

technologies. For example, definition of time of estrus onset based on increased walking activity is one of the areas that needs refinement for improved accuracy and determination of AI timing. Measurements of baseline activity, time of the day of estrus onset, and sources of variation among and within animals have not been substantially reported in the literature and are key components to determine relative and absolute increase in activity and duration of estrus. Such measurements of estrus intensity have been described as possible indicators of fertility (López-Gatius et al., 2005b; Madureira et al., 2013) and perhaps markers for phenotypic selection for this trait.

Acceptance of mount (i.e. stand to be mounted) is the classical, and still the primary estrous behaviour commonly observed. However, this behaviour alone may not be as useful as believed for estrus detection (van Eerdenburg et al., 1996). Secondary behaviours that occur at greater frequencies could be as important and more likely to be detected.

In this study, estrous behaviour of heifers was described in detail using automated measurements of walking activity as well as video observations of behaviour. Our objectives were to a) identify absolute and relative measurements from sensors and behaviours from video observations during baseline and estrus periods, and b) evaluate the variation in estrus expression between and within heifers and the possible sources of variation (e.g.: number of heifers simultaneously in estrus, season, time of day at estrus onset), using sensors to measure estrus expression. Our hypotheses were that estrous behaviour would be characterized by greater frequency of behavioural display and increased walking activity, and that factors such as a smaller number of heifers in estrus, pubertal estrus, and summer would be associated with reduced expression of estrous behaviour, whereas time of estrus onset would not influence behavioural estrus.

2.1.2 Materials and Methods

2.1.2.1 Heifers and Housing

This study was conducted at the University of British Columbia's Dairy Education and Research Centre (Agassiz, BC, Canada) from March 2012 to July 2013. The experimental herd is

closed and had an average 305-d mature equivalent milk yield of 12,236 ± 2,219 kg/cow in 2013. The local Institutional Animal Care Committee, following the requirements of the Canadian Animal Care Council (CACC), approved all experimental procedures.

Holstein heifers (n = 57) were housed in a sand-bedded free stall barn with rubber flooring on the feed bunk alley from 6 to 13 mo of age. Heifers were managed in groups of seven to 12 heifers/pen, where the maximum age difference was 3 mo. Pens were 6.7 m x 12 m and had 13 stalls each. Total mixed ration was offered once/d (0900 h) and pushed up three times/d (at approximately 1100 h, 1800 h, and 2200 h). Water was available *ad libitum* from one water bin/pen. All heifers were visually checked for signs of disease or injuries twice/wk at the time of ultrasonography. No major occurrences of disease or injury were registered during the experimental period.

2.1.2.2 Estrus Events and Sensors

Ovarian dynamics were determined by twice/wk ovarian scans with ultrasound equipped with a 7.5 MHz linear rectal transducer (Ibex Pro; E. I. Medical Imaging, Loveland, CO). Puberty was determined based on presence of a CL at ovarian ultrasonography. Pubertal estrus was identified in 46 of the 57 heifers (9.0 ± 1.0 mo old and 309.3 ± 34.3 kg body weight at puberty onset; mean ± SD). The remaining 11 heifers had a CL at one or more of the first four ovarian scans (8.6 ± 1.2 mo old and 295.1 ± 33.0 kg body weight), therefore these could not be confirmed as the CL of the pubertal estrus.

Walking activity was measured with accelerometers (IceTag sensors, IceRobotics Ltd., Edinburgh, Scotland) attached with a custom flexible plastic strap to the metatarsal region on one of the hind limbs during the whole period. The sensor's output consisted of number of steps, lying and standing time, and frequency of lying bouts per minute. The effects of estrus on lying and standing patterns are presented in a sister research paper (Silper et al., 2015a)[1]. Data were downloaded from these devices once/wk while heifers were restrained on headlocks, and processed with the manufacturer's software (IceTag Analyzer 2011; IceRobotics Ltd., Edinburgh, Scotland). Download and re-activation were performed without removal of the

[1] This citation refers to the manuscript presented in Section 2.2 of this thesis.

sensor from the heifer's limb resulting in a recording gap of approximately 10 min. IceTags have been validated for lying behaviour (Trénel et al., 2009), step counting by comparison with video recording (Nielsen et al., 2010), and estrus detection with research-developed algorithms (Jónsson et al., 2011). IceTags accurately measure number of steps, although false steps may occur (Nielsen et al., 2010). In the current study, the accuracy of the sensors for step counting on growing heifers was confirmed by comparison with results obtained by two different observers counting steps from video recordings during two 24 h periods (data not shown). Files from the sensors provided number of steps/min. An Excel macro (Excel; Microsoft Corporation, Redmond, WA) was developed to generate a chart of the rolling sum of steps for every 24 h period beginning at each min (Figure 2.1). Activity peaks identified in those charts were validated against expected estrus date ranges obtained from the interpretation of ovarian ultrasonography. The existence of a preovulatory follicle before the activity peak followed by appearance of a CL in the same ovary in the subsequent scanning indicated ovulation and validated the activity peak as true estrus.

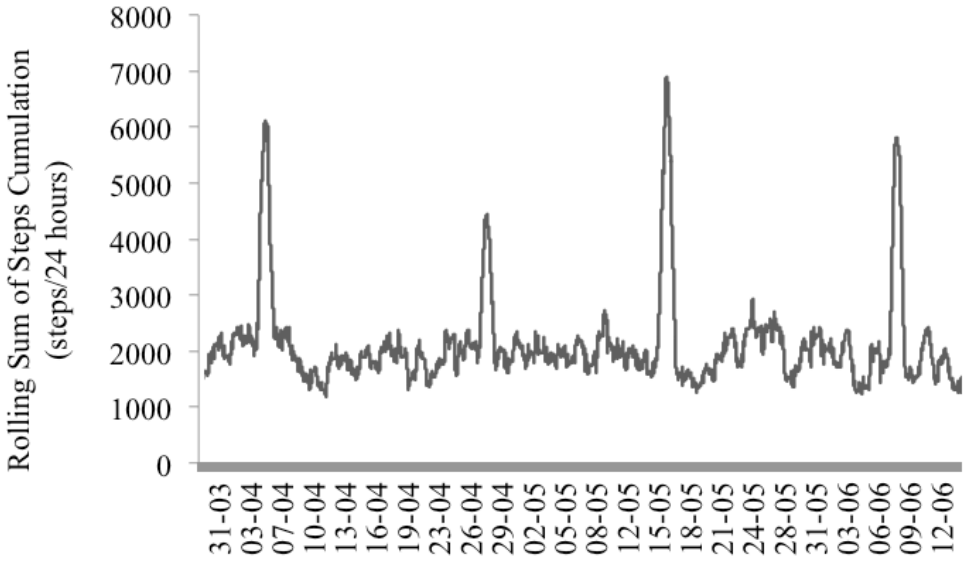

Figure 2.1 Rolling sum of steps cumulation for 24 h periods

Example of 24 h rolling sum of steps used to identify activity peaks; step counts were obtained from IceTag sensors attached to a hind limb of a Holstein heifer.

After determining the day of peak activity, the hour of start (estrus onset) and end of each estrus were identified based on the summed frequency of steps in 2 h-blocks. The 90th percentile

for the frequency of steps in all 2 h-blocks during a one-wk period (day of activity peak ± 3 d) was calculated ("90th percentile rule"). An estrus event was then identified as a sequence of at least two 2 h-blocks with a frequency of steps above the 90th percentile (cluster of estrus activity). The beginning of estrus was considered as the start hour of the first 2 h-block of the estrus cluster. An estrus event was considered to be finished when at least three 2h-blocks with a frequency of steps below the 90th percentile was observed following the estrus cluster. The end hour of the event was the end hour of the last 2 h-block of the estrus cluster. A maximum of two 2 h-blocks with a frequency of steps below the 90th percentile could be inside the estrus cluster. Estrus duration and total number of steps corresponded to the sum of all 2 h-blocks (including the ones below the 90th percentile) that composed the estrus cluster and the respective sum of steps. The number of baseline steps were then calculated as the mean number of steps for the same 2 h-blocks of the 3 d prior to the estrus cluster. The relative increase in walking activity during estrus was calculated as *[(Total estrus steps – Total baseline steps)/Total baseline steps] x 100%* and is presented in percent values. Season of the year, hour of estrus onset and number of heifers simultaneously in estrus in the same pen were recorded for each event.

Heifers were also classified as having high (above average; > 84 steps/h) or low (average or below average; ≤ 84 steps/h) level of baseline walking activity. This measurement was obtained from the mean steps/h taken in a 48 h-period 3 d before the estrus event that was closer to the age of 11 mo, including data from all 57 heifers. The estrus closest to 11 mo old was chosen as the earliest time point where data could be obtained from all heifers at the same age.

2.1.2.3 Estrous Behaviour and Video Recording

Behaviour was monitored continuously (24 h) during 12 estrus events from each of 12 heifers (one estrus/heifer) using two video cameras/pen, which were positioned on the barn ceiling to capture the whole area of the pen (CCTV camera, model WV-BP330, Panasonic, Osaka, Japan; with F1.4/2.5-18 6 mm lenses). Red lamps were positioned close to the cameras for low-light recording.

Heifers observed for estrous behaviour were 10.1 ± 0.5 mo old (mean ± SD), weighed 331.3 ± 31.3 kg and measured 125.7 ± 4.1 cm in the withers at the time of the evaluated event.

These 12 heifers were distributed in two groups of 11 and 12 heifers of similar age/pen. Estrus events occurred during April (n = 6) and July and August 2012 (n = 6). Continuous video reading was done for 15 h before and 15 h after a peak of activity (IceTag data). The frequency of each behaviour was registered in 30 min intervals. A total of 30 h of observation was used to ensure that the complete estrus event was included in the observation because duration of estrus has been reported to range from 3 to 24 h in primiparous Holstein cows (Roelofs et al., 2005). Baseline behaviour was evaluated in a corresponding 30 h interval, distributed over d -8, -7, and -6 relative to the day of estrus, with the objective of having a more representative sample than with only one day of baseline observations. Video evaluation of baseline was done in a "2 h-on/4 h-off design". Starting from the first hour, 2 h of video were watched, then the following 4 h were skipped, and the next 2 h were watched again. This pattern was repeated for the 3 d of baseline resulting in 30 h of video observation across three consecutive days. The two observers who watched the videos had a reliability of 0.89. Inter-observer reliability was measured in two 8 h periods of estrus (time of activity peak ± 4 h) for each of two different heifers.

Nineteen behaviours were defined (Table 2.1) and their frequency of occurrence counted from the continuously recorded videos. Videos were watched at 4x speed. The occurrence of each behaviour was recorded as a single event. If the same behaviour occurred twice in a row with an interval longer than 12 sec between them (3 sec counted on video at 4x speed), the second occurrence was counted as a new display. The methodology described above for IceTag data were used to obtain estrus and baseline steps, relative increase in walking activity, and duration of estrus based on increased walking activity for these 12 events. Duration of standing estrus determined from video observations was defined as the interval between first and last acceptance of front or back mount.

2.1.2.4 Data and Statistics

Descriptive statistics were calculated for the response variables (baseline and estrus total steps and steps/h, estrus relative increase in walking activity, estrus duration, and interval between consecutive events) using the UNIVARIATE procedure of SAS 9.3 (SAS Institute Inc., Cary, NC). Spearman Rank correlation was used to study the relationship between the relative

increase in walking activity and duration of estrus. Distribution of start hour (estrus onset) and end hour of estrus was tested with chi-square test.

Total estrus steps, estrus steps/h, estrus duration, and relative change in walking activity at estrus were individually analyzed by ANOVA with proc MIXED of SAS. The effects of day (estrus vs. baseline), season of the year, hour of estrus onset (sorted in six classes of 4 h each), number of heifers simultaneously in estrus, individual category of baseline walking activity (high vs. low), estrus order (pubertal estrus vs. second and greater estrus), and their 2-way interactions were tested. Only variables and interactions that had significant effects ($P < 0.05$) on the response variable were kept in the models. Visually observed behaviours were analyzed as the difference in frequency of expression from estrus to baseline and tested using the Signed Rank test of proc UNIVARIATE of SAS. Frequencies were often zero during the baseline period, resulting in non-normal distributions where transformations were not applicable.

2.1.3 Results

A total of 350 estrus events from 57 heifers were evaluated. Sixty eight events were excluded for the following reasons: data from IceTags were incomplete or missing (n = 27), the activity peak was not paired with ovulation (n = 10), the activity peak occurred during the luteal phase (n = 3), the presence of a CL without a corresponding activity peak (n = 15), the activity peak occurred before the first ultrasound (n = 4), repeated peaks within an interval were shorter than 4 d without the possibility of identifying which peak corresponded to estrus (n = 9). Eleven out of 15 events that had a CL but no activity peak correspond to pubertal estrus (i.e. first ovulation of a heifer). Other CL detected without activity peaks might be false negatives or estrus was expressed at a level lower than could be detected. Activity peaks without a CL, however, represent either false positives or ovulation failures. This experiment was not designed to assess these effects and therefore this remains an open question for research.

Despite missing data and activity peaks with intervals shorter than 4 d, the sensitivity of estrus detection was 95% (using ultrasonography as a reference). Specificity could not be calculated, but it should be noted that only 13 of the 295 activity peaks were not validated as estrus according to the data from ovarian ultrasonography.

Table 2.1 Definitions of behaviours evaluated on video

Behaviour	Definition
Chin Rest and Accept or Reject Chin Rest	The actor (standing) puts or rests her head on the back or rump of the receptor (standing). The receptor stays in the same place for at least 3 sec (acceptance of chin rest), or the receptor walks away (rejection of chin rest).
Crossover	One heifer (standing) walks through the alley that connects front and back of the pen (either direction). The event is recorded when the heifer, coming from one side, puts both feet down the step of the other side.
Eat and Drink	One heifer puts her head completely through the headlock (eating) or over the water bin (drinking) for more than three seconds.
Follow and Be Followed	The actor (standing) immediately follows the receptor as she walks away, and stops walking when she stops, keeping a maximum distance of one head length. Following has to be at least three steps for the actor (counted on the leg with the IceTag).
Head Butting	Two heifers (both standing) hit head with head at least one time from the front and after that either from the front or sides of the head, in an attempt to push the other heifer backwards. Both heifers have their heads down.
Lick and Be Licked	The actor (standing) licks the receptor (standing or lying down). While licking the actor does at least two vertical head movements of short amplitude.
Mount and Accept or Reject to be Mounted [a]	The actor (standing) does a quick movement towards the receptor (standing or lying down), taking at least one foot off the ground, but without resting her chest on the receptor's rump or withers (incomplete mount) or the actor (standing) stands on her rear legs with the chest touching and applying pressure on the receptor's rump or withers for 3 sec or more (completed mount). The receptor can either stand still (acceptance of mount) or walk away (rejection of mount).

Behaviour	Definition
Push/Be Pushed	The actor (standing) pushes the receptor (standing or lying down) with her head, at any part of the receptor's body with exception of the head. Pushing involves use of physical pressure or force and excludes rubbing. The receptor does not push back as observed in the head butting.
Sniff and Be Sniffed	The actor (standing) sniffs the receptor in the posterior region for at least three seconds. The receptor can be either standing or lying down. While sniffing, the muzzle has to touch the receptor or be at a maximum distance of one muzzle length, without licking.
Stand On Occupied Bed	One heifer (standing) stands with her front legs on the curb of an occupied stall.

[a] Behaviours related to mounting were classified as 'back' if the rump region was mounted, or 'front and side mounts' if the head or on the sides of the receptor's body were targeted.

2.1.3.1 Estrus Activity and Sensors

Hour of estrus onset and end were not evenly distributed along the day ($P < 0.0001$). Estrus events started most frequently in the morning (39% starting between 0600 h and 1000 h), and ended more frequently between 2000 h and 0000 h (Figure 2.2).

Expression of estrus as measured by sensors varied greatly within and between heifers. Table 2.2 presents the descriptive analysis of estrus characteristics. Coefficients of variation among all evaluated events for total estrus steps, relative increase in walking activity, duration, and interval between estrus events were 37%, 41%, 27%, and 12%, respectively. The variation within heifer (3 to 8 estrus events/heifer) was similar to the between-events variation. On average, within-heifer CV for total estrus steps, relative increase in walking activity, duration, and interval between estrus events were 27%, 39%, 24%, and 8%, respectively. The distribution of CV for relative increase in walking activity for each heifer is presented in Figure 2.3.

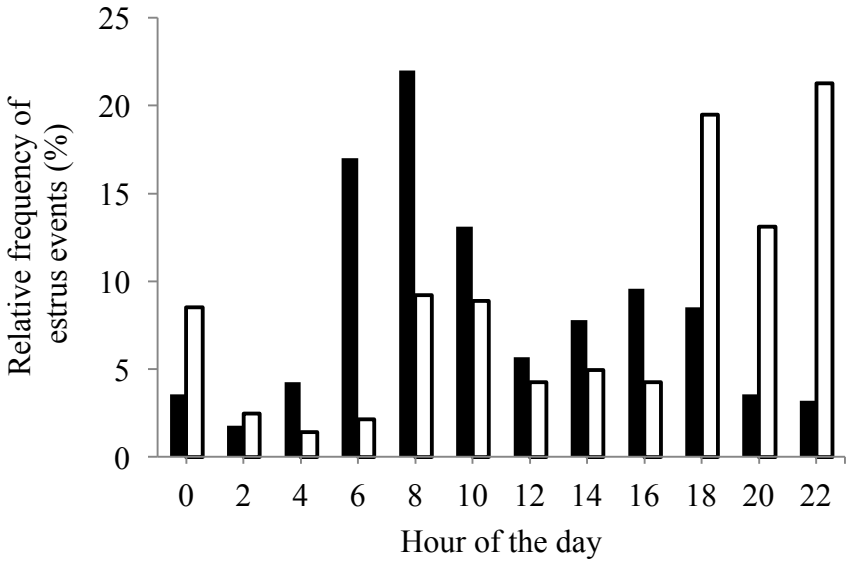

Figure 2.2 Relative frequency distribution of hour of onset and end of estrus

Estrus was determined as periods of increased walking activity from sensor data and confirmed by ovarian ultrasonography. Black bars represent hour of estrus onset and white bars represent hour of end of estrus (n = 282 estrus events)

Table 2.2 Automated measurements of walking activity and estrus expression

Variable	Mean (SD)	Minimum	Maximum
Baseline			
Total number of steps[a]	1,319 (498)	292	3170
Number of steps/h[b]	99 (39)	24	317
Estrus			
Total number of steps	4,743 (1740)	837	10,070
Number of steps/h	343 (96)	129	969
Relative increase in walking activity, %[c]	290 (160)	30	1,190
Duration, h	13.8 (3.8)	4	26
Interval between estrus events, d	19.9 (2.4)	10	31

n = 282 estrus events.

[a] Total number of steps per estrus event; [b] Total number of steps divided by duration of estrus in hours; [c] *Relative increase in walking activity = [(Total estrus steps – Total baseline steps)/Total baseline steps] x100%.*

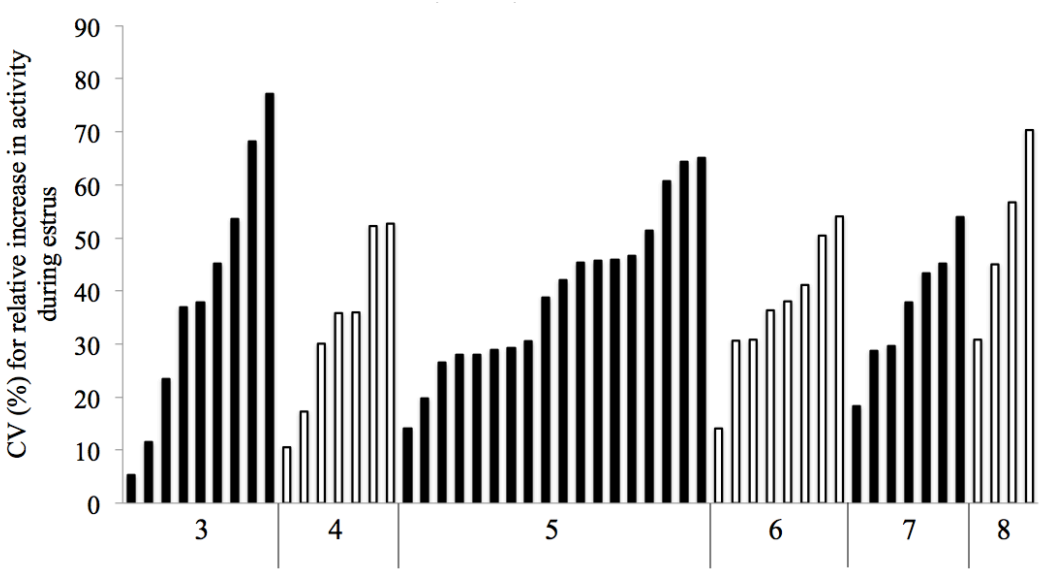

Figure 2.3 Within-heifer coefficient of variation for estrus relative increase in activity

Numbers from 3 to 8 on the x-axis represent number of events/heifer. Each bar indicates the coefficient of variation (CV; %) for the relative increase in walking activity of all estrus events of one heifer. Heifers with less than three estrus events were excluded from this analysis.

The studied fixed effects significantly influenced estrus characteristics. The only effect with no influence on estrus characteristics was the number of heifers simultaneously in estrus ($P > 0.05$). Pubertal estrus was markedly different from later events ($P < 0.01$). The total number of steps was 3,641 ± 238 for the first estrus and 5,072 ± 127 for second and greater events. Steps/h during the first and later events were, respectively, 296 ± 12 vs. 346 ± 6. The pubertal estrus also had shorter duration ($P = 0.03$) and smaller relative increase in walking activity (12.2 ± 0.7 h and 257 ± 27%, respectively; $P < 0.0001$) than the second and greater events (14.3 ± 0.3 h and 352 ± 17%).

Heifers classified as having high baseline walking activity had greater total steps and steps/h at estrus ($P < 0.05$; Figure 2.4) than heifers with low baseline walking activity. However, no effect of category of baseline walking activity was observed for duration and relative increase in walking activity during estrus ($P > 0.05$).

Relative increase in walking activity was the only estrus measurement influenced by season ($P = 0.03$). Relative increase in walking activity was greater for winter estrus events (356

± 33%) than for events occurring in the spring and summer (260 ± 21% and 286 ± 22%, respectively), but it was not different between these seasons and fall season (318 ± 27%). Hour of estrus onset also influenced the relative increase in walking activity. It was observed that estrus events with onset between 1600 h and 0300 h had greater relative walking activity increase than those starting at other times of the day ($P < 0.0001$; Figure 2.5). The interval between estrus events was not influenced by estrus order nor by season ($P > 0.05$). The two measurements characterizing estrus expression - relative increase in walking activity and duration - were positively correlated (r = 0.38; $P < 0.0001$).

2.1.3.2 Visual Observation of Estrous Behaviour

The 12 video-recorded estrus events had median steps/h for the 30 h baseline and estrus periods of 90 and 206 steps/h, respectively, with mean relative increase in walking activity of 289% and duration of 14.3 h. Duration of standing estrus was 9.1 ± 5.5 h (interval from first to last accepted mount). One estrus had no duration since only one acceptance of mount was observed during the 30 h observation period.

Frequency of behaviour display increased greatly from baseline to estrus (Table 2.3). The average difference in display frequency between estrus and baseline was different from zero for all behaviours ($P < 0.05$) with exception of reject chin rest, reject back mount, eat, and drink ($P > 0.05$). There was a large variation in frequency of behaviour display between estrus events, as evidenced by the quartiles on Table 2.3. Behaviours with greatest difference in display from estrus to baseline were chin rest, sniff, back mount, crossover, accept chin rest, and follow.

Twelve of the 19 behaviours (Table 2.1) were observed during the observation period at all events. Back mount, front mount, accept and reject front mount, reject back mount, lick, and stand on an occupied bed were not observed during one or more estrus periods. During baseline periods, 12 behaviours were not displayed at one or more events. Only push and be pushed, be licked, chin rest, and – naturally – eat, drink, and crossover were displayed during baseline periods by all heifers.

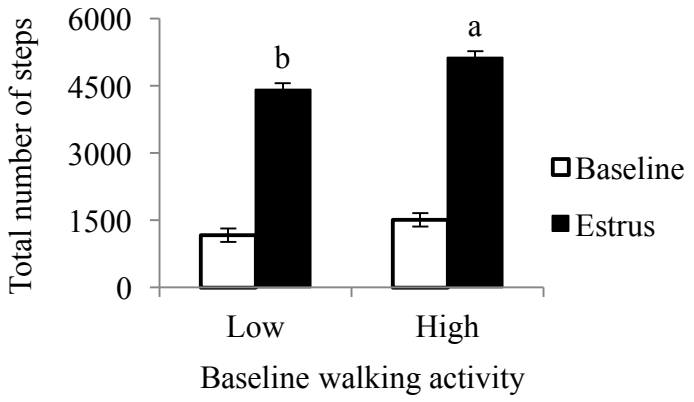

Figure 2.4 Baseline and estrus total steps by category of baseline walking activity

Black bars indicate estrus periods and white bars indicate corresponding baseline periods. Baseline walking activity was classified as 'high' if > 84 steps/h and 'low' if ≤ 84 steps/h at two baseline days at 11 mo old. Total number of steps is presented as means ± SEM. Within estrus periods, different letters differ ($P = 0.04$).

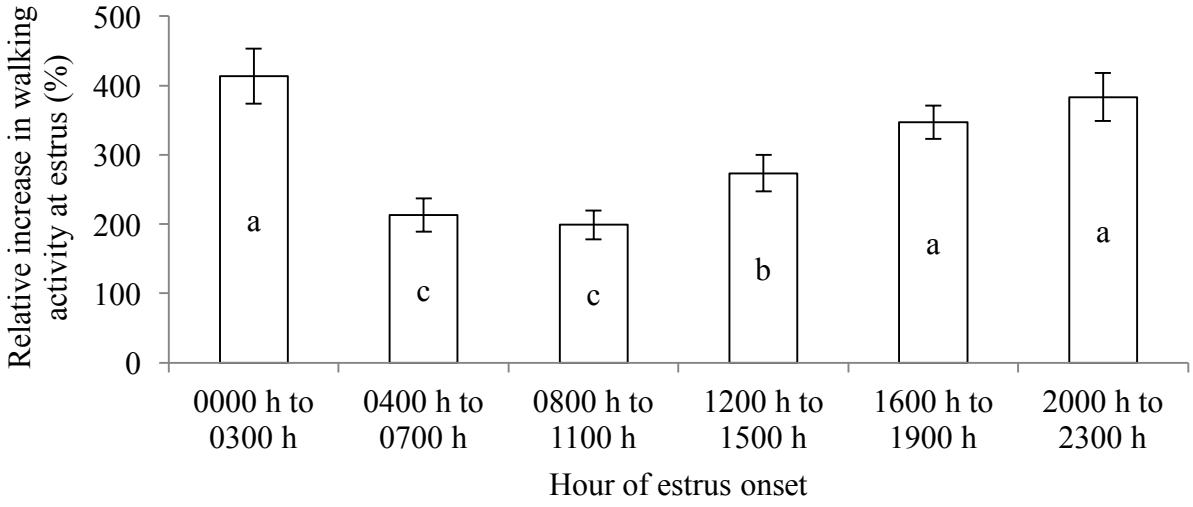

Figure 2.5 Estrus relative increase in activity according to hour of estrus onset

Relative increase in walking activity (%) is presented as means ± SEM. Different letters indicate difference among categories of hour of estrus onset ($P < 0.0001$).

Table 2.3 Frequency of behaviour display by Holstein heifers during estrus and baseline

	Estrus					Baseline				
Behaviours	Minimum	Q1	Median	Q3	Maximum	Minimum	Q1	Median	Q3	Maximum
Front Mount	0	2	5	14	63	0	0	0	0	15
Back Mount	0	43	53	80	125	0	0	1	2	5
Accept Front Mount	0	1	4	11	23	0	0	0	0	1
Accept Back Mount	1	8	26	33	59	0	0	0	0	4
Reject Front Mount	0	0	1	2	9	0	0	0	0	2
Reject Back Mount	0	0	1	6	42	0	0	0	1	16
Chin Rest	74	109	132	192	226	3	5	8	13	26
Accept Chin Rest	4	25	39	53	127	0	0	2	4	27
Reject Chin Rest	1	4	8	18	55	0	1	2	5	46
Sniff	22	37	57	91	191	0	2	4	7	9
Be sniffed	8	13	14	19	50	0	2	4	6	17
Lick	0	9	30	49	102	0	2	5	9	27
Be Licked	4	9	13	25	36	1	3	6	9	15
Head Butt	4	8	11	20	36	0	2	4	8	15

	Estrus					Baseline				
Behaviours	Minimum	Q1	Median	Q3	Maximum	Minimum	Q1	Median	Q3	Maximum
Push	9	14	33	46	72	2	8	14	20	34
Be Pushed	11	23	41	51	81	1	13	18	24	43
Stand on occupied bed	0	2	5	10	42	0	0	1	1	2
Follow	6	23	30	70	89	0	0	0	1	9
Be Followed	2	6	9	10	32	0	0	0	1	7
Crossover	18	40	49	66	90	5	8	9	11	23

Behaviour data were obtained from 30 h of continuous video observation of 12 estrus events and corresponding baseline periods (one estrus/heifer). Estrus periods were previously identified using walking activity data obtained with leg-mounted accelerometers and confirmed by ovarian ultrasonography.

2.1.4 Discussion

This study describes detailed metrics of estrus expression, as measured by sensors and video recording cameras. Large variation between estrus events and within heifers was demonstrated and the factors with the greatest impact on this variation were identified. Category of baseline walking activity, hour of estrus onset and behaviours frequently displayed during estrus, such as alley crossover, could improve estrus intensity calculations and detection by automated activity monitors or by a combination of different methods. Further, the current study was able to demonstrate these measurements in an appropriate number of heifers and estrus events, with several repetitions within heifers. Measurements of walking activity during estrus varied more than our initial expectations, raising further questions on the possible sources of variation, and on the potential use of this information to improve current technologies or to select individuals for optimal estrus expression. The study on nulliparous heifers should provide a reliable reference for further studies on the effects of age, lactation and management on expression of estrus and perhaps indicate the potential of each heifer for estrus expression.

Descriptions of estrous behaviour have been done mostly in lactating cows, using a variety of methods such as visual observation (Sveberg et al., 2011), mount detectors (At-Taras and Spahr, 2001; Rivera et al., 2010), pedometers (Roelofs et al., 2005), activity monitors (Løvendahl and Chagunda, 2010), or combinations of these (Holman et al., 2011). Also, there are not many recent studies reporting detailed absolute measurements. Arney et al. (1994) reported around 100 steps/8 h of baseline, and 400 steps/8 h of estrus. These values are similar to those observed in one hour in the current study, indicating differences in pedometer technology and/or animal's baseline and estrus activity levels. It is important to take into account the differences between systems and methodologies when comparing results of estrous behaviour studies, as well as differences in estrus expression due to genetic selection over time. Criteria used for determining duration of estrus, or type of automated estrus detection system will likely yield different results. In addition, given the demonstrated variation, studies of estrus expression should include a large number of animals or events to minimize data dispersion. Variation from day to day, or between non-estrus and estrus days, has been studied and shown to be large enough to justify its use for estrus detection (Kiddy, 1977; Schofield et al., 1991), but the literature lacks discussion regarding the variation in estrus expression within individuals.

The mean relative increase in walking activity was 290%, a value similar to that observed by Løvendahl and Chagunda (2010) also in heifers. These authors reported estrus duration of 9.2 h, whereas in our study the mean duration was of 13.8 h. It is clear from this study that individual estrus events vary significantly (4 to 26 h duration; 30 to 1,190% relative increase of walking activity) in animals of a similar age and not influenced by hoof problems, health issues, or milk production. Even more surprising is the size of this variation within the same heifer over several estrus events. The average within heifer CV for relative increase in walking activity (Figure 2.3) was 39.3% and had a large variation itself (range from 5% to 89%). The CV within and between-heifers for duration and number of estrus steps was lower than for the relative increase in walking activity during estrus, but still represents great variation. Selection of cows with high and relatively constant expression of estrus could be possible, but data collection for such traits would require information from several estrus events given the variation observed for measurements of estrus expression. Associations between estrus activity of the lactating cow and reproductive parameters from the heifer-rearing period could also be useful.

Interestingly, there was a high frequency of estrus events starting in the morning and ending at late night. Aungier et al. (2012) reported no difference in time of estrus onset along the day for lactating cows. The highest frequency of estrus onset in the morning and the highest frequency of end of estrus at night is coherent with the daily pattern of activity, since duration was determined based on periods of increased walking activity. Hurnik et al. (1975) reported the greatest number of cows in estrus is between 0600 h and 0800 h and greater frequency of estrus onset from 1800 h to 0000 h. This is consistent with observations by Gwazdauskas et al. (1983), who reported a greater frequency of mounts in the morning than in the afternoon period of visual observation.

Events starting between 0400 h and 1100 h and between 1600 h and 1900 h, which are times of greater circadian activity, had the lowest total number of estrus steps. This cannot be associated to estrus of shorter duration, since there was no effect of time of onset on estrus duration. The smaller total number of steps observed for events occurring at hours of higher circadian activity resulted in smaller relative increase of walking activity at those times. Estrus events with onset at night (1600 h to 0300 h) had the greatest relative increase in walking activity.

The category of baseline walking activity, used to indicate if heifers were routinely more or less active than average, might be an important factor in selecting cows that have a greater level of estrus expression. Heifers classified as having high baseline walking activity took approximately 15% more steps during estrus than those with low baseline walking activity. In addition to a greater total number of steps, the number of steps/h at estrus was also greater for heifers with high baseline walking activity, while this variable did not influence the duration of estrus or its relative increase in walking activity. It has been reported that cows with lower basal activity had estrus of lower activity, but of greater proportional activity increase (Phillips and Schofield, 1989). To the best of our knowledge, there is no recent study clearly suggesting an association of baseline levels and absolute and relative increase in estrus intensity. The category of baseline activity did not influence the measurements obtained here for intensity (relative increase in walking activity) and duration of estrus, but the effect on number of steps during estrus suggest an important factor to be considered for automation of estrus detection. Collectively, the effect of time of day when estrus is initiated and the inclusion of baseline categories could be new additions to improve calculations of estrus intensity. The relative and absolute intensity of estrus seem to provide promising associations with fertility (López-Gatius et al., 2005b; Madureira et al., 2013) and the possibility for further selection of this trait.

The relative increase in walking activity was greater during the winter months, likely due to lower environmental temperature leading to a greater level of activity. Another possible hypothesis is that there was an effect of photoperiod on activity, which has been described by Phillips and Schofield (1989) but was not tested in the present study. The difference in estrus expression from winter to spring and summer events was of 96 and 70 percent units, respectively, and is in agreement with previous reports (Peralta et al., 2005). Mounting activity has been observed to increase with increasing environmental temperature up to 25°C, but to reduce at higher temperatures (Gwazdauskas et al., 1983).

The pubertal estrus was 2.3 h shorter, had relative increase 100 percentage units smaller, and around 30% less total estrus steps than second and greater events. In addition to the lower expression of the detected pubertal estrus, 11 of the 46 heifers with detected first ovulation did not show a corresponding activity peak. The absence of exposure to progesterone prior to the first ovulation is probably the cause of the silent and low intensity estrus (Allrich, 1994).

Among the video-observed behaviours, chin rest, sniff, back mount, crossover, accept chin rest, and follow had the greatest difference in display from baseline to estrus. Chin rest and back mount are commonly evaluated at estrous behaviour studies. Among the behaviours not frequently mentioned in relation to estrus, follow and crossover could be important components of estrous behaviour and activity increase. Crossover between front and back of the pen was also much more frequent during estrus than baseline and is related to the increased number of steps/h seen at estrus. This increased use of the pen alley (crossover) could represent an opportunity for simple automation of passage records by radiofrequency identification tags. Furthermore, proximity loggers could be useful tools to detect increased frequency of following, behaviour that was also highlighted by Sveberg et al. (2011).

As much as the value of relative increase in walking activity depends on how baseline activity is measured, duration of estrus also depends on the criteria used to define its onset and end. If increased walking activity is chosen as the criteria, the 12 events described from video recording had an approximate duration of 14 h. However, if duration is based on observed behaviour (standing estrus), duration for the same events would be of 9 h. Researchers have identified low frequency and duration of mount acceptance by lactating Holstein cows (At-Taras and Spahr, 2001; Lopez et al., 2004; Rivera et al., 2010), but this is the most reliable visual sign of estrus (Galina and Orihuela, 2007). The ratio of accepted to rejected mounts, for example, could provide information about the relationship of standing and being mounted. Helmer and Britt (1985) reported that 86% of attempts to mount are performed by heifers in proestrus and estrus and that the number of mounts and stands to be mounted increased when two heifers were in estrus instead of only one at a time. Walking activity could also be influenced by the presence of a sexually active group. However, the number of heifers simultaneously in estrus did not have a significant effect on any of the variables obtained from IceTag sensors. Estrus walking activity is probably more dependent on the individual and thus not dependent on availability of other heifers for behavioural interactions. Other factors such as plasma estradiol concentration, expression of estradiol receptors in the brain, and level of progesterone from the previous cycle likely also influence the behavioural expression of estrus (Roelofs et al., 2010).

Front and back mounts and stands to be mounted accounted for 88 events per 30 h period per heifer (approximately three mounts and standings/h) and could be inefficient as the estrus sign to be detected in programs with poor visual observation schedules (e.g.: 30 to 60 min

twice/d). High producing dairy cows were reported to mount only six to eight times/estrus (Lopez et al., 2004; Rivera et al., 2010). The frequencies reported here for heifers are greater than those reported for dairy cows (Lopez et al., 2004; Rivera et al., 2010), but still not high enough to guarantee detection based simply on this behaviour if visually observed for such short periods of time. Previous reports (Britt et al., 1986; van Eerdenburg et al., 1996) have suggested that performing front mounts and rejecting mounts are discriminative estrous behaviours. We did not observe increased frequency of rejected back mounts from baseline to estrus, in contrast with van Eerdenburg et al. (1996). Weight, size, and hoof health might account for differences in standing behaviour among young and adult cows. Frequent rejection of mounts should not be discarded as an important component of visual estrus detection, as it can be an indicator of imminent estrus onset (Hurnik et al., 1975).

The most frequent behaviour during estrus was chin rest. This secondary behaviour is easy to be correctly identified and occurred at the lowest frequency of 74 times during a 30 h interval. Other secondary behaviours that had considerably greater frequency during estrus were sniff, lick and follow (Table 2.3). Behaviour display was also analyzed as frequency/h of estrus duration (based on walking activity). The most frequent behaviours were the same as reported for the complete 30 h period. Chin rest, back mount, sniff, acceptance of chin rest and crossover occurred an average frequency of eight, four, four, three, and three displays/h, respectively. Acceptance of back mounts averaged two displays/h (range from 0 to 4), and acceptance of front mounts averaged zero displays/h, varying from 0 to 1. The behaviour display/h of estrus duration reinforces the importance of secondary estrous behaviours for visual estrus detection. The reported values are means, and thus there is no guarantee that these behaviours will be displayed at times of visual observation, usually done for 30-min periods twice/d. Evidence from the literature (Galina and Orihuela, 2007; Roelofs et al., 2010) is in agreement with the current results, but probably more emphasis should be placed on high-frequency secondary signs, as mount and stand to be mounted occur sparsely.

2.1.5 Conclusions

The current study demonstrated the large variation of estrus expression existing within and between heifers. The time of estrus onset and the category of baseline walking activity

influenced estrus duration and activity increase. Baseline steps and relative walking activity increase during estrus could be possible phenotypical targets to predict fertility and to assist selection for this trait. Behaviours such as chin rest, crossover, and follow had the greatest difference is expression from baseline to estrus and should be emphasized in visual and automated estrus detection programs and technologies. The association of different measurements is a resource that should be further explored for improvement of estrus detection sensitivity and specificity.

The results presented here are important as a base for comparison with lactating cows and raise the question on potential for genetic selection of cows with a more constant and higher level of estrus expression. Further studies with focus on individual variation of behavioural estrus and its relationship with other reproductive parameters and physiological measurements will improve our understanding of estrus expression and estrus detection tools.

2.2 Standing and Lying Patterns

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2.2.1 Introduction

The recent development of sensors that can automatically monitor animal behaviour allows for more complete and precise measurements of estrus expression. Pedometers started being tested in the 1980's as an alternative to visual observation of estrus and to improve estrus detection rates (Rutten et al., 2013). Since then, different kinds of sensors have become available to monitor animal activity, focusing mostly on step count or acceleration of movement. Research to validate sensors and identify factors affecting behavioural expression of estrus is key for proper estrus detection.

Restlessness is commonly cited as one of the main behavioural changes at estrus (van Eerdenburg et al., 1996; Roelofs et al., 2010). Increased walking activity, which is the most

common behavioural change measured by estrus detection devices, and the behaviour of following other cows are indicators of restlessness (Diskin and Sreenan, 2000). An increased number of changes in position (Walton and King, 1986; Kerbrat and Disenhaus, 2004) and a decrease in the total daily lying time at the time of estrus have been suggested as alternatives that could be automatically measured (Kerbrat and Disenhaus, 2004). Restlessness, however, is subjective when visually evaluated (van Eerdenburg et al., 1996).

There has been little research on the application of lying (LY) and standing (ST) behaviour towards improvements in estrus detection rate and accuracy. Rutten et al. (2013) reviewed 48 papers but only two reported LY or ST data (Brehme et al., 2008; de Mol et al., 2009). The first analyzed six cows and one estrus, and the latter 10 cows and 40 estrus events. Sensors that measure LY time have the potential to be used for estrus detection but there is a dearth of information on how LY and ST times change during estrus. Given the increase in walking activity during estrus (Kiddy, 1977), it is logical to expect increased ST time during the same period. While describing estrous behaviour of heifers (Silper et al., 2015b)[2], it was noticed that heifers spend long periods of time exclusively ST when in estrus.

If changes in LY and ST measurements from baseline to estrus are consistent and of sufficient magnitude, they could represent potential additions to estrus detection systems. Sensors using a combination of measurements would not only have improved sensitivity and specificity for estrus detection, particularly for events of low intensity, but also greater accuracy for AI timing.

Many factors are known to influence estrous behaviour (Roelofs et al., 2010). Understanding how these factors influence activity measurements is essential to set threshold levels for automated detection. Here, effects of season, estrus order, number of heifers simultaneously in estrus, baseline levels of activity, and time of estrus onset were tested with various measurements of ST and LY. Research on estrous behaviour of Holstein heifers provides an opportunity to understand estrus without the influence of milk production, hoof and hock injuries, and variable metabolic states, all of which are known to influence estrus expression in lactating dairy cows (Galina and Orihuela, 2007; Walker et al., 2008; Roelofs et al., 2010).

[2] This citation refers to the manuscript presented in Section 2.1 of this thesis.

Our objectives were to 1) quantify changes in patterns of LY and ST between d -7 and d +2 relative to estrus, 2) assess factors contributing to its variability, and 3) identify which measurements have potential for automation of estrus detection and precise determination of time of estrus onset. We hypothesized that estrus would be associated with altered lying and standing patterns, as well as that factors such as pubertal estrus, smaller number of heifers simultaneously in estrus, and warmer weather would be associated with reduced behavioural change at estrus.

2.2.2 Materials and Methods

The study was performed at the University of British Columbia's Dairy Education and Research Centre (Agassiz, BC, Canada) from March 2012 to July 2013. The experimental procedures followed the requirements of the Canadian Animal Care Council (CACC) and were approved by the local Institutional Animal Care Committee.

2.2.2.1 Heifers and Housing

Holstein heifers (n = 57) born between May 2011 and August 2012 were group-housed from 6 to 13 mo of age in a sand-bedded free stall barn with rubber flooring on the feed bunk alley. Heifers were housed in groups of seven to 12 heifers/pen. Pens were 6.7 m x 12 m and had 13 stalls each. Heifers were fed TMR formulated for weight gain of 1.0 kg/d. Feed was offered once/d and pushed up three times/d. Water was available ad libitum.

Heifers reached puberty (first CL detected at ovarian ultrasonography) at 9.0 ± 1.0 mo of age and body weight of 309.3 ± 34.3 kg (mean ± SD). Thirteen percent of the estrus events occurred from 7.2 to 9.0 mo of age, 48% from 9.1 to 11.0 mo, and 36% from 11.1 to 13.8 mo.

2.2.2.2 Sensor Recording Procedures and Estrus Definitions

Accelerometers (IceTag sensor, IceRobotics Ltd., Edinburgh, Scotland) were attached with a custom flexible plastic strap to the metatarsal region of one of the heifers' hind limbs for the whole trial. The sensor's output consisted of number of steps taken, LY and ST time, and LY

bouts on a per minute basis. Data were downloaded from the sensors once/wk while heifers were restrained on headlocks, using a specific reader unit (IceReader, IceRobotics Ltd., Edinburgh, Scotland), and processed with IceTag Analyzer 2011 software. Download and re-activation of IceTags were done without removing the device, so that two consecutive files have a gap between recordings of approximately 10 min. During this time, the heifers were always standing by the feed bunk. IceTags had been previously validated by comparison with video recordings to effectively measure LY behaviour (Trénel et al., 2009) without influencing the animal's behaviour (Gibbons et al., 2012).

Estrus events were identified based on increased count of steps/h and confirmed as true estrus by ovarian ultrasonography, which was done twice/wk (Ibex Pro; E. I. Medical Imaging, Loveland, CO, equipped with a 7.5 MHz linear rectal transducer). The complete methodology used for identification of estrus is described in Section 2.1.2.2. Briefly, peaks of activity (i.e. estrus events) were identified in a chart of the rolling sum of steps over 24 h periods. After detection of activity peaks, start and end of each event were determined based on the summed frequency of steps in 2 h-blocks. The 90^{th} percentile for the number of steps for all 2 h-blocks during a one-wk period (day of activity peak ± 3 d) was calculated and 2 h blocks that met or exceeded the 90^{th} percentile were included to make up the estrus period and determine time of onset and time of end of estrus. Duration and total number of steps during estrus were calculated as the sum of all 2 h-blocks that comprised the estrus cluster. Baseline steps were then calculated as the mean frequency of steps for the same 2 h-blocks 3 d prior to the estrus cluster. Relative increase in walking activity was defined as *[(Total estrus steps – Total baseline steps)/Total baseline steps] x100%.*

The estrus day is referred to as d 0. Since many estrus events stretched over two calendar days, the day where the majority of the estrus occurred was considered to be d 0. When events were evenly split over two days, the day that included the peak of activity was considered d 0. Day -7 represents an average of d -8, -7, and -6 relative to estrus. Other non-estrus days (d -2, -1, +1, and +2 relative to estrus) were also analyzed.

2.2.2.3 Sensor Corrections

IceTag sensors counted many LY bouts of extremely short duration and occasionally more than one or two LY bouts per minute resulting in an overestimated number of bouts/d. Macros (Microsoft Excel, Microsoft Corporation, Redmond, WA) were developed to delete false LY bouts (i.e. one or more bouts/min for a number of consecutive minutes) and to identify ST bouts. The macro corrected up to three consecutive errors by converting the seconds of ST or LY to the position detected during the minutes surrounding the error. With this, any minute containing both ST and LY where the previous and following minutes were fully spent on one of the two positions was converted to the correct position. The applied correction is in agreement with validation by Ledgerwood et al. (2010). Macros were also used to calculate start day, start time, and duration of each bout (min/bout). An example of ST and LY bouts summarized by day is presented in Figure 2.6. Macro-edited files were validated with videos (d 0 of five estrus events from different heifers, by one observer). Mean bout duration and duration of the longest bout (ST and LY) measured from videos or obtained from macro-edited files were comparable at a ratio of 1.1. Macro-edited files counted 10% less ST bouts than observed on video. All bouts observed on video and not counted in macro-edited files were shorter than 1 min.

2.2.2.4 Statistical Analyses

Data were summarized as frequency and as mean, minimum and maximum duration of ST and LY bouts on a daily basis (midnight to midnight) and processed with proc MEANS (SAS version 9.3, SAS Institute Inc., Cary, NC). Number of ST bouts/d, total daily ST time (min/d; obtained from the sum of bouts started on each day), mean ST and LY bout duration, and duration of the longest ST and LY bout of each day were calculated from the daily summaries.

The data set linked bout duration only to the day it started. Therefore, a ST bout that extended from one day to another had its full duration added to the total daily ST time of the day in which it started. The same is true for LY bouts. This meant that individual days did not have exact 1,440 min, but consecutive days added up to 24 h/d. Hour of start and end of the longest bout of a day (ST and LY) were recorded and are reported as medians.

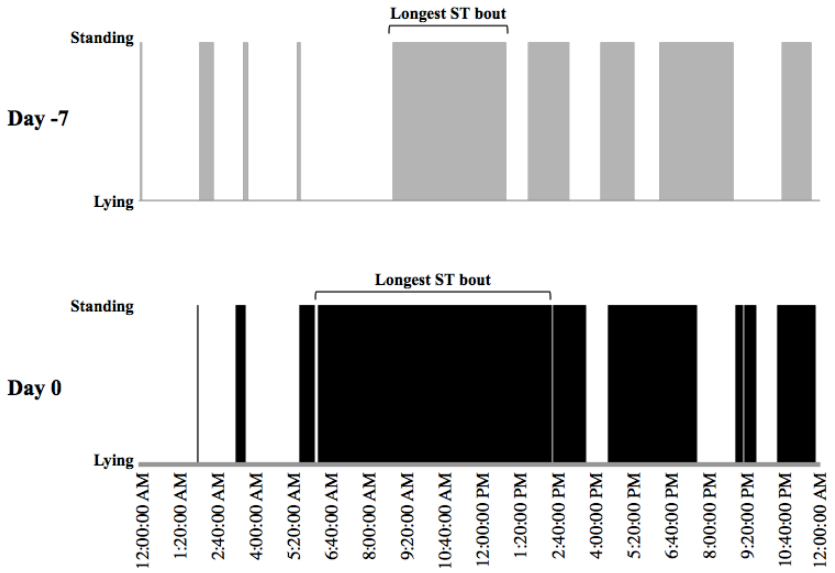

Figure 2.6 Standing and lying bouts summarized by day during estrus and baseline periods
Example of standing (ST) and lying bouts for one period of estrus (d 0) and its corresponding baseline (d -7). Full bars represent standing bouts and empty bars represent lying bouts.

A total of 283 activity peaks were confirmed as estrus by ovarian ultrasonography (detailed information on baseline and estrus number of steps, relative increase in walking activity and duration of estrus are presented in Silper et al. (2015b). Lying and standing data were available from 269 of those events (4.8 ± 1.6 events/heifer). Seventeen events had one or more days (except for d 0) partially or completely missing, totaling 20 missing days. Data from these days were deleted, but the event was kept in the dataset.

Estrus events were identified as pubertal (35 of the 57 heifers had their first estrus recorded) or as second and greater events. They were also classified according to season of the year (warm – April to September; cold – October to March; mean low and high temperatures were 5 to 24 °C and -1 to 15 °C, respectively), according to time of estrus onset (morning – 0400 h to 1100 h; afternoon – 1200 h to 1900 h; night – 2000 h to 0300 h), and according to the number of heifers in estrus simultaneously (one, two, or three) in the same pen. Heifers were classified as presenting high or low baseline walking activity. Categories of baseline walking activity were determined from the total number of steps taken on d -3 and -2 prior to the estrus

that occurred closest to the age of 11 mo. Heifers were classified as having high (above average; > 84 steps/h) or low (average or below average; ≤ 84 steps/h) level of walking baseline activity.

Descriptive statistics (mean, standard deviation, coefficient of variation, Q1 and Q3) for LY and ST bout variables at d -7 and d 0 were obtained with proc UNIVARIATE. Repeated measures ANOVA (proc MIXED) was used to assess the effects of estrus order, season of the year, time of estrus onset, number of heifers in estrus simultaneously, category of baseline walking activity (high vs. low), and its 2-way interactions on LY and ST bout response variables. Only variables and interactions with $P < 0.05$ were kept in the model. The mixed model included day as fixed effect, heifer as random effect, and days within event as repeated measures. Contrasts were analyzed for all days to evaluate effects of estrus on d -1 and d -2 (late proestrus), and to determine if behaviour returned to basal (d -7) within the first 2 d post-estrus.

Equivalence between distribution of start and end hour of the longest ST bout of d -7 vs. d 0 was analyzed with Friedman's chi-square test using proc FREQ. Start and end hour of the longest ST bout are reported as medians for d -7 and d 0. The relationship between LY and ST variables at d 0 and measurements of walking activity (estrus steps/h, duration, relative increase in walking activity, and hour of estrus onset and end) obtained previously from the same estrus events (Section 2.1) was tested with Spearman Rank correlations. Correlations were also performed between start and end of the longest ST bout across days, for start of longest ST bout vs. time of estrus onset, and for end of longest ST bout vs. end of estrus. The difference in hours from start of longest ST bout and time of estrus onset, and from end of longest ST bout and end of estrus are reported as means ± SD and Q1 and Q3.

2.2.3 Results

2.2.3.1 Summary of Standing and Lying Patterns

Estrus (d 0, defined as the day of peak walking activity) significantly influenced all variables ($P < 0.05$; Table 2.4). The ratio between total daily ST time and total daily LY time was of 0.7 for d -7, - 2, +1 and +2. It increased to 1.0 at d -1 and 1.9 at d 0 ($P < 0.0001$). Among the tested univariable fixed effects, only the number of heifers simultaneously in estrus did not

influence any of the variables ($P > 0.05$). Coefficients of variation were, in general, greater for ST than for LY measurements, as well as higher at d 0 than at d -7 (Table 2.4).

2.2.3.2 Total Daily Standing Time

Overall, d 0 was characterized by increased ST time compared to d -7 ($P < 0.0001$; Table 2.4). At the day of estrus, ST time was approximately 6 h greater than at d -7. Estrus order, category of baseline activity, and time of estrus onset significantly influenced total daily ST time.

At d 0 of the pubertal estrus, total daily ST time was shorter than at d 0 of second and greater estruses (753.8 ± 36.9 vs. 879.7 ± 16.1 min/d, respectively; P = 0.04) whereas non-estrus ST time/d did not differ between first and later estrus events ($P > 0.05$). Heifers classified as having high baseline walking activity had greater total ST time than those with low baseline walking activity (Figure 2.7; $P < 0.01$). No interaction with day relative to estrus was observed for this effect ($P > 0.05$).

There was an interaction between time of estrus onset and day relative to estrus for total daily ST time ($P < 0.001$). Total daily ST time, which was 588 ± 10 min on d -7, increased to 915 ± 22 min/d on d 0 for morning-onset events ($P < 0.0001$). While the behavioral change was restricted to d 0 for morning-onset events, those starting in the afternoon or at night resulted in increased total daily ST time on both d -1 and d 0 (afternoon: 761 ± 23 and 752 ± 25 min/d, night: 757 ± 37 and 784 ± 40 min/d, d -1 and d 0, respectively; $P < 0.0001$). Total ST time for d -1 was not different from d 0 for afternoon and night events ($P > 0.05$). This effect of time of onset was characterized by smaller changes from d -7 to d 0, but an intermediary value at d -1 was also observed for number of bouts/d, mean duration of ST bouts, and duration of the longest ST bout, and are reported in the next sections.

2.2.3.3 Frequency of Standing Bouts

There were fewer ST bouts on d 0 compared to d -7 ($P < 0.05$; Table 2.4). Interactions between day and season, and day and time of estrus onset significantly influenced ST bout frequency. Overall, the number of bouts/d at d 0 was greater for events occurring in the warm

season than in the cold season (8.7 ± 0.3 vs. 7.7 ± 0.4 bouts/d, respectively; $P < 0.0001$). The number of bouts/d was not different between seasons for the non-estrus days (11.0 ± 0.3; $P > 0.05$).

An interaction between bout frequency and time of estrus onset resulted in greater bout frequency observed for events that started in the morning than for those starting at other times of the day. Estrus events starting in the afternoon or at night had a significant reduction in frequency of bouts at d 0 ($P < 0.0001$), but that was not different from the frequency observed at d -1 ($P > 0.05$; Figure 2.8a).

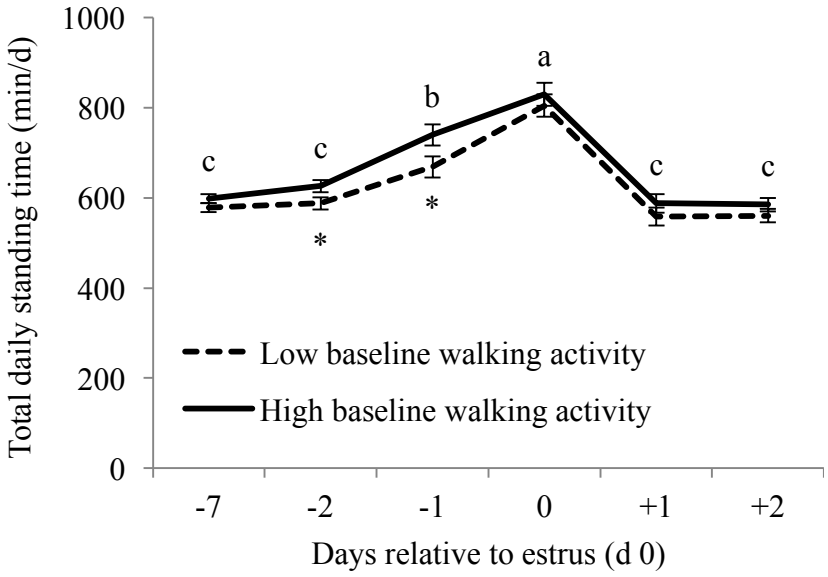

Figure 2.7 Daily standing time by category of baseline walking activity

Total daily standing time is presented as means ± SEM for d -7 to d +2 relative to estrus (d 0). Days with different letters differ within category of baseline walking activity ($P < 0.01$). Days with asterisks differ between high and low baseline level of walking activity ($P < 0.01$). Baseline walking activity was classified as 'high' if > 84 steps/h (continuous line) and 'low' if ≤ 84 steps/h (dashed line) at two baseline days at 11 mo old.

Table 2.4 Lying and standing behaviour of Holstein heifers at baseline and estrus

Measurement	Baseline (d -7)			Estrus (d 0)		
	Mean (SD)	Q1 - Q3	CV (%)	Mean (SD)	Q1 - Q3	CV (%)
Standing						
Frequency, bouts/d	11.3 (2.2)[b]	9.7 – 12.3	19.4	8.4 (3.0)[a]	6.0 -10.0	35.4
Mean bout duration, min	55.1 (12.2)[b]	46.0 – 62.5	22.2	124.7 (65.8)[a]	79.4 – 154.2	52.8
Duration of the longest bout, min	232.1 (75.5)[b]	184.9 – 259.7	32.5	488.8 (258.2)[a]	271.5 – 658.0	52.8
Daily total time, min	591.1 (64.0)[b]	549.9 – 624.8	10.8	892.4 (191.8)[a]	768.5 – 1008.0	21.5
Lying						
Mean bout duration, min	79.0 (15.0)[b]	70.5 – 88.8	18.9	71.3 (25.0)[a]	51.4 – 85.5	35.0
Duration of the longest bout, min	233.2 (82.5)[b]	180.2 – 269.5	35.4	180.5 (85.5)[a]	128.3 – 205.6	47.4

n = 269 estrus events. Day of estrus was determined as a period of high walking activity from sensor data and confirmed by ovarian ultrasonography. Different lowercase letters indicate differences between d -7 and d 0 for each measurement ($P < 0.05$).

2.2.3.4 Mean Duration of Standing Bouts

There was an increase in mean duration of ST bouts on d 0 in comparison with d -7 ($P < 0.05$; Table 2.4). Estrus order and time of estrus onset significantly affected the mean duration of ST bouts. Mean duration of ST bouts was shorter at d 0 of pubertal estrus than at d 0 of second and greater events (79.9 ± 12.4 min vs. 116.4 ± 5.3 min; $P < 0.05$). Time of estrus onset influenced this measurement in the same fashion described for total daily ST time and number of ST bouts/d (Figure 2.8b).

2.2.3.5 Duration of the Longest Standing Bout of the Day

The longest ST bout of a day was longer on d 0 (488 ± 258 min) compared to d 7 (232 ± 76 min) ($P < 0.0001$). Figure 2.6 exemplifies the difference observed between d -7 and d 0. Season, time of estrus onset, estrus order and category of baseline walking activity significantly influenced this measurement.

Pubertal estrus was characterized by shorter duration of the longest ST bout at d 0 than observed at d 0 of second and greater events (313.9 ± 47.8 vs. 472.7 ± 20.9 min, respectively; $P < 0.05$). Duration of the longest ST bout was different only at d 0 between first and second and greater events.

The longest ST bout at d 0 was greater ($P < 0.0001$) for morning events (564 ± 29 min) than for afternoon and night events (320 ± 32 and 296 ± 51 min, respectively). However, at d -1, estrus events with afternoon and night onset had greater longest bout (308 ± 23 and 324 ± 36 min, respectively) than morning events (176 ± 21 min). In addition, a high level of baseline walking activity determined overall greater longest ST bouts ($P < 0.05$), but no interaction with day was observed ($P > 0.05$). Cold season events had greater longest ST bout on d 0 (Figure 2.9; $P = 0.02$). Duration of the longest ST bout on non-estrus days was not different between seasons ($P > 0.05$).

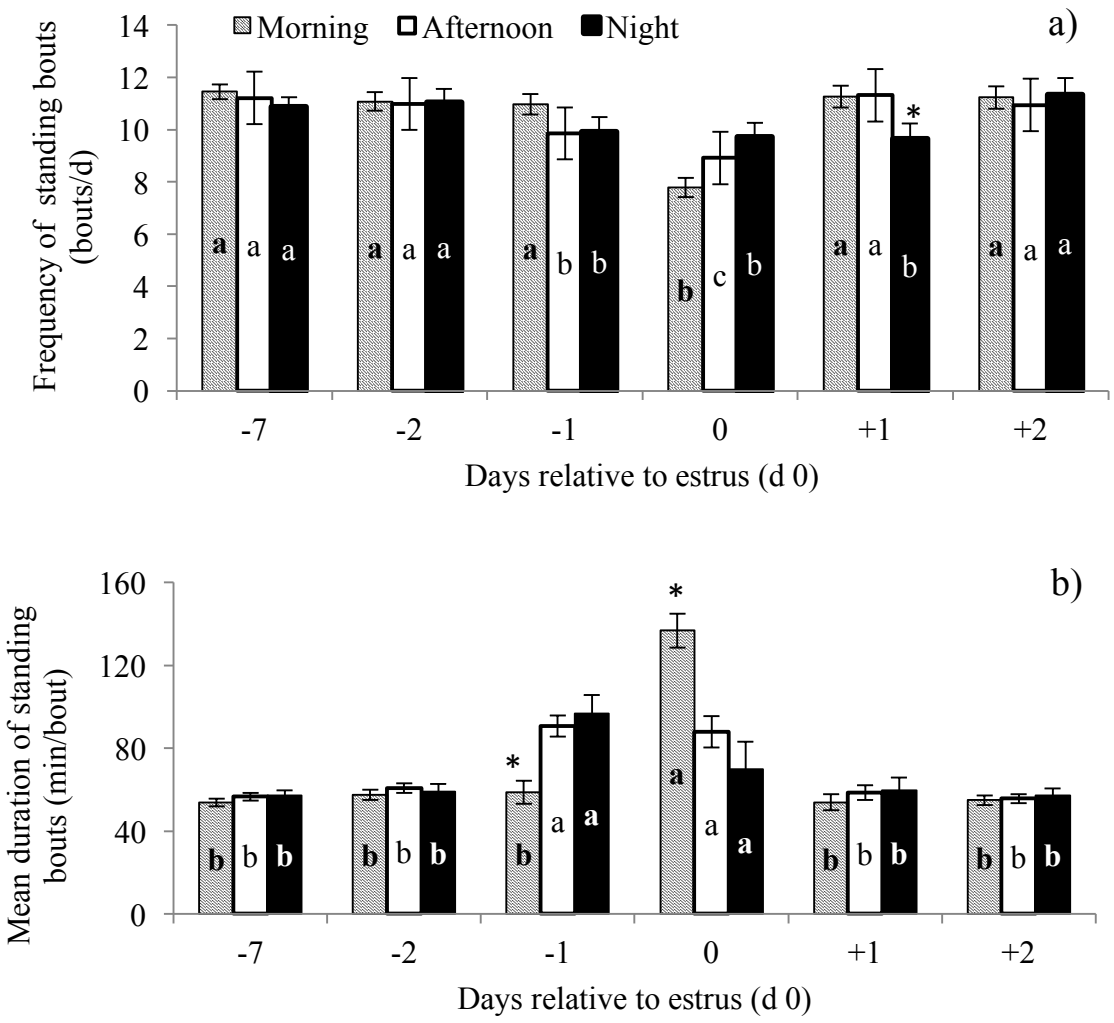

Figure 2.8 Frequency and mean duration of standing bouts

Frequency of standing bouts (bouts/d) and mean duration of standing bouts (min/bout) presented as means ± SEM for d -7 to d +2 relative to estrus (d 0). Time of estrus onset (morning [0400 h to 1100 h; dashed bars], afternoon [1200 h to 1900 h; white bars], night [2000 h to 0300 h; black bars). Different letters indicate differences within each category of time of estrus onset for each figure. Asterisks indicate difference among categories of time of estrus onset within each day ($P < 0.001$).

2.2.3.6 Relationship between Standing and Lying Measurements and Walking Activity

Step-related measurements were mostly negatively correlated to LY bouts measurements and positively correlated to ST bout measurements (Table 2.5). There were moderate correlations between duration of longest ST bout or daily ST time and estrus steps/h.

Table 2.5 Spearman Rank correlations between estrus characteristics

	Walking activity-based estrus characteristics[1]		
Measurement	Steps/h	Relative increase in walking activity	Estrus duration
Standing			
Frequency of bouts	-0.05^{NS}	0.13*	-0.07 ^{NS}
Mean bout duration	0.17*	-0.14*	0.09 ^{NS}
Duration of longest bout	0.25**	-0.04 ^{NS}	0.09 ^{NS}
Total daily duration	0.37**	-0.01 ^{NS}	0.17*
Lying			
Mean bout duration	-0.15*	-0.12*	-0.10 ^{NS}
Duration of longest bout	-0.04 ^{NS}	0.03^{NS}	0.02 ^{NS}

n = 269 events. *$P < 0.05$; **$P < 0.001$; ^{NS} $P > 0.05$

[1]Estrus steps/h, intensity and duration of estrus refer to the same estrus events reported in Section 2.1. Relative increase in walking activity was calculated as *[(Total estrus steps – Total baseline steps)/Total baseline steps] x 100%*. Baseline steps are an average of the three days previous to estrus, corresponding to the same time interval as estrus.

In agreement with the effect of estrus on duration of the longest ST bout, its hour of start and end were different for d -7 and d 0 ($P < 0.0001$). Median hour of start and end of the longest ST bout were 0800 h and 1200 h on d -7, but 0700 h and 1400 h on d 0. Start and end hour of the longest ST bout were positively correlated at all non-estrus days (r = 0.52, 0.66, 0.23, 0.70, and 0.80 for d -7, -2, -1, 1, and 2, respectively; $P < 0.001$), but were not correlated at d 0 (r = 0.09; P

= 0.14). Hour of start of the longest ST bout on d 0 had a correlation of 0.46 ($P < 0.0001$) with the time of estrus onset, but the correlation between the end hour of estrus and of the longest ST bout was of only 0.12 ($P = 0.05$). The difference between hour of start of the longest ST bout (d 0) and the hour of estrus onset was of 1.7 ± 6.1 h (Q1 and Q3 were -2 and 4 h, respectively). The difference between end hour of the longest ST bout (d 0) and end hour of estrus was of -3.5 ± 5.1 (Q1 and Q3 -7 and -1 h, respectively).

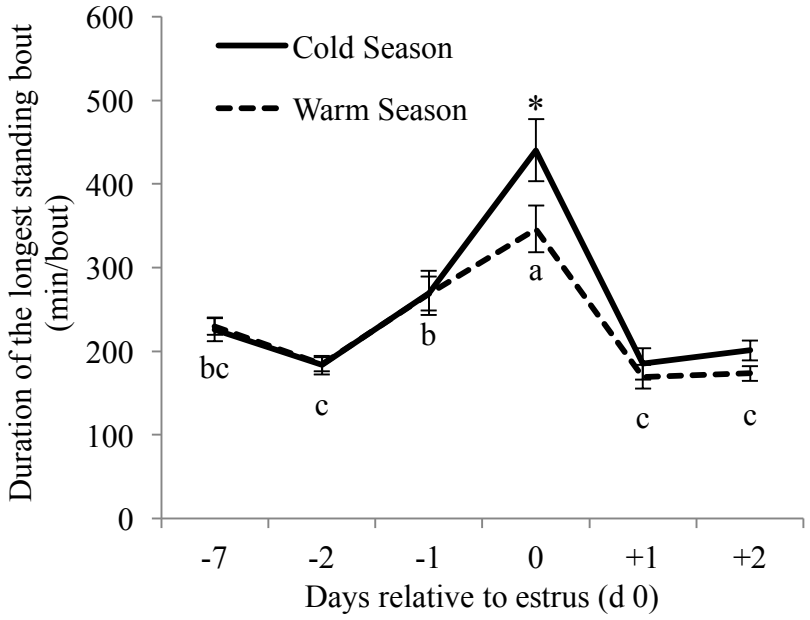

Figure 2.9 Duration of the longest standing bout of a day by season of the year

Duration of the longest bout (min/d) is presented as means ± SEM for d -7 to d +2 relative to estrus (d 0). Seasons were determined as 'warm' (April to September, dashed line), and 'cold' (October to March, continuous line). Days with different letters differ within seasons. Days with asterisks differ among seasons ($P = 0.02$).

2.2.3.7 Mean Duration of Lying Bouts and Duration of the Longest Lying Bout of the Day

Mean duration of LY bouts was shorter on d 0 than on d -7 ($P < 0.05$; Table 2.4) but there was an interaction between day and time of estrus onset ($P = 0.05$). Mean duration of LY bouts tended to be lower on d 0 for events starting at night than for those starting in the afternoon (63 ± 5 min and 75 ± 3, respectively), while events with morning onset had intermediate values (72 ± 2

min). However, at d +1, mean duration of LY bouts was greater for events with onset at night than for afternoon onset (89 ± 5 and 78 ± 3 min, respectively). Mean LY bout duration at d +1 for morning-onset events was not different from other start times (84 ± 3 min; $P > 0.10$). The longest LY bout of d 0 was not different among pubertal and second and greater estrus. However, the longest LY bout was greater at d +1 than at d 0 for second and greater events but not for pubertal estrus (Figure 2.10; $P < 0.01$). Heifers with low level of baseline walking activity tended to have greater mean duration of LY bouts and greater longest LY bouts ($P = 0.08$), but there was no significant interaction between day and category of baseline walking activity.

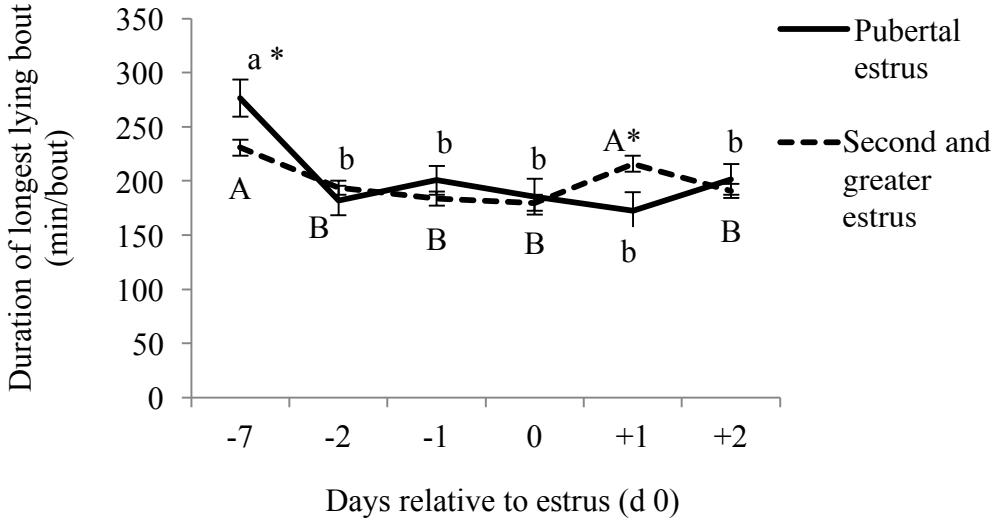

Figure 2.10 Duration of the longest lying bout of a day by estrus order

Duration of the longest lying bout of a day (min/d) is presented as means ± SEM for d -7 to d +2 relative to estrus (d 0). Estrus order corresponds to pubertal (continuous line) vs. second and greater estrus (dashed line). Days with different lowercase letters differ within pubertal events. Days with different uppercase letters differ within second and greater estrus. Days with asterisks differ among pubertal and second and greater estrus ($P < 0.01$).

2.2.4 Discussion

The results confirm our hypothesis of altered ST and LY patterns during estrus. Measurements such as the longest ST bout on estrus day and longest LY bout on d +1 have potential to help improve estrus detection and timing accuracy in automated systems. Most LY

and ST bout measurements at d 0 were correlated with estrus walking activity (Table 2.5) and could be related to increased display of behaviours such as mounting, chin resting, and following.

Duration of LY and ST bouts had large variation (Table 2.4) particularly on the day of estrus, indicating the need for proper calculation of the sample size (animals and events). This is in agreement with the results from a sister study evaluating walking activity and frequency of behaviours during estrus (Section 2.1). Most of the variation of mean ST bout duration at d 0 is likely due to the longest ST bout of that day. It was expected that ST time would increase on day of estrus, but the weight of the longest ST bout on this measurement was surprising. The association between the longest ST bout start time and time of estrus onset determined by walking activity suggests that start time of the longest ST bout could be an important addition to automated estrus detection systems for improved accuracy.

It has been reported that cows in tie-stalls change positions more frequently when in estrus (Walton and King, 1986), and a negative correlation was reported between LY time and frequency of posture change (Kerbrat and Disenhaus, 2004). However, we observed that heifers housed in a free-stall barn had decreased bout frequency at the day of estrus. It is possible that a reduced bout frequency was compensated by an increased ST time and walking activity when cows were housed in free-stalls.

In addition to reduced bout frequency, d 0 was characterized by increased total daily ST time and greater mean ST bout. Most of the increase in mean duration of ST bouts at d 0 was due to increased duration of a single bout, the longest ST bout of that day. At d -7, heifers stood up for approximately 4 h (39% of total) without lying down, whereas at d 0 the longest ST bout was two times greater (8 h or 55% of the total). To our knowledge there is only one report (Brehme et al., 2008) describing the absence of LY time over long periods during estrus. However, it does not provide detailed information about this measurement nor about factors that affect its duration.

At d 0, the longest ST bout started around 1 h earlier and ended 2 h later than at d -7. This difference could be large enough to allow automation based on a heifer standing when she would likely be lying down, or if standing for longer than predicted. The absence of a correlation

between start and end hour of the longest ST bout on d 0, as was observed for the other days, is further evidence of an altered pattern of ST during estrus. Time of estrus onset and start hour of the longest ST bout were moderately correlated. The mean difference between these two measurements was only 1.7 h and Q1 and Q3 for this time difference were -2 and 4 h, respectively, evidencing the proximity between these events.

Time of estrus onset determined by walking activity level was associated with most studied variables. The effect observed was similar for frequency of ST bouts, mean ST bout duration, duration of the longest ST bout, and total daily ST time. Measurements of ST bout duration were markedly greater on d 0 for events starting in the morning. Estrus starting in the afternoon and night had a less marked effect, because the difference observed from d -7 to d 0 was split between d 0 and d -1. Day 0 was determined as the 24 h period starting at 0000 h where the peak of walking activity was detected. It is possible that the moment of onset of high walking activity largely influences the degree of estrus expression. One of the challenge with automated estrus detection systems is use of a single threshold for the whole herd. A low threshold will favour sensitivity but it will also increase the false positive rate. The opposite is true for a high threshold. Inclusion of the effect of time of estrus onset on activity level as a component of automated estrus detection algorithms might be an opportunity for use of variable thresholds according to time of the day.

On d 0, duration of the longest LY bout was not different from other days, but on d +1 it was longer than on all other days. This could be a result of increased need to rest on the day after estrus. Time of estrus onset is usually determined based on onset of increased walking activity or first observation of stand to be mounted (Walton and King, 1986). Decision of time of AI depends on the methodology used to determine estrus onset, but still there is large variation in AI to ovulation interval when it is done based on onset of high activity (Kerbrat and Disenhaus, 2004). Since the longest LY bout occurred the day after peak activity, it could potentially signal that estrus has ended.

Heifers with high baseline walking activity (number of steps/h during non-estrus days) had greater longest ST bout and total daily ST time on all days. These heifers also had a tendency to have a smaller mean and longest LY bout duration. Baseline walking activity could be a potential selection trait when focusing on improvement of estrus expression.

All variables related to duration of ST bouts were influenced by estrus order. Day 0 of pubertal estrus was characterized by shorter mean duration of ST bouts and longest ST bout, and smaller total daily ST time than observed at later estrus events. There was no effect of estrus order on frequency of ST bouts or mean duration of LY bouts. The pubertal estrus was confirmed for 46 of the 57 heifers, but 11 did not have an activity peak associated to the first corpus luteum. Besides the 24% pubertal estruses not detected, those that were detected were characterized by a lower level of expression.

The number of heifers simultaneously in estrus within the same pen was the only fixed effect with no association with LY and ST patterns. It was expected that the formation of a sexually active group would enhance automated measurements of estrus expression. Helmer and Britt (1985) related increased mounting activity when the number of heifers in estrus increased from one to five. Formation of sexually active groups was also identified by Sveberg et al. (2013) as a reliable sign of estrus. It seems that more cows in estrus at a time facilitates behavioural interactions, but does not affect ST and LY patterns when recorded by the current automated device for groups of two or three heifers simultaneously in estrus.

Only frequency of ST bouts and duration of the longest ST bout of a day differed between seasons. During the warm season, the day of estrus had lower frequency of ST bouts, but non-estrus days did not differ between seasons. The longest ST bout of d 0 of warm season events was around 25% shorter than during the cold season. This implies that detection rates could be improved by the use of variable thresholds within herd according to the time of year. Heat stress is known to influence estrus detection in lactating dairy cows (Peralta et al., 2005), and it seems likely that it also influences estrus expression of heifers, although with a smaller impact. If estrous behaviour is affected by environmental factors such as season, which did not affect basal behaviour, measurements of relative intensity will vary during the year; further studies are required for this matter.

2.2.5 Conclusions

Measurements such as the longest ST bout at the day of estrus, its relationship to time of estrus onset, and longest LY bout on the day after estrus provided important insights on how

specific ST and LY measurements could be used to further improve estrus detection and timing. The results of this study also revealed great variation on these patterns, similar to that previously reported for walking activity during estrus.

Standing time clearly increased at the day of estrus. The length of the longest ST bout of each day and its effects on total ST time and mean ST bout duration are of great interest for future research. Automated measurements of start time of the longest ST bout and time of estrus onset were correlated and occurred within a short window of time from each other, revealing a potential new approach for activity monitoring systems. The longest LY bout occurred on the day after estrus as the likely consequence of fatigue and could be a reference point for the end of estrus and assist in determining time to AI. Season of the year, baseline walking activity and number of heifers in estrus seem to be minor factors to explain the observed variability on ST and LY patterns.

Chapter 3: Comparison of Estrus Characteristics in Holstein Heifers by Two Activity Monitoring Systems

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3.1 Introduction

Accurate detection of estrus is a key factor for good reproductive performance of dairy herds (Nebel et al., 2000; Roelofs et al., 2010). Traditional methods for detection of estrus include observation of standing to be mounted behaviour and secondary signs such as mounting other cows, restlessness, clear vaginal mucus, and swollen vulva (Rae et al., 1999; Roelofs et al., 2005; Roelofs et al., 2010). Visual observation of estrus, however, has high labor demands, besides generally low detection rate (e.g. 54% reported by At-Taras and Spahr (2001)). Failure to detect estrus and detection of false estrus can result in missed or untimely inseminations, which lead to poor reproductive efficiency and economic losses (Rae et al., 1999).

Focusing on the characteristic restlessness and increased physical activity displayed by cows in estrus, tools for automated measurement of movement have been under development for almost 40 years (Kiddy, 1977). These automated detection systems have the potential to detect estrus more effectively and precisely than visual observation alone. The investigation of characteristics such as duration of estrus, time of estrus onset, breed differences, synchronized estrus expression, and ideal timing of AI in relation to onset of estrus is possible with the use of automated detection systems (Rae et al., 1999; Nebel et al., 2000). The most commonly used automated detection systems are pedometers, accelerometers, and mount detection devices (Senger, 1994; At-Taras and Spahr, 2001; Roelofs et al., 2010).

Heatime (SCR Engineers Ltd, Israel) is a commercial collar-mounted activity monitoring system, which transfers data wirelessly to a station every 2 h and generates real time high activity alerts for individual cows. IceTag (IceRobotics Ltd, Scotland) is a leg-mounted research-

based sensor that measures number of steps and standing and lying times on a per minute basis. Previous research reported that Heatime correctly identified 72% of preovulatory phases, but it also identified some activity peaks during high-progesterone periods (Aungier et al., 2012). IceTag sensors have been validated for step counting by comparison with video recording (Nielsen et al., 2010). These authors recognized that IceTags accurately estimated steps during walking periods, although some "false steps" may occur. The use of IceTags for detecting estrus has been validated with research-developed algorithms with up to 93% sensitivity (McGowan et al., 2007). Jónsson et al. (2011) also developed algorithms for detection of estrus with IceTag step counts that resulted in 89% sensitivity and 99% specificity. The objectives of this study were to assess detection rate and quantify the degree of estrus expression according to these 2 activity monitoring sensors worn simultaneously by Holstein heifers at breeding age, as well as to investigate the associations between estrus characteristics and physiological measurements. We hypothesized that larger preovulatory follicle diameter and greater plasma estradiol concentration would be associated with increased estrus intensity and duration.

3.2 Materials and Methods

The present study was conducted at the University of British Columbia's Dairy Education and Research Centre (Agassiz, BC, Canada) from May 2012 to August 2013. The local Institutional Animal Care Committee following the requirements of the Canadian Animal Care Council (CACC) approved all experimental procedures.

Fifty-seven Holstein heifers were group-housed in a sand-bedded free-stall pen (13.0 x 14.5 m) with rubber flooring on both alleys. The group was composed by 24 heifers, which were moved into the pen at 12 mo of age and were moved out at the first positive pregnancy diagnosis. Total mixed ration was offered once daily at 0900 h and pushed up 3 times/d (approximately at 1100 h, 1800 h, and 2200 h). Water was available at all times.

The activity monitoring systems used were Heatime (SCR Engineers Ltd., Israel) and IceTag (IceRobotics Ltd., Scotland). Heatime tags were attached to the upper left side of a collar worn at the cranial portion of the cow's neck. These tags are accelerometers that send data wirelessly every 2 h to a receiving unit connected to a computer. A cow's activity is translated

into a proprietary index that represents deviations from each cow's own basal activity. Index values range from 0 to 100; the threshold for an event of high activity was set at 35 index value. Data were exported from the Heatime software and converted into Microsoft Excel files (Microsoft Corporation, Redmond, WA) using the Heatime batch tool. An Excel macro was developed to identify events of high activity. Estrus onset hour, end hour and duration according to the timeframe above the threshold were obtained for each high activity event from the macro-edited files. The maximum index value observed at estrus was defined as "peak index value" and represents the measurement of estrus intensity for Heatime.

IceTag sensors were attached with custom flexible plastic straps to the metatarsal region of one of the heifer's hind limbs. Data were downloaded once/wk with the IceReader unit connected to a laptop using the IceTag Analyzer 2011 software (IceRobotics Ltd., Scotland), therefore providing retrospective data. Because download and re-activation did not require removal of the sensor from the heifer's leg, there was a recording gap of only 10 min between two consecutive files.

Estrus events were identified from IceTag step counts following the methodology described by Silper et al. (2015b). In summary, peaks of activity (i.e., estrus events) were identified in a chart of the rolling sum of steps over 24-h periods. After detection of activity peaks, start and end time of each estrus were determined based on the summed frequency of steps per 2-h blocks. The 90^{th} percentile of the number of steps for all 2-h blocks during a 1-wk period surrounding the day of estrus (day of activity peak ± 3 d) was calculated. The 2-h blocks that met or exceeded the 90^{th} percentile were used to compose the estrus period and determine the hour of onset and end of the estrus period. Duration and total number of steps during estrus were calculated as the sum of all 2-h blocks that comprised the estrus cluster. Baseline steps were calculated as the mean frequency of steps for the same 2-h blocks of 3 d preceding the estrus cluster. Total estrus steps and total baseline steps divided by estrus duration are presented as estrus steps/h and baseline steps/h. Relative increase in walking activity was defined as *[(Total estrus steps – Total baseline steps)/Total baseline steps] x 100%.*

Estrus characteristics were evaluated at each high activity event identified by Heatime. The Heatime software was checked for new estrus alerts thrice daily. Once a new estrus alert was observed, a blood sample was collected immediately. Blood samples (10 mL) were collected

from the coccygeal artery or vein in K2/EDTA vacuum tubes (Becton & Dickinson Vacutainer Systems, Rutherford, NJ) to determine plasma estradiol concentration. Samples were centrifuged (1,565 x g for 15 min); plasma was harvested and stored frozen at -80^{o}C. Estradiol concentration was determined by radioimmunoassay (Kirby et al., 1997). Ovaries were scanned by ultrasonography (Ibex Pro; E. I. Medical Imaging, Loveland, CO, equipped with a 7.5 MHz linear rectal transducer) to describe and measure ovarian structures at the a.m. or p.m. period after Heatime generated a new high activity alert. Signs of estrus were assessed at the time of reproductive examination (clear vaginal mucus, uterine tone, visual mounting activity, standing to be mounted, and/or rump showing signs of repeated acceptance of mounts). Heifers were artificially inseminated if on-farm evaluation judged the alert as true estrus. Peak index value, days from last estrus, ovarian structures, and estrus signs were considered for this decision. Precision of estrus detection was retrospectively determined from ovarian dynamics and plasma estradiol concentration. Because only estrus events detected by the Heatime system were evaluated, performance could only be measured by positive predictive value (PPV = true detected events/all detected events). Events were classified as true estrus or false positive alert, and as detected by Heatime only or by Heatime and IceTag.

Means ± SD and distributions were obtained with proc UNIVARIATE (SAS version 9.3, SAS Institute Inc., Cary, NC). Intensity and duration of estrus were studied as the variables characterizing the degree of estrus expression. Estrus intensity is represented by peak index value (Heatime) and relative increase in walking activity (IceTag). Number of steps/h during estrus and during baseline (mean of 3 d before estrus) are also reported. Timing of estrus detection was assessed by hour of onset and end of the high activity periods. Period of overlapped high activity and differences of duration, onset and end hour were used to compare timing among systems. Correlations between measurements of intensity and duration within and between systems were determined with proc CORR. Correlations were also determined for the relationship between physiological measurements (diameter of preovulatory follicle and plasma estradiol concentration) and automated measurements (intensity and duration of estrus measured by either system). False positive estrus alerts were excluded from the correlation analysis between physiological and automated measurements.

The relationships between automated measurements of estrus expression and signs of estrus (clear vaginal mucus, uterine tone, visual mounting activity, standing to be mounted, or rump showing signs of repeated acceptance of mounts), preovulatory follicle diameter, and plasma estradiol concentration were analyzed by ANOVA using proc GLM with heifer as the random variable. The following measurements were analyzed as categorical variables: number of estrus signs (0 to 3), diameter of preovulatory follicle (≤ 15 mm: small; ≥ 16 mm: large), and plasma estradiol concentration (≤ 11.2 pg/mL: low; ≥ 11.3 pg/mL: high). Continuous variable results were described as mean ± SD when purely descriptive statistics and mean ± SEM when comparisons were calculated (e.g. estrus intensity and duration according to the number of estrus signs).

3.3 Results and Discussion

A total of 119 estrus events from 57 Holstein heifers at breeding age were evaluated. Heatime (n = 111) gave real time activity information, thus allowing for ultrasound scanning of ovaries, collection of blood sample, and assessment of signs of estrus at the beginning of the event. Data from IceTag sensors were evaluated retrospectively and compared to Heatime measurements of estrus expression.

Intensity of estrus recorded by Heatime was 77.3 ± 19.5 (mean ± SD) peak index value. Peak activity ranged from 35, which was the predetermined threshold for high activity, to 100, which is the maximum possible value. It was expected that heifers would have estrus events of high intensity. Lactating cows in the same herd had an average estrus intensity of 72.8 ± 20.2 peak index value (Madureira et al., 2013). Intensity of estrus can be influenced negatively by lactation and lameness, among other factors (Galina and Orihuela, 2007; Roelofs et al., 2010). Estrus peak activity has been reported to have a negative linear relationship with milk production by some (López-Gatius et al., 2005b; Valenza et al., 2012). Factors influencing estrus expression are mainly absent in heifers, resulting in generally better reproductive performance for this category of cattle.

As observed with Heatime, IceTag data also recorded estrus with high intensity in this study. Number of steps/h was 371 ± 91 (mean ± SD) during estrus and 87 ± 28 during baseline.

The mean relative increase in walking activity during estrus was 360 ± 170% baseline walking activity. Fold increase in activity of heifers during estrus, measured by neck-mounted tags was reported to be 2.75 (Løvendahl and Chagunda, 2010). The methodology used to obtain estrus intensity from IceTag sensors is based on raw data. Heatime, conversely, reports an index value that is weighted based on the activity of the previous week and can only increase up to 100 index value. Use of raw activity might be a better measurement of intensity, but it is more sensitive to day-to-day variations.

Although differences exist between systems, measurements of intensity were correlated ($r = 0.63$; $P < 0.01$; Figure 3.1). Estrus intensity measurements were comparable for lower intensity events, but those with peak index value > 80 were equivalent to a wide range of relative increase in walking activity. Heatime algorithmic processing imposes an upper limit for activity. Because of this characteristic, the greater the peak index value, the greater was the observed range of corresponding relative increase in walking activity.

Mean duration of estrus measured by Heatime was 14.3 ± 4.1 h (mean ± SD), ranging from 4 to 22 h. Aungier et al. (2012), using Heatime, reported mean estrus duration of 8.4 h and 10.8 h for the first and subsequent post-partum ovulations, respectively. If visual observation or mount detectors are used as the method for detection of estrus, duration is determined as the period of acceptance of mount, lasting 5.6 to 7.6 h for lactating cows (At-Taras and Spahr, 2001; Lopez et al., 2004; Rivera et al., 2010) and 9.2 h for heifers (Silper et al., 2015b). The relationship between duration of standing estrus and of increased activity should be further studied. Peralta et al. (2005) observed that onset of estrus of lactating cows varies with time of day when detected by pedometers, but not when mount detectors were used. At-Taras and Spahr (2001), also working with lactating cows, observed that duration of standing estrus (approximately 6 h) and high activity counts (approximately 10 h) overlapped by 3.6 or 6 h, according to 2 experiments. Neither of these authors reported the difference in hour of onset between the detection methods.

It is speculated that the number of mounts per estrus has decreased over the last decades. It has been reported that only 45% of cows show standing behaviour during estrus (Roelofs et al, 2004). Lopez et al. (2004) observed 6 mounts/estrus in lactating cows and Rivera et al. (2010), 4 mounts/estrus. A low number of mounts/estrus and a low percentage of cows showing standing

behaviour suggest that the use of secondary signs of estrus or of changes in walking activity might be important to achieve desirable levels of estrus detection. Walking activity is likely a more accurate tool for determining start and end of estrus than visual observation of mounting and standing behaviour.

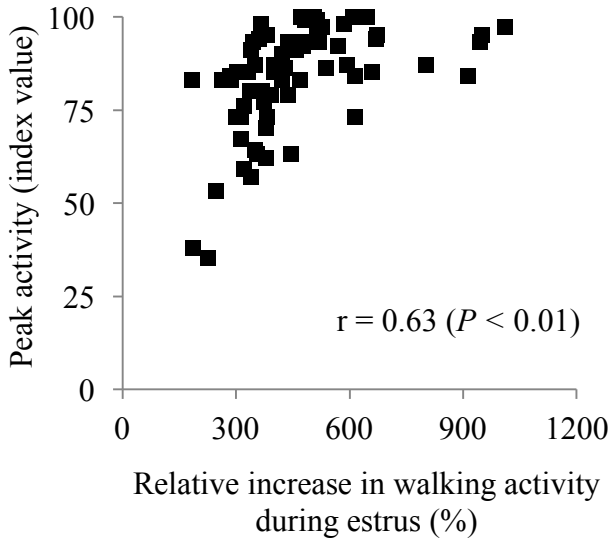

Figure 3.1 Correlation among measurements of estrus intensity

Intensity of estrus measured by collar-mounted accelerometer as peak activity (index value) and by leg-mounted pedometer as relative increase in walking activity (%).

Duration of estrus measured by IceTag (15.0 ± 4.0 h; mean ± SD) was correlated with duration measured by Heatime (r = 0.60; $P < 0.01$). The difference in estrus duration (IceTag – Heatime) is presented in Figure 3.2. Seventy-five percent of the duration differences ranged from -2 to +2 h, supporting the agreement between systems. In addition to the similarity in duration of estrus, the systems also recorded estrus onset and end hour with small differences. IceTag recorded estrus onset 3.5 ± 4.3 h earlier than Heatime, and end of estrus 2.9 ± 4.9 h earlier. Knowledge about the time of estrus onset is of main importance to determine AI timing. This is in fact one of the main advantages of using automated estrus detection technologies. With visual observation of standing to be mounted it is possible to determine if a cow is in estrus, but little can be said about whether that is the start or the end of estrus. Once the time of high activity onset is known, interval to ovulation can be estimated and AI time determined (Stevenson et al.,

2014). There is, however, a need for more research with diverse estrus detection systems. For Heatime, Valenza et al. (2012) reported mean interval of 28.7 h from high activity onset to ovulation and 16.4 h from peak weighted activity to ovulation. It is important to note that Valenza et al. (2012) observed a large variation on the interval between estrus onset and ovulation, which makes the determination of optimal AI timing less predictable. Given the differences in reproductive performance between heifers and lactating cows, it can be hypothesized that the interval from estrus onset to ovulation would be less variable in heifers than in lactating cows. The agreement between systems concerning timing of estrus detection was also evidenced by the period of overlapping high activity. Considering time of onset and duration of estrus, estrus events determined by IceTag and Heatime overlapped by 9.4 ± 5.1 h.

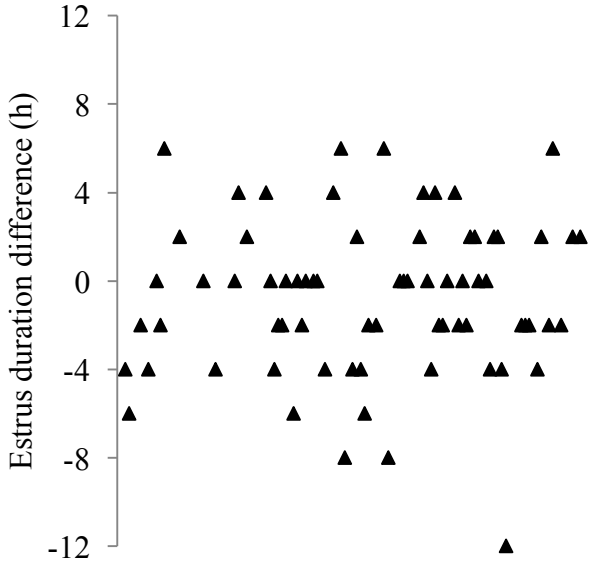

Figure 3.2 Difference in estrus duration measured by two activity monitoring systems
Duration difference in hours calculated as duration by leg-mounted pedometer minus duration by collar-mounted accelerometer.

Duration and intensity are important components of estrus expression that directly influence AI submission rate. Heatime measurements of intensity and duration were correlated (r = 0.64; $P < 0.01$), suggesting that estrus is usually either well expressed or poorly displayed in both Heatime measurements. In contrast, intensity and duration measured with IceTag data were

not correlated (r = 0.13; P = 0.26). Analysis of walking activity from IceTag data had minimal transformation compared to Heatime data. Perhaps corrections for circadian walking activity would smoothen the data variability and improve the relationship between estrus characteristics.

The number of baseline steps/h measured by the IceTag system had a negative correlation with intensity of estrus in both sensors (r = -0.37 and -0.70 for Heatime and IceTag, respectively; $P < 0.01$). Previous research reported that heifers with greater level of baseline walking activity at 11 mo old had increased number of steps during estrus (Silper et al., 2015b). In contrast, relative increase in walking activity and duration of estrus were not different among heifers with high vs. low level of baseline activity. This might be not only an important indication that some heifers are more predisposed to better estrus expression than others, but also that estrus expression could be linked to pattern of activity during non-estrus periods.

Mean diameter of the preovulatory follicle was 15.7 ± 2.6 mm (mean ± SD) and mean plasma estradiol concentration was 11.2 ± 4.6 pg/mL. These are similar to results by Sartori et al. (2004), who reported greater plasma estradiol concentration in heifers than in lactating dairy cows, even though cows had larger preovulatory follicles. Accordingly, Wiltbank et al. (2006) highlighted a role of elevated metabolic rate on reduction of circulating estradiol in dairy cows, resulting in heifers having longer estrus and greater pregnancy rate than lactating cows.

Estrous behaviour is induced mainly by the effect of estradiol from the preovulatory follicle on the brain (Forde et al., 2011). Correlations between preovulatory follicle diameter, plasma estradiol concentration, and degree of estrus expression could have been expected. Surprisingly, no correlation was observed between preovulatory follicle diameter and plasma estradiol concentration (r = -0.02; P = 0.87). According to measurements of either system, preovulatory follicle diameter was not correlated ($P > 0.05$) with duration of estrus (Table 3.1), and only moderately correlated with intensity of estrus ($P < 0.05$). Plasma estradiol concentration, however, was not correlated with duration or intensity of estrus, independent of system (Table 3.1). Measurements of intensity and duration from either sensor were not associated with categories of preovulatory follicle size nor with categories of estradiol concentration at time of estrus onset ($P > 0.05$).

Few reports identified correlations between measurements from activity monitoring systems with plasma estradiol concentration or preovulatory follicle diameter. The results of the present study were contrary to our initial hypothesis that larger preovulatory follicle diameter and greater estradiol concentration would be associated with increased physical activity during estrus. One possibility for the lack of correlation could be that the true onset of estrus, and thus the best time for blood sample collection, is earlier than what is captured by activity monitors. This would result in inconsistent sampling time and possibly estradiol concentration lower than peak concentration for some samples. Another possibility would be the randomness of collections in relation to feeding time as they were entirely dependent on the Heatime information. Vasconcelos et al. (2003) described acute changes in progesterone catabolism after feeding. Nonetheless, the lack of correlation may suggest that other factors such as estradiol receptors in the brain or genetic traits could be key determinants of estrous behaviour.

Table 3.1 Spearman Rank correlations between physiological and automated measurements of true estrus

	Heatime intensity	Heatime duration	IceTag intensity	IceTag duration	Estrus steps/h
Preovulatory follicle diameter	0.20^{*} (94)	0.02^{NS} (94)	0.23^{*} (73)	-0.09^{NS} (73)	0.11^{NS} (73)
Plasma estradiol concentration	0.04^{NS} (77)	0.01^{NS} (77)	0.01^{NS} (65)	0.01^{NS} (65)	0.15^{NS} (65)

Values in table are Spearman Rank correlation coefficients with number of observations in parenthesis. False positives were excluded from this analysis (17 out of 111 estrus events detected by Heatime). $^{*}P < 0.05$; $^{NS}P < 0.05$

Signs of estrus, measured herein as mounting and standing to be mounted, clear vaginal mucus and uterine tone, are important for on-farm assessment of the validity of activity peaks as true estrus. An increase in the number of estrus signs was related to greater duration and intensity of estrus as measured by Heatime only ($P < 0.001$; Figure 3.3).

It is important to consider the percentage of false positive and false negative alerts when evaluating automated estrus detection systems. In this trial we could not determine false negatives for Heatime, thus only PPV was calculated. True estrus was determined from analysis of ovarian dynamics and plasma estradiol concentration. Positive predictive values were high for both systems (Table 3.2), implying high precision of estrus detection (low frequency of false positive alerts).

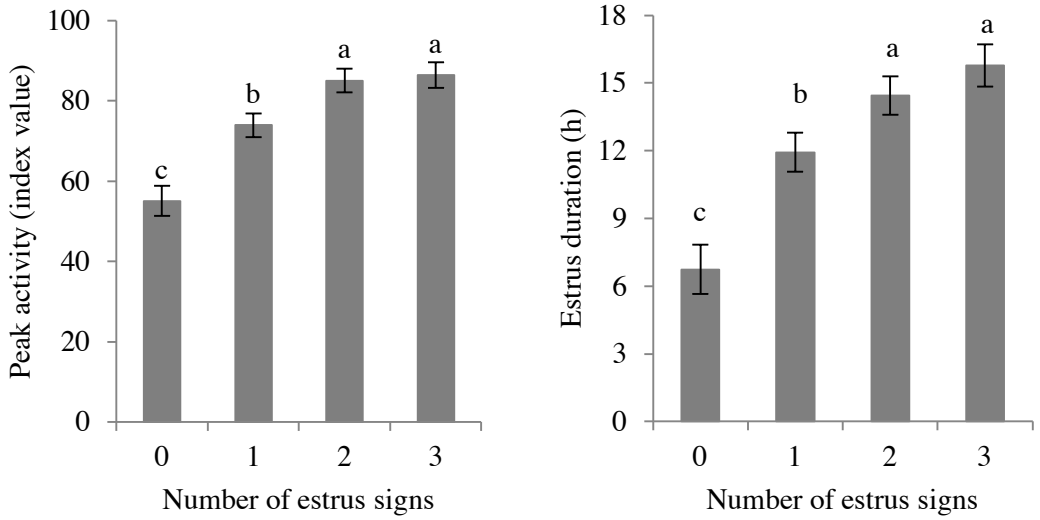

Figure 3.3 Estrus intensity and duration according to number of estrus signs

Estrus intensity measured as peak activity (index value) and duration measured as period of high activity (h) are presented as means ± SEM ($P < 0.001$). Estrus characteristics were measured by a collar-mounted accelerometer. Estrus signs were evaluated at time of estrus onset (clear vaginal mucus, uterine tone, and visual mounting activity and/or standing to be mounted).

Table 3.2 Performance of two activity monitoring systems and characteristics of estrus expression

	IceTag (leg-mounted)	Heatime (collar-mounted)
PPV[1], % (n/n)	98.7 (74/75)	84.7 (94/111)
Estrus intensity, mean ± SD	360 ± 170%[2]	77.3 ± 19.5[3]
Estrus duration, mean ± SD	15.0 ± 4.0 h	14.3 ± 4.1 h

[1] PPV (*Positive Predictive Value = true detected events/all detected events*); [2] Relative increase in walking activity from baseline activity = *[(Total estrus steps – Total baseline steps)/Total baseline steps] x100%;* [3] Peak index value.

3.4 Conclusions

The systems had good agreement regarding determination of estrus characteristics and high precision of estrus detection. Heifers had estrus events of high intensity and duration within expected values. The relationships between preovulatory follicle diameter, plasma estradiol concentration, and characteristics of estrus expression were not consistent between the two systems. Although Heatime data presented generally better correlations than IceTag data, the latter provided valuable information on raw activity during estrus and non-estrus periods. Variables such as concentration of estradiol and estrus signs should be analyzed independently for research purposes. Future research should involve assessing the interval between estrus onset and ovulation as measured by different types of sensors.

Chapter 4: Daily Lying Behaviour of Lactating Holstein Cows during an Estrus Synchronization Protocol and its Associations with Fertility

A version of this Chapter has been submitted for publication: B.F. Silper, A.M.L. Madureira, L.B. Polsky, S. Soriano, A.F. Sica, J.L.M. Vasconcelos, and R.L.A. Cerri. Daily lying behavior of lactating Holstein cows during an estrus synchronization protocol and its associations with fertility.

4.1 Introduction

Current precision dairy farming technologies allow for automated detection of estrus using tools that record different aspects of animal behaviour. In a review of the literature, Rutten et al. (2013) listed 48 types of sensors applied to fertility management (e.g. estrus detection), where 61% measured aspects of cow activity. Restlessness is an important component of behavioural estrus (Roelofs et al., 2010). Although subjective if visually evaluated, restlessness can be quantified through measurements of steps, neck movements, lying time and bout frequency, for example. Estrus walking activity and neck movements have been researched regarding patterns (Løvendahl and Chagunda, 2010; Valenza et al., 2012) and associations with environmental and cow factors, endocrine profiles, ovulation timing, and fertility (López-Gatius et al., 2005a; Stevenson et al., 2014; Madureira et al., 2015; Aungier et al., 2015). Lying behaviour, conversely, is more frequently employed in cow comfort and welfare assessments (von Keyserlingk et al., 2012; Charlton et al., 2014). Even though reports of its application towards estrus detection are rare (e.g. see Table 2 in Rutten et al. (2013)), lying behaviour has recently been deemed as a useful predictor of estrus (Dolecheck et al., 2015). Research from our group reported a decrease in lying time of 36% at the day of estrus in nulliparous animals (Silper et al., 2015a).

Expression of estrus (compared to absence of it) at the moment of TAI has been associated with greater pregnancy per artificial insemination (P/AI; Cerri et al., 2004: Heatsynch protocol;

Souza et al., 2007: Ovsynch protocol with injection of 1 mg of estradiol 17-β 8 h before the last GnRH). Among cows that expressed estrus, those with higher degree of intensity measured by AAM were associated with greater P/AI compared to poorly expressed events (Madureira et al., 2015). Pregnancy per AI was also greater among cows with confirmed ovulation after TAI (Pereira et al., 2014). Estrus expression and fertility likely share endocrine regulators. Possible mechanisms could involve progesterone exposure and the regulation of endometrial estradiol receptor-α (Spencer and Bazer, 2004), LH concentration and follicular growth (Cerri et al., 2011a; Cerri et al., 2011b), in addition to plasma estradiol concentration.

Estradiol and progesterone based protocols are more likely to induce expression of estrus when compared to GnRH-based protocols (Pereira et al., 2013), providing an opportunity to study the associations between intensity of estrus and fertility under controlled conditions. Automated activity monitors allow grading of behavioural estrus, measurements that can be further used as real-time predictors of fertility and reproductive states. The objectives of this research were to measure the changes in lying behaviour during an estradiol and progesterone-based synchronization protocol and to assess risk factors associated with the degree of behavioural change at estrus, ovulation, and P/AI. We hypothesized that lying time would decrease and bout frequency would increase at estrus, and that the degree of change would be associated with ovulation rate and P/AI.

4.2 Materials and Methods

4.2.1 Cows, Housing and Management

This study took place at a commercial dairy located in São Paulo, Brazil, between July 2013 and June 2014. At the time of the experiment, the herd had an average of 1,700 lactating cows and 305 d average yield of 11,438 kg. Cows were housed in a cross-ventilated free-stall barn in groups of 300 animals and milked three times daily (at approximately 0500, 1300 and 2100 h). The barn had grooved concrete floors and 2 rows of deep sand-bedded stalls. Fresh TMR balanced to meet or exceed the nutritional requirements of lactating dairy cows producing 40 kg of 3.5% fat corrected milk/d (NRC, 2001) was provided thrice daily. Water and TMR were

available for ad libitum intake. Experimental procedures followed requirements and practices outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999). Procedures were performed while the cows were restrained in headlocks at the feed bunk after the first daily milking.

4.2.2 Synchronization Protocol, Exams and Blood Sampling

Eligible cows (first AI or after negative pregnancy diagnosis, determined apt for breeding by the herd veterinarian) were enrolled onto an ovulation synchronization protocol (Figure 4.1). Timed AI was done using commercial frozen-thawed semen. Ovaries were examined by ultrasonography at d -11 (presence or absence of CL), d 0 (assessment of largest pre-ovulatory follicle) and d +7 (presence or absence of CL to confirm ovulation). Positive pregnancy diagnosis required presence of amniotic vesicle with viable embryo (visible heartbeat). Body condition score (1 to 5 scale at 0.25 increments; Wildman et al., 1982) was recorded at TAI. Milk production was recorded at each milking with automated milk meters (AfiLite, Kibbutz Afikim, Israel).

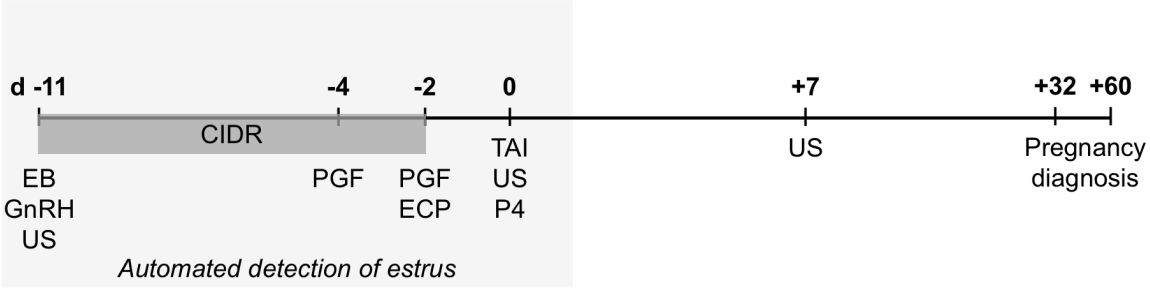

Figure 4.1 Experimental estrus synchronization protocol

EB (estradiol benzoate - 2 mg, Gonadiol, Zoetis, São Paulo, Brazil), GnRH (gonadorelin diacetate - 100 µg, Cystorelin, Merial, São Paulo, Brazil), PGF (dinoprost tromethamine - 25 mg, Lutalyse, Zoetis, São Paulo, Brazil), ECP (estradiol cypionate - 1 mg, E.C.P., Zoetis, São Paulo, Brazil), CIDR (intravaginal progesterone implant - 1.9 g progesterone; CIDR, Zoetis, São Paulo, Brazil), TAI (timed AI), US (examination of ovaries with ultrasonography), P4 (collection of blood sample for analysis of progesterone concentration). Automated detection of estrus was done with Afimilk Pedometer Plus Tags and AfiFarm software (Afimilk, Kibbutz Afikim, Israel).

Blood samples were collected at TAI by puncture of the medial coccygeal artery or vein into 10 mL non-treated Vacutainer tubes (BD, São Paulo, Brazil), placed on ice, and centrifuged at 3000 *g* at 4 °C for 30 min. Serum was harvested and stored in microtubes at -20°C until analysis. Progesterone concentration was determined using a chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA). Intra- and inter-assay CV were, respectively, 5.1 and 5.2%. The minimum detectable concentration was 0.1 ng/mL.

4.2.3 Behavioural Data

All cows carried an accelerometer (Afimilk Pedometer Plus Tag, Afimilk, Kibbutz Afikim, Israel) attached to one of the hind limbs within 1 wk of calving. Data were recorded in 2-h time bins, downloaded thrice daily by an electronic scanner at the milking parlor's entrance. Data were later retrieved from AfiFarm Dairy Farm Management Software (Afimilk, Kibbutz Afikim, Israel) and compiled using Excel (Microsoft Corporation, Redmond, WA). Lying behaviour was recorded on a 24 h basis (0000h to 2359 h) and summarized as total lying time (L_time; min/d), bout frequency (bout_N; bouts/d), average lying bout duration (L_dur; min/bout), ratio of daily total lying time by total standing time, and restlessness, an Afimilk proprietary calculation measured in arbitrary units.

4.2.4 Data and Statistical Analyses

These data are the result of an observational cohort study. Pregnancy diagnoses were performed for all 1,411 TAI events. Lying behaviour and ovulation data were available for at least 1,209 events and 677 events, respectively. Statistical analyses were performed with SAS Studio University Edition ver. 3.1 (SAS Institute Inc., Cary, NC). Significance was set at a probability of type I error of 5%, and tendencies between 5 and 10%. Data were trimmed at the 1st and the 99th percentiles of L_time for each day (1st percentile: 279, 168, 126, 126, and 141 min for d -7, -2, -1, 0 and +1; 99th percentile: 1019, 1029, 1008, 1044, and 1074 min for d -7, -2, -1, 0, and +1).

Descriptive statistics were performed for each lying behaviour variable. For objectivity of results and discussion, lying behaviour was further analyzed regarding only L_time and bout_N, given that the calculation of daily total lying time by total standing time ratio includes L_time and that of the restlessness variable is not known, and because L_dur was constant during the evaluated period. Least square means and standard errors were obtained with proc MIXED of SAS (TAI event as subject and days as repeated measures). Three subgroups were present regarding the day when behaviour suggestive of estrus occurred (d -2, -1, or 0). For this reason, we present L_time and bout_N based on the lowest daily value among d -2, -1 and 0. The relative change between estrus and baseline for L_time% and bout_N% were calculated as *[lowest L_time / baseline L_time] * 100* and *[lowest bout_N / baseline bout_N] * 100*, respectively.

Associations between baseline L_time or bout_N and season, parity, milk yield, and BCS were tested with SAS proc MIXED using cow as repeated measures and event as the experimental unit. After testing for univariable associations and two-way interactions, those with $P \leq 0.20$ were presented to final models. Results are presented as LSM ± SEM.

Ovulation rate and P/AI at d 32 after AI were calculated for categories of L_time% and bout_N% (15-29%, 30-44%, 45-59%, 60-74%, 75-89%, 90-104%, and ≥ 105%) using proc FREQ. Given the observed ovulation rate and P/AI within these categories, changes in L_time% and bout_N% were further classified as large if < 75% and small if ≥ 75%. Events with lowest L_time (n = 133) or bout_N (n = 201) occurring at d -2 were excluded from the following analyses because of apparent poor response to the protocol and lack of synchronization with time of insemination, which would create a confounding effect especially within P/AI results. Table 4.1 exemplifies protocol response and estrus lying behaviour according to day of lowest L_time. Multivariable logistic regression models (proc LOGISTIC) were built to test the probability of large change in lying behaviour according to L_time% or bout_N% (< 75%), and probability of confirmed ovulation, pregnancy at d 32, or pregnancy at d 60, where TAI event was the experimental unit. Effects of parity (1st vs. 2nd and greater), DIM (1-60, 61-120, 121-250, and ≥ 251 d), milk yield (mean of d -3 to d +3, tested as continuous and categorical forms [≤ mean or > mean]), CL at start of protocol (yes or no), BCS (≤ 2.5, 2.75-3.00, and ≥ 3.25), progesterone concentration at TAI (tested in continuous and categorical forms [≤ 0.09 ng/mL, 0.10 to 0.21

ng/mL, or ≥ 0.22 ng/mL]), and season (colder: June to August; hotter: September and February to May) were tested. In addition to the explanatory variables mentioned above, the categories of estrus intensity (according to degree of change in L_time% and bout_N%) were individually tested as explanatory variables in models where the outcome was ovulation or pregnancy. Explanatory variables were presented to multivariate logistic models using backward stepwise elimination at $P \leq 0.10$. Odds ratio (OR) and 95% confidence limits were obtained. Associations between explanatory and individual response variables were tested with chi-square test (proc FREQ).

4.3 Results

4.3.1 Cows and Inseminations

At the TAI events (n = 1,411), cows were 137 ± 93 DIM (Q1-Q3: 72-170 DIM), had mean ± SD milk production of 43.6 ± 11.0 kg/d and median BCS of 2.75 (17% of cows ≤ 2.5, 67% with BCS 2.75 or 3.00, and 16% with BCS ≥ 3.25). Primiparous represented 40.8% and multiparous (maximum 8 lactations) 59.2% of the enrolled cows. Fifty-two percent of TAI corresponded to first AI, 16% to second AI, and 32% to third or greater AI.

4.3.2 Daily Lying Behaviour During Estrus and Baseline

At baseline (d -7), L_time was 695 ± 124 min/d and bout_N was 13 ± 5 bouts/d (mean ± SD). Baseline L_time was affected by the interaction of parity and DIM (Figure 4.2a; $P = 0.01$), and decreased by 1.22 min with every 1 kg increase in milk yield ($P = 0.002$). Cows with high BCS had greater L_time (728 ± 10 min/d vs. 693 ± 6 and 663 ± 10 for high, medium and low BCS, respectively; $P < 0.001$) as well as greater bout_N at baseline (Figure 4.2b; $P < 0.001$). Primiparous had greater baseline bout_N than multiparous (14.6 ± 0.31 vs. 13.2 ± 0.27; $P < 0.0001$).

Daily L_time and bout_N within subgroups (lowest L_time or bout_N at d -2, -1 or 0) are presented in Figure 4.3. Overall lowest L_time was 446.7 ± 157.1 min/d, equivalent to 64.9 ±

21.4% of baseline L_time. Lowest bout_N occurred at d -2, -1 and 0 for 18.0%, 51.0% and 31.0% of events. Overall lowest bout_N was 8.5 ± 4.0 bouts/d, representing 64.9 ± 23.8% of baseline bout_N. Seventy-three percent of events had lowest L_time and lowest bout_N occurring at the same d.

Table 4.1 Distribution, realized fertility, and characteristics of estrus events

	Day of lowest total lying time		
	-2	-1	0
Number of events (% of all events)	133 (11.8%)	569 (50.7%)	421 (37.5%)
Ovulation rate (%)	66.7	91.2	84.0
P/AI d 32 (%)	16.5	32.0	31.6
P/AI d 60 (%)	15.8	27.8	27.7
Total lying time			
min/d	495.6 ± 192.5	423.1 ± 142.5	463.2 ± 158.4
% of baseline[1]	73.8 ± 25.4	61.2 ± 19.3	67.1 ± 21.7
Bout frequency			
bouts/d	9.7 ± 5.1	8.2 ± 3.8	8.6 ± 4.0
% of baseline[2]	73.3 ± 27.7	61.7 ± 21.6	66.6 ± 24.5

[1] Relative decrease in lying time: *L_time% = (lowest L_time / baseline L_time) * 100*, where L_time = daily total lying time. [2] Relative decrease in bout frequency: *bout_N% = (lowest bout_N / baseline bout_N) * 100*, where bout_N = bout frequency. Lying behaviour measurements were obtained with leg-mounted accelerometers.

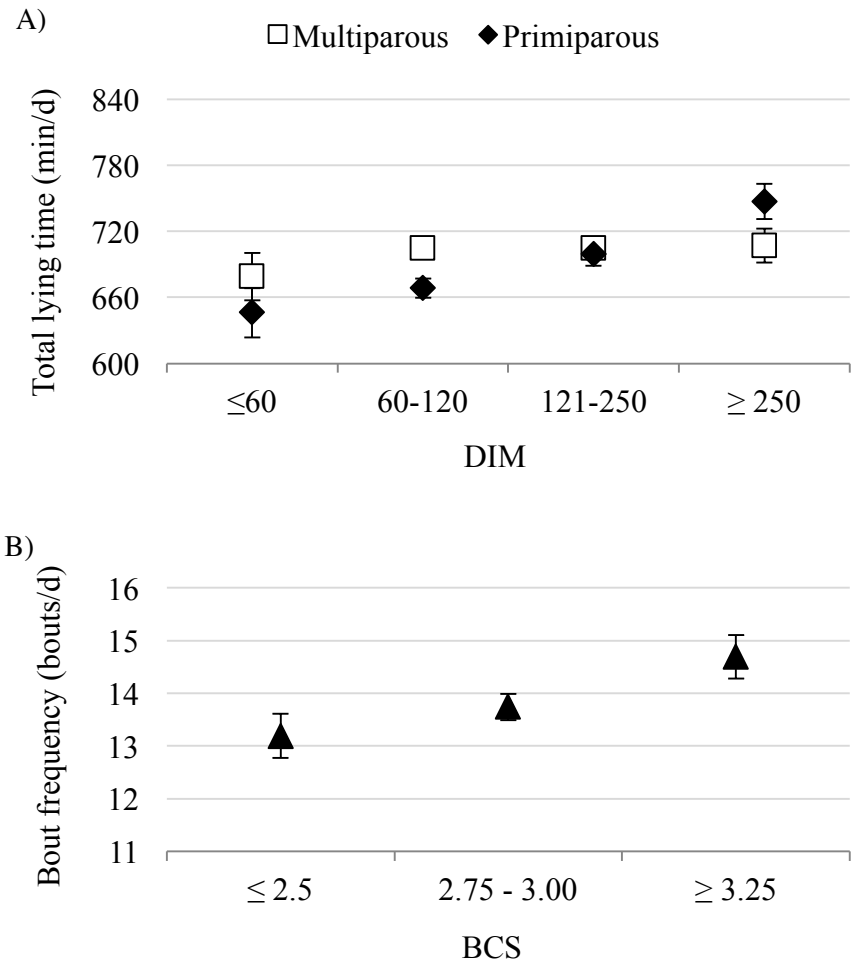

Figure 4.2 Baseline lying behaviour of lactating Holstein cows

Baseline measurements obtained 7 d before day of timed AI with leg-mounted accelerometers. A) Total lying time (min/d) according to parity (primiparous [white squares]; multiparous cows [black diamonds]) and categories of days in milk (DIM; ≤ 60, 61-120, 121-250, and ≥ 251 DIM; P = 0.01). B) Bout frequency (bouts/d) according to body condition score (BCS, graded 1 [severe undercondition] to 5 [severe overcondition]; P = 0.01).

4.3.3 Risk Factors for Ovulation and Pregnancy

Ovulation rate and P/AI across 7 categories of lowest L_time% and lowest bout_N% are presented in Figure 4.4. Using these data as reference, we classified estrus into categories of large and small change in lying behaviour (large if L_time or bout_N < 75% of baseline; small if L_time or bout_N ≥ 75% of baseline).

Based on L_time%, odds of ovulation were greater when a CL was present at start of protocol (vs. absence of CL at start of protocol; OR [95% confidence limits] = 3.62 [1.96-6.69], $P < 0.0001$), when milk yield was above average (vs. below average; OR = 2.03 [1.11-3.71], $P = 0.02$), and when the change in L_time% at estrus was large (vs. small change; OR = 4.86 [2.69-8.80]; Figure 4.5). Odds of ovulation was also affected by DIM ($P = 0.04$).

A large change in bout_N% was associated with greater likelihood of ovulation (OR = 2.06 [1.11-3.83]*;* $P = 0.02$; Figure 4.5). Within the bout_N% model, ovulation was more likely among cows with a CL at start of protocol (OR = 4.17 [2.18-7.98]; $P < 0.0001$) and for estrus occurring in the colder season (OR = 1.97 [1.02-3.83], $P = 0.04$). Ovulation tended to be more likely within the group of cows with milk yield above average (OR = 1.76 [0.96-3.22], $P = 0.07$).

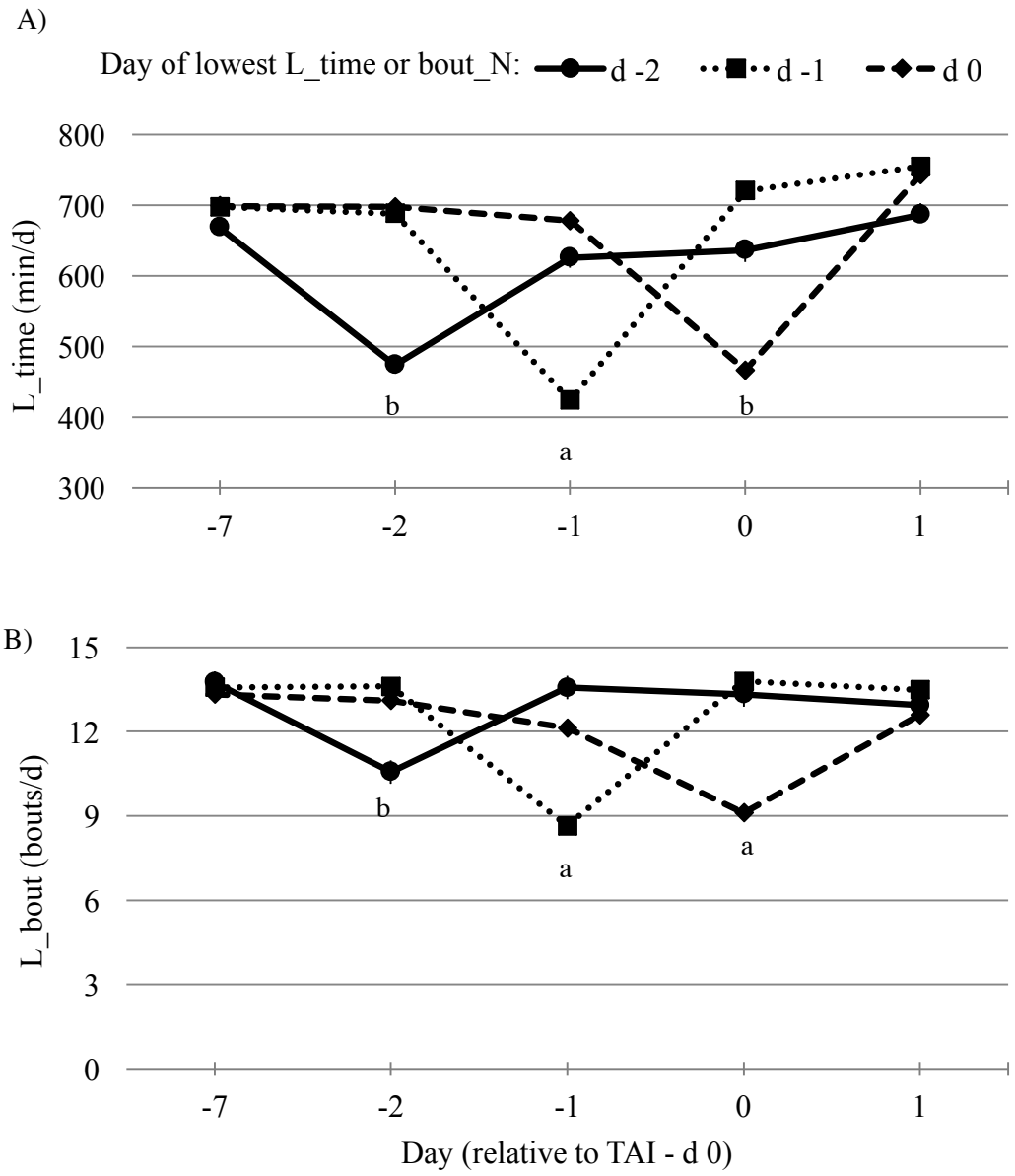

Figure 4.3 Daily lying time and bout frequency

L_time (total lying time; min/d) and bout_N (frequency of lying bouts/d) are presented as means ± SEM for d -7 and d -2 to d+1 relative to timed AI (d 0). Different curves represent 3 subgroups of cows with lowest L_time or bout_N at d -2 (continuous line), -1 (dotted line), or 0 (dashed line). P-values for effect of day, subgroup and their interaction were < 0.0001 for L_time, and < 0.0001, 0.03, and < 0.0001 for bout_N. Different lowercase letters within chart indicate statistical difference between the lowest value of each subgroup.

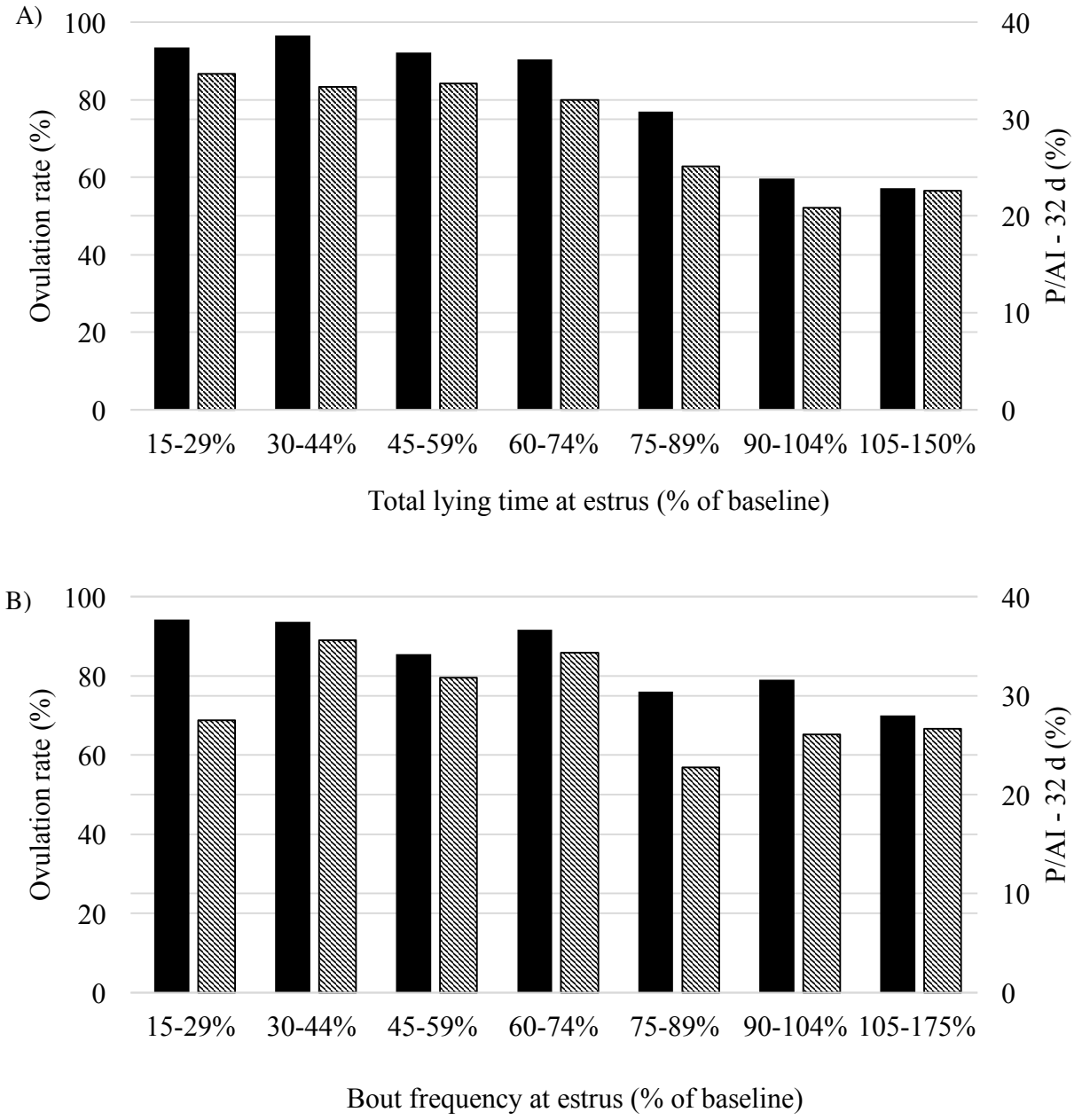

Figure 4.4 Distribution of ovulation rate and pregnancy according to relative change in lying behaviour at estrus

Ovulation rate (%; dark bars) and pregnancy per artificial insemination (P/AI assessed 32 d after AI, %; dashed bars) according to estrus total lying time (A) and bout frequency (B) relative to baseline measurements.

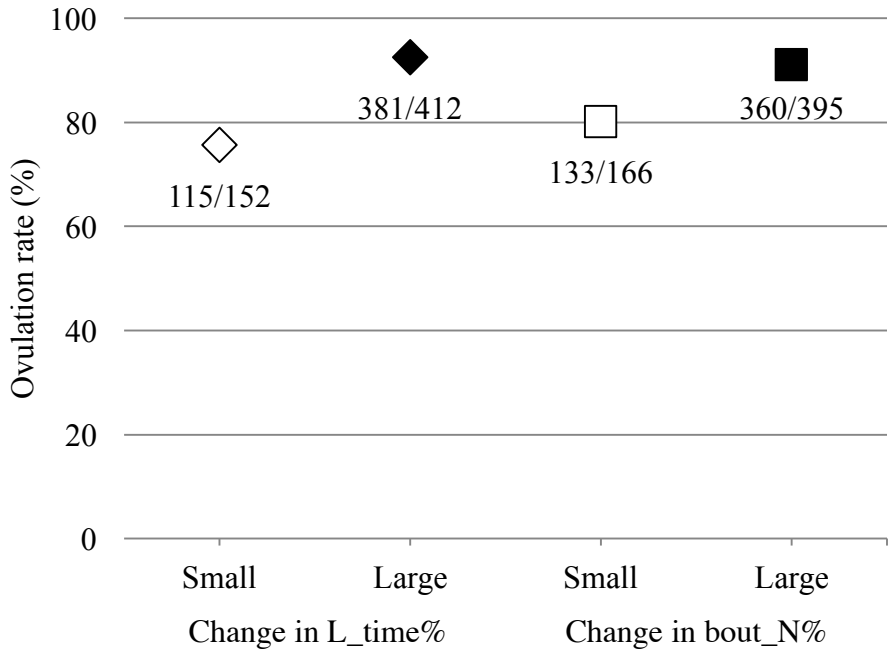

Figure 4.5 Ovulation rate according to degree of change in lying behaviour

Degree of change in lying behaviour according to relative change in lying time (L_time%; diamonds) and bout frequency (bout_N; squares) categorized as small if ≥ 75% baseline (white makers) and large (black markers) if < 75% baseline. $P < 0.0001$ (L_time%) and $P = 0.0003$ (bout_N%).

Table 4.2 contains OR and 95% confidence limits for variables affecting P/AI at d 32 in the model composed with L_time%. Similar to the model with L_time%, odds of P/AI at d 32 in the model with bout_N% were influenced by parity, DIM, season, and milk yield ($P < 0.05$), as well as by bout_N%, where a large decrease in bout_N% was associated a likelihood of P/AI of 1.57 [1.05-2.34] ($P = 0.03$). Greater odds of P/AI at d 32 were observed following estrus with large change in both L_time% and bout_N% (Figure 4.6a). There was a tendency for an association between large change in lying behaviour and greater likelihood of P/AI at 60 d (L_time%: OR = 1.46 [0.99-2.15]; $P = 0.06$; bout_N%: OR = 1.50 [0.99-2.26]; $P = 0.06$; Figure 4.6b). Parity, DIM and milk yield also influenced P/AI at d 60 ($P < 0.05$). When only cows that had a confirmed ovulation were included in the analysis of risk factors for P/AI at 32 and 60 d,

there were significant effects of parity, DIM and bout_N%, but L_time% did not affect the likelihood of pregnancy in this circumstance.

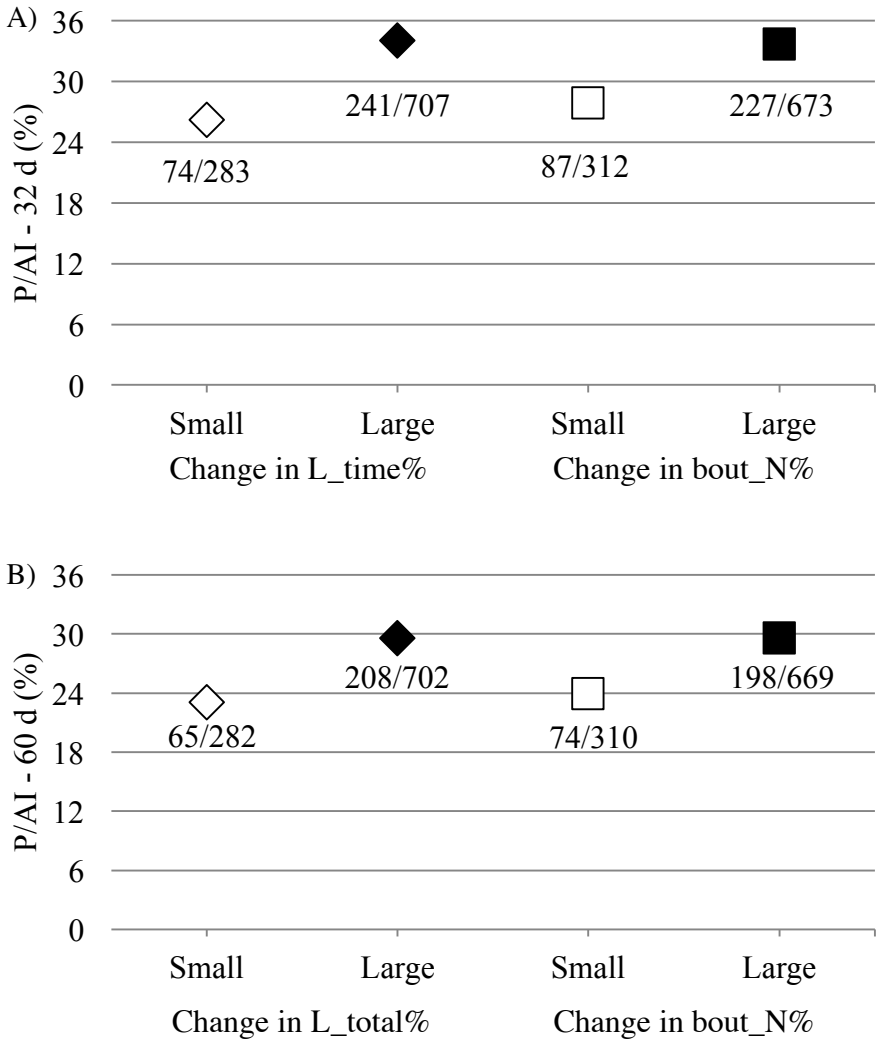

Figure 4.6 Pregnancy per artificial insemination according to degree of change in lying behaviour

Pregnancy per artificial insemination (P/AI) assessed at 32 and 60 d after timed AI according to the relative change in lying time (L_time%; diamonds) and bout frequency (bout_N; squares) as small if ≥ 75% baseline (white makers) and large (black markers) if < 75% baseline. P/AI at d 32: $P = 0.02$ [L_time%] and $P = 0.06$ [bout_N%]; P/AI at d 60: $P = 0.04$ [L_time%] and $P = 0.06$ [bout_N%].

Table 4.2 Factors affecting pregnancy diagnosis 32 d after insemination

Variable	Pregnancy per artificial insemination		
Level	% (n pregnant/total n)	OR (95% CL)	P-value
Parity			
1st	38% (159/423)	1.67 (1.20-2.32)	0.003
2nd or greater	28% (156/567)	Ref.	-
DIM			
≤ 60	32% (17/53)	Ref.	0.005
61-120	34% (180/525)	0.75 (0.24-2.33)	-
121-250	33% (100/301)	0.72 (0.23-2.27)	-
≥ 251	16% (18/111)	0.20 (0.05-0.75)	-
Milk yield (kg/d)			
≤ 43.6	31% (146/477)	Ref.	0.06
> 43.6	33% (169/512)	1.38 (0.99-1.92)	-
Season			
Warm	27% (126/468)	Ref.	0.01
Cold	36% (189/522)	1.57 (1.10-2.26)	-
Degree of change (based on L_time%)			
Large (< 75%)	34% (241/707)	1.61 (1.10-2.34)	0.01
Small (≥ 75%)	26% (74/283)	Ref.	-

Pregnancy per artificial insemination, frequencies, odds ratio (OR and 95% confidence limits; CL) and P-values refer to logistic regression model that included relative change in lying behaviour measured as *L_time% = (lowest L_time / baseline L_time) * 100*, where L_time = daily total lying time.

4.3.4 Factors Affecting the Likelihood of Large Change in Lying Behaviour

Milk yield above average reduced the likelihood of large change in L_time% at estrus (OR = 0.66 [0.47-0.92]; P = 0.0003; 75.1% vs. 68.2% large change in L_time% for below-average and above-average yield cows), but other potential risk factors were not significant (P > 0.05).

Primiparous were more likely to show a large change in bout_N% (OR = 1.75 [1.21-2.53]; *P* = 0.003; 77.2% of primiparous vs. 68.1% of multiparous had a large change in bout_N% at estrus). Likelihood of large change in bout_N% tended to be greater for estrus occurring during the warmer season compared to the colder season (OR = 1.45 [0.98-2.15]; *P* = 0.06).

4.4 Discussion

The results presented here provide supporting evidence for the association between the degree of behavioural change (intensity) of estrus expression and realized fertility, which has been recently reported for AI performed after detection of spontaneous estrus (Madureira et al., 2015). Although restlessness is widely recognized as one of the main signs of estrus (Roelofs et al., 2010), its lying behaviour component has not been extensively employed in the measurement of estrous behaviour. Factors influencing the relative decrease in lying time or bout frequency were similar to those affecting intensity of spontaneous estrus (Madureira et al., 2015) and behavioural display of standing to be mounted (Lopez et al., 2004). The association between estrus expression and fertility suggests underlying physiological mechanisms where hormones such as estradiol and progesterone would coordinate estrus expression and also influence fertility-related parameters such as ovulation, oocyte quality, uterine environment, and CL function.

Changes in daily lying time of lactating cows during periods of estrus were similar to those observed for dairy heifers (Silper et al., 2015a), although baseline bout frequency was apparently greater and baseline daily lying time shorter for lactating cows. Lying behaviour of lactating cows analyzed in min/h and bouts/h (Dolecheck et al., 2015) indicated a larger difference between baseline and estrus than that reported here. Although different AAM often quantify the same measurement, we assume that there are differences in the outcome due to equipment precision, algorithms and data processing, among other possible sources of variation.

Lying time and bout frequency at baseline were influenced by factors such as parity, DIM, and BCS. This contributes to the variation in estrous behaviour that is observed among cows, supporting the use of relative change as the measure of choice for automated estrus detection. These sources of variation should be investigated regarding a potential contribution to estrus

expression: provision of conditions for expression of baseline behaviour in the opposite direction from estrous behaviour could increase relative intensity of expression. For example, if cows with low BCS are likely to have reduced daily lying time, it cannot be expected that these cows will show a large relative decrease in lying time when in estrus.

The development of new technologies enables obtainment of diverse measurements from a single sensor and potentially greater accuracy of detection. As an example, Jónsson et al. (2011) reported increased probability of estrus detection and reduced error rate when combining walking activity and lying behaviour. More studies are needed to address the applicability of monitoring lying behaviour for real-time estrus detection and determination of AI-timing.

Display of estrus behaviour has been associated with greater P/AI when cows had their estrous cycles synchronized (Cerri et al., 2004; Souza et al., 2007). The present analysis suggests that the magnitude of the relative decrease in lying time or bout frequency at the time of estrus is associated with fertility in a similar fashion to the relationship between walking activity and fertility reported by Madureira et al. (2015). These results reinforce the role of estrus expression on fertility of high producing dairy cows; such measurements could be employed in fertility prediction and benchmarking of estrus expression among herds.

The physiological mechanisms linking estrus expression and fertility are not yet fully understood. Lower concentration of progesterone during the luteal phase preceding estrus has been associated with precocious endometrial expression of estradiol receptor-α, leading to increased $PGF_{2\alpha}$ secretion and short estrous cycles (Cerri et al., 2011a). In addition, a lower pre-ovulatory estradiol concentration has been associated with precocious luteolysis (Mann and Lamming, 2000). Conversely, endometrial and CL gene expression in Nelore cows at d 19 of gestation was more favourable to pregnancy maintenance in cows that expressed behavioural estrus at the moment of TAI (Davoodi et al., 2016). Ovulation timing, uterine environment, and diameter and age of pre-ovulatory follicle should also be investigated concerning the association between estrus expression and fertility.

Pre-ovulatory estradiol and previous luteal phase progesterone concentrations are involved in regulation of estrus expression (Reames et al., 2011). Estradiol concentration at onset of estrus has been observed to be approximately 1 pg/mL greater in cows expressing high intensity estrus

(Madureira et al., 2015), but correlations between estradiol concentration and estrus activity levels are weak (Aungier et al., 2015, Madureira et al., 2015). It is interesting to note that duration of standing to be mounted has been reported to be associated with estradiol concentration (Reames et al., 2011; Aungier et al., 2015). Progesterone concentrations were not timely measured in this experiment, but cows bearing a CL at start of synchronization protocol (d -11) were more likely to ovulate.

The synchronization protocol used in this experiment relied on ECP injection for induction of estrous behaviour and ovulation. Estrus onset and ovulation have been reported to occur 29 ± 2 h and 55 ± 3 h after ECP injection as part of a Heatsynch protocol (Pancarci et al., 2002). Therefore, estrus events occurring at d -2 (day of ECP injection) were not influenced by the exogenous estradiol and they likely correspond to lack of synchronization in response to the protocol. All cows were inseminated at d 0, independently of estrus expression. Neves et al. (2012) reported that 26% to 44% of AI within an Ovsynch protocol were done after visually observed estrus in spite of fixed time AI. Using a Heatsynch protocol, Cerri et al. (2004) observed greater P/AI for cows expressing estrus and inseminated before TAI, compared to those that did not express estrus and were inseminated at TAI. Twenty-two percent of cows expressing estrus in response to a Heatsynch protocol did so before TAI (Cerri et al., 2004), in accordance with data presented here. Moreover, estrus expression near AI can decrease pregnancy losses and significantly improve fertility in cows receiving embryo transfers (Pereira et al., 2016). The observations by Pereira et al. (2016) suggest that the beneficial effects of estrus are beyond improvements in ovulation rate, most likely affecting the endometrial environment and consequent maintenance of the embryo and fetus. In the present study, we could not determine if the realized fertility of d -2 estrus events would be improved if insemination had been performed in a timely manner. Determination of insemination time according to estrus during a synchronization protocol could have potential to improve overall results, especially with estradiol and progesterone-based protocols, which induce estrus expression in a significant proportion of cows. Concomitant use of synchronization protocols and AAM should allow for such improvements.

One aspect regarding the study of intensity of estrus expression is the generation of practical information for on-farm use. These results suggest that estrus intensity could be used to

predict fertility when incorporated to decision-making features on AAM user interfaces. Research on cow and environment-related effects on baseline behaviour and their effects on magnitude of automated measurements is another area for further research. Limitations of this study that remain to be investigated are the use of lying behaviour as a primary tool for generation of estrus alerts and its potential combination with other measures of physical activity. A second aspect of measurement of estrus intensity concerns its association with fertility. In this experiment, lying behaviour proved to be similarly associated with fertility as previously observed with walking activity measurements. Evaluation of daily circulating estradiol and progesterone concentrations and of endometrial gene expression might contribute to the understanding of physiological mechanisms linking magnitude of estrus expression and fertility.

4.5 Conclusions

The assessment of lying behaviour in association with fertility, where a larger decrease in lying time or bout frequency at estrus was associated with greater likelihood of ovulation and P/AI, provides evidence for the role of estrus expression in reproductive physiology. The relationship between estrus intensity and fertility still requires further understanding; nonetheless, application of intensity classification as part of decision-making tools represents an opportunity for improving the use of sensors and reproductive performance. Baseline lying behaviour was influenced by factors such as BCS and DIM. Because of its direct effect on the calculation of relative behavioural change, baseline behaviour should be investigated regarding alternatives for increased estrus detection.

Chapter 5: General Discussion and Conclusions

5.1 Thesis Findings

The overall goal of this thesis was to contribute to the understanding of automated measurements of estrus, their variability and associations with fertility. The literature on neuroendocrine control of estrus, estrous behaviour, and automated detection of estrus was reviewed in Chapter 1. Based on the literature review and field observations during research conducted at the University of British Columbia's Dairy Education and Research Centre, specific objectives were outlined and organized into experiments presented in Chapters 2, 3 and 4 of this thesis. The research presented in these chapters addressed areas such as variability and factors affecting automated measurements, agreement between estrus characteristics, and possible use of AAM data to predict fertility. This is a current and applicable research field, as AAM technologies are now widely available and are reportedly efficient at detecting estrus. The next step involves maximization of data usage and refinement of technology application.

Section 2.1 presented the first investigations, which aimed to describe visual and automated measurements of estrous behaviour in heifers and the variation among estrus events. It was observed that, on average, heifers expressed estrus with high intensity, despite having the same genetics and housing conditions of high yield lactating cows. It was interesting to observe that, although heifers were healthy (reproductively sound, metabolically unchallenged, and with adequate gait) there was a large variation in behavioural expression. This would be positive for selection according to estrus expression, but the lack of repeatability indicates that there is large environmental influence on the degree of estrus expression.

During video analysis (Section 2.1), it came to my attention that heifers in estrus spent extremely long periods of time without lying down. One mention to this fact was found in the literature (Brehme et al., 2008), but as with most of the literature regarding lying behaviour during estrus, sample sizes are limited and data are not clearly presented. Thus, the objectives of the study presented in Section 2.2 were to describe how estrus affected lying behaviour of heifers. This study was done with heifers because of availability of data without need to enrol additional animals, as well as to understand effects of estrus in absence of lactation. Our results

reinforced previous observations by Brehme et al. (2008). Heifers spent 488 ± 16 min standing without changing position during estrus (longest standing bout), in contrast to 232 ± 5 min during baseline). The large increase in longest standing bout, its correlation with walking activity, and occurrence within -2 to 4 h of estrus onset indicate possible application of this measurement for improved accuracy of estrus detection.

Chapter 2 and the literature review presented intensity and duration of behavioural change as characteristics used to quantify estrus expression. The various AAM available differ in placement, target behaviours, algorithms, and frequency of data summarization and transfer. Knowledge about AAM agreement is important for research and on-farm application. The experiment described in Chapter 3 aimed to compare estrus characteristics between a collar-mounted and a leg-mounted sensor. Good correlations between measurements and similar determination of estrus onset time were observed. This provided important background for future standardization of recommendations such as AI timing (which depends on interval from estrus onset to ovulation) and for meta-analysis of associations between level of estrus expression and physiology, fertility, or reproductive management. Detection of estrus from both systems was precise, but we could not evaluate sensitivity. We observed a positive association between estrus intensity, as well as duration, and visual signs of estrus (clear vaginal mucus, uterine tone, visual mounting activity, and standing to be mounted). Conversely, plasma estradiol concentration was not associated with estrus characteristics.

The last study, presented in Chapter 4, aimed to investigate the association between relative change in lying behaviour, an alternative measurement of estrus expression, and fertility of dairy lactating cows. We confirmed that changes in lying behaviour during estrus in lactating cows are similar to those observed in heifers (Section 2.2), although visual behavioural expression by cows was not measured. The most important result of this study was an association between magnitude of estrus expression (measured by relative change in lying behaviour) and fertility. Cows that reduced their lying time further than 75% of baseline lying time were 1.6 times more likely to be diagnosed pregnant at 32 d post-AI. Similar results were observed for events where bout frequency was < 75% of baseline bout frequency. These results suggest potential of automated measurements of estrus for fertility prediction and potential use as a phenotypical marker for selection of more fertile cows.

In conclusion, results presented in Chapters 2, 3, and 4 enhanced our understanding of expression of estrus by heifers and lactating cows and provided evidence that data from automated systems can be applied further than for estrus detection. The associations between estrus measurements and fertility raise questions about the physiological basis of this relationship. In addition, it underlines investigation areas such as control of fertility via induction of estrus expression and application of real-time estrus information to breeding decisions that optimize reproductive management and increase performance.

5.2 Implications for Dairy Cattle Reproductive Management

Automation of estrus detection represents an opportunity to improve AI submission rate and herd reproductive efficiency. Recent literature has reported estrus detection rates of approximately 70% by AAM (Aungier et al., 2012; Valenza et al., 2012), performance comparable to TAI (although herd-dependent; Neves et al., 2012; Burnett et al., 2017) and possible use in reproductive programs that also include TAI (Stevenson et al., 2014; Fricke et al., 2014). As a tool that quantifies estrus expression, AAM allows identification of risk factors for poor expression, determination of optimal AI timing, and investigation of associations with behavioural display and hormonal milieu. Potentially, data from AAM could be employed in fertility prediction and reproductive management practices, and even as genetic selection traits (Løvendahl and Chagunda, 2010; Ismael et al., 2015).

Results presented in Section 2.1 evidenced the reduced opportunity for estrus detection by visual observation of standing to be mounted. Despite the experimental animals being heifers, the median number of acceptance of mounts was 30 per 30 h of continuous observation. Duration of standing estrus was shorter than high-activity duration. There was large expression of secondary behaviours, similarly to conclusions from a study with low-yield cows (Sveberg et al., 2011). Detailed analysis of expression of secondary behaviours in high-yield cows, as well as the synchrony between behavioural expression and activity patterns are still to be evaluated. Among the observed secondary behaviours, heifers had large increase in frequency of commonly reported behaviours such as mounting and chin resting. Other less known behaviours, for example follow and crossover, were also increased during estrus. Crossover, defined as a heifer

walking through the alley that connects front and back of the pen, represents an example of simple automation that could be tested for estrus detection. Proximity loggers could also be attempted for detection based on following or formation of sexually active groups as described by Sveberg et al. (2013).

The variability observed for behavioural expression was also present among automated measurements of estrus expression. Coefficients of variation among estrus events were greater than 25% for estrus intensity and duration. Among estrus events of the same heifer, average CV was greater than 20% for those same characteristics. Because we were studying estrous behaviour in heifers, thus without influence of lactation, metabolic issues, or daily management routines, we had expected smaller variation. Among factors contributing to this variation, we identified effects of season, time of estrus onset, and basal activity on estrus duration and intensity. In a co-authored project studying high-yield cows, we observed that multiparity, BCS ≤ 2.5, and DIM ≤ 45 were associated with reduced intensity and duration of estrus (Madureira et al., 2015). Cows with milk yield ≤ 31.3 kg/d at the day of estrus had greater expression than cows with yield > 31.3 kg/d (Madureira et al., 2015). Others have reported reduced duration of standing estrus and number of standing events in cows with yield ≥ 39.5 kg/d (Lopez et al., 2004).

Another resource that can be obtained from AAM is the measurement of basal activity. In our study with young heifers, basal activity at 11 mo old was associated with greater number of steps, greater standing time and longer standing bout at estrus, although it did not affect estrus intensity or duration. Phillips and Schofield (1989) indicated an association between basal and estrus activity, but this had not been further investigated. Future research could assess factors that affect basal activity and its associations with estrus expression in lactating cows.

Section 2.2 and Chapter 4 presented evaluations of lying and standing behaviour, measurements that have been poorly investigated in association with estrus until this moment. Both heifers and lactating cows had significant reduction in lying time, of approximately 36% or 4 to 5 h/d, as well as a reduction in bout frequency. Measurements of lying and standing behaviour had fair correlation with walking activity. Total daily standing time of the heifers in estrus was correlated with steps/h and duration of estrus ($r = 0.37$ and 0.17). Although not frequently employed in estrous behaviour research, its use combined with walking activity

reduced the error rate and increased the probability of estrus detection (Jónsson et al., 2011). Jónsson et al. (2011) worked with development of complex mathematical models, from a computer science rather than animal science approach. Collaboration between these two areas is key to develop the next generation of sensors for estrus detection and general monitoring of cattle behaviour. Peralta et al. (2005) suggested that combinations of systems, including visual observation, are the best alternative to enhance detection and conception rates when challenges such as heat stress are present. Rumination has also been recently researched regarding its application towards estrus detection. Rumination time and feeding time, rate and intake have been reported to be reduced at the day of estrus (Reith and Hoy, 2012; Pahl et al., 2015). Reith and Hoy (2012) alerted for the high variability in rumination time across cows and need for assessment of its value for detection alone or in combination with other automated measurements. Combination of measurements within one system is likely to increase detection accuracy, partially overcoming poor expression and large variability.

It was interesting to observe the effect of estrus on the longest standing bout of a day. This was mentioned once in the literature (Brehme et al., 2008). The sensor used in Chapter 4 does not measure standing time, thus we could not investigate changes in the longest standing bout. Nonetheless, lying behaviour measurements such as relative change in lying time and in bout frequency were markedly decreased at the day of estrus and were associated to risk factors for decreased expression of estrus similar to those reported for walking activity.

Another result that suggests opportunities for refinement of AAM is the effect of time of estrus onset on daily standing time, where events starting in the afternoon and night hours had daily change of smaller magnitude, but spread across two consecutive days. Conversely, estrus starting in the morning had changes in standing behaviour that were larger and concentrated in a single day. Time of onset also affected relative increase in activity, which was greater when estrus started at night, i.e. the time of lower basal activity. Knowledge on factors affecting magnitude of estrus measurements could be applied towards improved detection with use of customized thresholds (e.g. by category, herd, season, time of onset), or towards management actions aiming for increased estrus expression. Establishment of minimum duration thresholds (6 to 8 h), in addition to the standard intensity threshold, are options for reduction of false positive alerts (Aungier et al., 2012). Collectively, changes in walking activity and lying and standing

behaviour, including the longest standing bout, reinforce the concept that behavioural estrus can be quantified, although there is substantial variation. This emphasizes the need for large number of events in studies of estrus expression.

It is important to acknowledge that, in addition to measuring diverse characteristics, AAM differ in measurement methodologies. Studies with different systems are needed to obtain a comprehensive characterization of estrus expression. Research and on-farm use of AAM will benefit greatly from the knowledge on estrus characteristics measured by different sensors and across animal categories, as well as on agreement between measurements. To address this topic, we investigated the association between data obtained from two AAM commercially available (Chapter 3). Positive predictive values (*true and detected estrus / [true detected estrus + false positive alerts]*) were 84.7% and 98.7% for a collar-mounted and a leg-mounted AAM attached to heifers at breeding age. In lactating cows of the same herd, Madureira et al. (2015) reported PPV of 89.6% and 85.5% for the same collar-mounted sensor and a different leg-mounted sensor, indicating similar precision of detection for both categories of cattle. The complementary values to PPV are false positive alerts, thus interpretation of PPV should highlight that roughly 2 to 15 out of 100 alerts would be false. False positive alerts might be identified due to distinguishable characteristics (Aungier et al., 2015), examination of the cow (Roelofs et al., 2010), or improved detection systems that monitor more than one characteristic (Firk et al., 2002). Calculation of false negatives, or sensitivity of AAM, requires monitoring of a gold standard reference such as milk progesterone. Under these circumstances, AAM have been reported to miss approximately 30% of estrus, but low detection rates with AAM are likely a consequence of poor expression of estrus by cows, rather than detection failure by the systems (Valenza et al., 2012; Giordano et al., 2015). Among cows that were not detected in high activity within 7 d of induced luteolysis, only 35% ovulated (Valenza et al., 2012).

Intensity and duration were fairly correlated among systems, supporting a potentially broader interpretation of research across different AAM. Determination of time of estrus onset, which occurred with 3.5 ± 4.3 h difference between systems, is perhaps the most important comparison. Time of estrus onset is important because of its association with ovulation timing, and thus determination of AI timing. According to Neves and LeBlanc (2015), most producers using AAM were inseminating cows 7 to 12 h after estrus onset. This is in agreement with

research that identified interval from estrus onset to ovulation between 24 and 29 h (Valenza et al., 2012; Stevenson et al., 2014; Aungier et al., 2015). Considering a period of 10 to 12 h until sperm enters the oviducts and capacitates (Hunter and Wilmut, 1984), optimal time for AI has been determined as 9 to 15 h after estrus onset (Aungier et al., 2015), or 13 to 16 h for primiparous and maximum 13 h for multiparous (Stevenson et al., 2014). Variation in estrus duration and interval from onset to ovulation have been hypothesized as causes of low conception rates in cows (Valenza et al., 2012). Comparison of time of estrus onset by other AAM, in addition of factors that can influence the onset-ovulation relationship, should be further investigated.

The experimental conditions of the study described in Chapter 3 permitted investigation of associations between preovulatory follicle diameter, estradiol concentration, and estrus intensity and duration. Given the role of preovulatory follicle in estradiol synthesis and the function of estradiol on induction of estrous behaviour, some degree of association between these factors was hypothesized. However, preovulatory follicle diameter and estradiol concentration were not associated with estrus characteristics, except for correlations of 0.20 and 0.23 between preovulatory follicle diameter and estrus intensity measured by two AAM. Lacking or weak associations between follicle size, estradiol concentration and estrus activity have been reported elsewhere (Aungier et al., 2015; Madureira et al., 2015), although Madureira et al. (2015) observed a 1 pg/mL greater concentration of estradiol in plasma when high-intensity estrus was compared to low-intensity estrus. Estrus has been determined to be an "all-or-none" response to elevated circulating estradiol (Allrich, 1994), but others have observed increasing duration of standing estrus with higher dosages of exogenous estradiol administered to ovariectomized cows (Reames et al., 2011). Supporting a greater effect of estradiol on estrus duration rather than on intensity, Aungier et al. (2015) reported greater estrus duration when concentration of estradiol during the 8 h that preceded the LH surge were higher.

The challenge in determining existence of associations between estradiol concentration and estrous behaviour measured by AAM could be attributed to timing of blood sampling and variation in time of estradiol peak relative to estrus onset. Steroid concentrations in plasma are reduced in lactating cows due to increased metabolic clearance (Sangsritavong et al., 2002). In addition, progesterone concentration has been reported to decrease after feed intake and to

fluctuate during the day as a function of feeding frequency (Vasconcelos et al., 2003). Sampling at time of estrus onset results in blood collection dispersed over all hours of the day, thus the concentration of some samples might not reflect peak values. Lack of representability of peak values might also result from variable estradiol peak to estrus onset timing. Estradiol peak has been reported to precede estrus onset by 8 h or to follow estrus onset by 7 h (Saumande and Humblot, 2005; Aungier et al., 2015). Because estradiol decreases to 50% of peak concentration 5 h after LH surge, and LH surge and estrus onset occur closely in time, the interval from estrus alert to actual sampling might account for lower-than-peak estradiol concentration and lack of correlation with estrus expression.

It has been suggested that greater estradiol concentration at estrus enhances uterine function and favours fertility even if it is assumed that estradiol does not enhance estrus expression (Allrich, 1994). Interestingly, estrus expression has been linked to increased fertility at different experimental settings. Greater conception rate at TAI has been observed when cows expressed estrus at time of AI (Cerri et al., 2004; Souza et al., 2007; Pereira et al., 2014). In addition, Pereira et al. (2016) recently reported that estrus expression near AI, measured by removal of paint from a tail-head device, favours conception and reduces pregnancy losses after embryo transfer. These results reinforce the hypothesis that expression of estrus is associated with differential regulation of uterine environment and pregnancy maintenance.

Associations between standing estrus and fertility (Garcia et al., 2011), and between high estrus score on a behavioural scale and fertility (Gilmore et al., 2011) have been observed, although less than 120 cows were enrolled in these two studies. Others have reported significant associations between activity level measured by pedometers and fertility, but without further details (López-Gatius et al., 2005b). Within our group's research, we have observed an increase in P/AI of 10 to 12 percent units when peak activity was greater than 90-index (maximum value is 100-index; threshold for estrus detection is 35-index) or 300% relative increase in walking activity (Madureira et al., 2015).

Further research is needed to understand the association between magnitude of estrus expression and fertility. A recent study observed that beef cows (Nelore breed) expressing estrus at TAI had endometrial gene expression more favourable for pregnancy establishment and maintenance (Davoodi et al., 2016). In further support of these results, we observed an

association between estrus measured from the novel standpoint of relative change in lying behaviour and fertility in high yield cows (Chapter 4). A large decrease in lying time was associated with increased likelihood of ovulation and P/AI by 4.9 and 1.6-fold. Daily and peak progesterone and estradiol concentrations could not be evaluated in this experiment, but we hypothesize that these factors influence the relationship between estrus and fertility. Higher progesterone at time of induction of luteolysis has been associated with greater likelihood of estrus expression and of being inseminated after estrus detection instead of at a fixed time (Giordano et al., 2015). Complementarily, we observed increased ovulatory response if cows bore a CL at start of TAI protocol. While the main focus has been on associations with estradiol, effects of progesterone on estrus activity should be further investigated (Løvendahl and Chagunda, 2010). Another significant aspect that requires further investigation is the combined use of relative change in lying behaviour and walking activity, regarding possible employment as alert generators and reduction of false positives. It should be highlighted that, together, the results of Chapter 4 and the work of Madureira et al. (2015) represent evidence of associations between estrus expression and fertility where estrus characteristics were measured by three AAM in two herds.

Although there is large variation in automated measurements of estrous behaviour and an undefined association with physiological aspects such as estradiol and preovulatory follicle diameter, the degree of estrus expression is associated with greater likelihood of ovulation and P/AI. The large variability can be interpreted as opportunity for genetic selection or for improvements in housing and management practices that could enhance estrus expression and thus detection. It remains to be investigated if hypothetical improvements in estrus expression through enhanced health, metabolism or hormonal profile translate into greater fertility. Selection for fertility via magnitude of estrus expression addresses the root of the problem (low expression), while increased detection, a currently achievable outcome, provides a partial solution.

Among three components of reduced fertility, namely delayed resumption of estrus cycles, greater incidence of abnormal cycles, and poor conception rates (Garnsworthy et al., 2008), AAM have potential to monitor the first two and contribute to improve the latter. AAM can also provide information on interval from calving to first post-partum estrus. Shorter intervals from

calving to the first ovulation have been associated with increased fertility (Santos et al., 2009). The heritability reported for this interval is 0.13 to 0.28 (Darwash et al., 1997). Resumption of cyclicity can be determined by on-line progesterone meters, but AAM are generally more accessible. One could argue that the first post-partum estrus has lower expression and that the AAM would miss a large portion of first post-partum ovulations, as demonstrated by (Johnson et al., 2012). First estrus or not, Ismael et al. (2015) concluded that interval from calving to first high activity event is heritable and associated with calving to first AI interval, estrus intensity and duration. In further support of a genetic component of estrus expression, Cummins et al. (2012) observed greater estrus intensity and duration in Holstein-Friesian cows bearing a "Fertility +" genotype.

5.3 Thesis Strengths and Limitations

The main subject of this thesis, i.e. the characterization of estrus from AAM data, is a current and prevalent research topic. While I developed this work, a large amount of information has become available with regard to validation of AAM, its integration into reproductive management programs that also use TAI, and associations with cow physiology and fertility (Fricke et al., 2014; Dolecheck et al., 2015; Madureira et al., 2015; Neves and LeBlanc, 2015). In addition, we are witnesses to increasing accessibility to and adoption of these technologies by dairy producers. The research presented herein contributed to this body of literature, where the greater goals are to improve AAM sensitivity and PPV, and to use data to its maximum potential for improvements in reproductive performance and sustainability of dairy farms.

Our research presented a thorough approach to estrus lying and standing patterns in heifers and cows, including investigation of risk factors, correlations with walking activity, and associations with ovulation rate and P/AI that had not been previously presented. We have also explored the variation in automated measurements of walking activity in heifers and the agreement among two AAM, providing applicable information for those developing estrus detection sensors. It is crucial that automated measurements of estrous behaviour are characterized across categories of cattle and production systems. Research is still warranted to

improve reproductive performance, especially if consumer concerns about administration of pharmacological products to dairy cows is considered.

The fact that most of this thesis research was done in nulliparous heifers can be seen as a limitation, as it is known that prevalence of reproductive issues is a major concern in lactating cows (Sartori et al., 2004). By studying heifers, we have approached behavioural expression and reproductive function without the influence of lactation or of metabolic and health challenges. Nonetheless, these studies contributed to the literature providing knowledge and new questions about estrous behaviour and physiology.

Another limitation was the observational rather than randomized controlled approach. Chapter 2 resulted from a larger study of effects of weaning strategy on heifer growth, which allowed evaluation of reproductive cycles of heifers with great detail. The research of Chapter 4 required a large number of inseminations to draw conclusions about ovulation rate and P/AI, which was obtained in a large commercial farm (approximately 1,700 lactating cows), with the drawback of restricted intervention opportunities. At this stage of AAM research, I believe that these observational studies were able to provide valuable information regarding factors associated with estrus expression. The association between estrus expression and fertility was observed within a single herd and with data from one AAM. Results should be considered with caution and in face of supporting literature.

Among features that I would like to have added to these experiments, I would highlight routine blood sampling for estradiol in heifers enrolled in Chapter 2, as well as routine sampling for serum progesterone to complement interpretation of data from Chapters 3 and 4. This thesis would also have benefited from an analysis of standing to be mounted behaviour to contrast with lying behaviour data from Chapter 4. Regarding visual behavioural expression, it would have been valuable to compare timing of events related to automated and visual detection in more than 12 heifers, but this was a time-consuming activity and not the major objective of my research. Even so, video evaluation was extremely valuable for my understanding of estrous behaviour and interpretation of the literature and the data presented here.

Finally, there were some restrictions regarding validation of automated lying behaviour data. In Section 2.2, we had video available for verification of extreme lying behaviour data

points. In contrast, in Chapter 4, we did not have video nor the possibility of validating the data obtained from the commercial AAM. As expected, extreme observations were present in that data set. The literature supports the observed large variation in basal lying time and bout frequency (Chapinal et al., 2009; Charlton et al., 2014). Our own video-validation showed that outliers (Section 2.2) were correctly measured. Considering these circumstances, we decided to proceed with the analysis of Chapter 4 by removing only observations that were smaller than the 1st percentile or greater than the 99th percentile of total lying time within each day. This limitation of research with AAM is counterweighed by the possibility of studying a much larger sample than would be possible with video-recordings.

5.4 Recommendations for Future Research

While working on this research topic, a frequent thought was: "Can we enhance estrus expression? If so, would that improve dairy cow fertility?". Some factors for reduced expression were presented here, but certainly there are sources of variation still unaccounted for. Nonetheless, optimally and poorly expressed estrus events differ in their association with fertility outcomes. This led me to identify three questions for future research, which will be individually developed below.

1) How can we use the knowledge on factors affecting estrus and basal behaviour towards enhanced estrus expression and detection?
2) What are the physiological bases of the association between estrus expression and fertility?
3) How can we apply estrus measurements in daily herd management?

5.4.1 How Can We Use the Knowledge on Factors Affecting Estrus and Basal Behaviour towards Enhanced Estrus Expression and Detection?

Once risk factors for poor expression of estrus have been identified, investigation of methods to reduce their impact on reproductive performance should follow. Management and housing are areas that could be acted upon in support of enhanced behavioural changes

associated with estrus. The minimum gain would be an increased rate of AI submission; greater achievements would be improvements in reproductive performance and possibly in fertility if the estrus expression-fertility association and the existence of common risk factors are considered.

Some factors, for example parity, cannot be mitigated. The resource in this case would be to test how different calculations and thresholds impact detection rate and PPV. In contrast, factors such as heat stress or lameness can be acted upon. It is not novel that reproductive function and estrus expression are impaired in those situations (Peralta et al., 2005; Walker et al., 2008). My approach would be to test measurements of estrus as response variables to assess efficacy of mitigating such risk-factors. Detailed investigations of the association between estrus expression and fertility within groups at risk of reproductive failure could also contribute to development of management practices.

Research from the 1980's reported greater frequencies of mounting and standing to be mounted than the current literature, as illustrated in Figure 1.2. Access to dry lots was a particularity of these older studies that is not present in the most current housing systems for dairy cows. Although I acknowledge that giving outdoor access to free-stall housed cows is challenging and not yet a practical solution, I would be interested in studying the reproductive performance, and especially the characteristics of estrus expression of high-yield cows housed in open packs or with access to dirt-floored spaces. An important feature of such studies would be to avoid confounding by possible impacts in hoof health.

In addition, I would hypothesize that fitness is an important feature in estrus expression. We have observed that heifers with naturally greater baseline steps/h had greater steps/h and greater standing time during estrus. Although this did not result in greater relative increase in walking activity or estrus duration, basal activity of cattle should be further researched. Basal activity is an important feature of AAM because these systems build alerts when behaviour deviates from normal. However, factors inducing differences in basal activity could reveal opportunities for increased estrus expression and detection, especially if we consider that conditions usually related to poor reproductive performance such as low BCS and lameness can influence basal level of activity. Play behaviour of calves is believed to improve muscle development, balance, fitness, and social interactions (Weary and Fraser, 2009). Further evidence for study of fitness of dairy cows is the observation that heifers stand for an average of

8 h without lying down on the day of estrus, followed by increased lying time the day after estrus. I believe this indicates that estrus expression requires a certain degree of fitness and that it leads to a degree of physical exhaustion. I would be interested in studying the presence of metabolites in blood or milk as markers of muscular damage. The longer lying time observed at d +1 requires further confirmation and understanding. The ideas for future research presented until here would benefit reproductive performance by increasing mostly the detection of estrus.

5.4.2 What Are the Physiological Bases of the Association between Estrus Expression and Fertility?

There is evidence from beef cattle that expression of estrus is associated with gene expression in endometrium, CL, and conceptus at d 19 after AI that is more favourable to pregnancy maintenance (Davoodi et al., 2016); similar studies are needed in dairy cows. As a chance speculation, increased behavioural expression of estrus might also affect reproductive function via neuroendocrine pathways, given that hypothalamic neurons participating in integration of afferent chemosensory stimuli and efferent motor responses have estradiol receptors and synapses with GnRH neurons (Petersen et al., 2003; Carlson 2013). Another area of special interest to me within this topic is the role of plasma estradiol concentration on induction of estrous behaviour and preparation of the reproductive tract for pregnancy establishment. It is evident that estradiol is associated with expression of estrous behaviours, but threshold concentration or extent of time above this threshold are unknown (Allrich, 1994). Individual differences in estradiol concentration requirement for estrus expression have been reported (Reames et al., 2011), what could explain the lack of clear association between estrus expression and estradiol levels. Presence of receptors for estradiol could be studied as an additional factor regulating the association between high expression and fertility. Among factors controlling receptor expression, progesterone in the previous luteal phase would be an important area of investigation. Manipulation of the estrous cycle with exogenous hormone administration to induce different periods of exposure and peak concentrations of estradiol and progesterone can be a valuable approach and have been recently investigated within our research group (Denis-Robichaud et al., 2016b; Silper et al., 2016).

Finally, a perspective I would like to take is an investigation of the time of blood sampling relative to estrus onset. Timing of sample collection according to estrus onset, taking into consideration periods of increased hepatic blood flow (Sangsritavong et al., 2002) needs to be reviewed. The first problem with current studies is that the dispersion of estrus onset along the day implicates in variable sampling times. For example, sampling could vary relatively to feeding, a factor known to reduce circulating steroids in cows (Sangsritavong et al., 2002; Vasconcelos et al., 2003). A second problem would be the timing between estrus onset and peak estradiol concentration. This interval has been reported to be between -8 and 7 h (Saumande and Humblot, 2005; Aungier et al., 2015), and estradiol concentration decreases by 50% within 5 h of LH surge (Chenault et al., 1975). Considering the lag between estrus onset, AAM alert, and blood sampling, it is likely that the peak estradiol will be missed, or at least that sampling will correspond to different stages for different cows. A third and final problem is that estradiol concentration is under influence of metabolic rate (Sangsritavong et al., 2002), which could vary among cows of different feed efficiencies. The fact that some cows of very high yield can achieve good reproductive performance (Santos et al., 2009) could be seen as evidence of an association between feed efficiency and reproductive performance.

5.4.3 How Can We Apply Estrus Measurements to Daily Herd Management?

Practical research directions relate to alternatives for use of information from AAM for decision-making, for example at breeding time, or for benchmarking between farms and identification of herd-level risk factors for poor reproductive performance. I have presented arguments for the role of estrus expression on fertility, and because AAM allows measurement of these characteristics, the potential for fertility prediction, assessment of reproductive potential, and herd management should be investigated. One factor that requires further investigations is the decision-making regarding estrus of low intensity or short duration. Possible actions would be to provide exogenous GnRH to induce ovulation at a known interval, or to inseminate at different timing considering that estrus to ovulation intervals are associated with estrus characteristics (Burnett et al., 2016). Differential AI timing according to estrus characteristics might contribute significantly to reproductive outcomes. Bloch et al. (2006) highlighted that if identification of cows at risk of prolonged estrus to ovulation interval was possible, this would

allow hormonal treatment of individual cows instead of applying treatments as the main reproductive program; AAM might be the tool that allow identification of cows at risk of delayed ovulation. Another possibility would be to evaluate the economic impact of skipping AI after estrus with poor characteristics and enrolling such cows in hormonal synchronization protocols, depending on DIM, parity, or health status. The challenge is to determine thresholds of estrus intensity or duration that impact fertility to an extent that justify interventions.

Among current alternatives for improving dairy cow reproductive performance, automated estrus detection technologies have an enormous potential. Research has reported that the AAM available are efficient at detecting estrus and can yield reproductive performance similar to TAI in some herds. Nevertheless, I believe that there are still plenty of opportunities to be explored regarding how to use these sensors and transform the data they provide into useful information.

5.5 Final Conclusions

The research presented in this thesis contributed to the knowledge on automated measurements of estrus expression, especially regarding patterns of estrus activity within repeated estrus events of the same heifers, characterization of lying behaviour, agreement between AAM, and association between estrus characteristics and fertility. The literature has reported that AAM are able to increase AI submission rates. The results from this thesis indicate that there are many more areas to be explored given the large quantity of data generated daily for individual cows and herds.

In support of maximizing the potential of AAM, I listed three questions for future research. These concern how to use AAM data to improve estrus detection and expression, what the physiological mechanisms behind the fertility association are, and how can we apply the AAM to daily routine decision-making. Use of AAM can be an alternative to hormonal synchronization in some herds as well as a tool to increase overall AI submission rates. A follow up action is to learn how to use AAM data for improved management, housing and health, ultimately resulting in increased expression of estrus and enhanced reproductive performance of dairy cows.

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