IMPROVING REPRODUCTIVE PERFORMANCE THROUGH THE
DEVELOPMENT OF A DAIRY HERD INDEX, INTRAUTERINE HORMONE
DELIVERY OR INSEMINATION TECHNIQUE IN LACTATING COWS

DISSERTATION

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By

Santiago Bas, DVM

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Dissertation Committee:

Dr. Gustavo M. Schuenemann, Advisor

Dr. Päivi Rajala-Schultz, Co-Advisor

Dr. Marco Coutinho da Silva

Dr. Michael L. Day
ABSTRACT

Improving reproductive performance of dairy cattle is paramount to maximize productivity and sustainability of dairy operations. The present dissertation includes three specific aims containing a series of five experiments (Chapters) aimed at improving reproductive performance in lactating dairy cows. The first specific aim (Chapters 3 and 4) evaluated the effectiveness of using protective plastic sheaths to minimize vaginal contamination of the AI catheter at the time of AI and pregnancies per AI in lactating dairy cows inseminated with or without the use plastic sheaths. In addition, the economic benefit of using plastic sheaths within a herd was estimated (Chapter 4). Using plastic sheaths at the time of AI resulted in reduced contamination of the AI catheter and improved pregnancies per AI for second or greater services in lactating dairy cows. Additionally, the use of plastic sheaths increased herd profitability by improving overall pregnancies per AI at a minimal cost. This first specific aim show the importance of reducing bacterial contamination at the time of AI, which should not be disregarded by AI technicians, in order to achieve consistent reproductive outcomes over time.

Specific aim 2 (Chapters 5 and 6) assessed the LH profile and the ovulatory response following the intrauterine administration of GnRH alone (100 vs 200 µg) or with the addition of glycerol (7% v/v). The first experiment (Chapter 5) was designed to
assess the LH profile and the ovulatory response following the intrauterine administration of GnRH (100 µg) in lactating dairy cows. Findings from this study revealed that GnRH administered intramuscularly resulted in higher serum LH concentrations when compared to cows receiving GnRH via the intrauterine route. Interestingly, despite the lower concentration of LH, cows receiving GnRH through the intrauterine route were able to ovulate. These results served as the basis for a subsequent study (Chapter 6) designed to enhance GnRH absorption after intrauterine administration. Thus, the study presented in chapter 6 was performed to evaluate whether the intrauterine administration of 200 µg of GnRH plus glycerol would result in the preovulatory release of LH comparable to the intramuscular administration of 100 µg of GnRH in lactating dairy cattle. Findings showed that increasing the concentration of GnRH (200 µg) and addition of glycerol (7% v/v) to enhance GnRH absorption did not result in increased release of LH when compared to cows receiving GnRH (100 µg) through the intramuscular route. However, despite the lower concentrations of LH, ovulation was observed in cows where GnRH was infused into the uterus. Thus, based on these results, delivery of GnRH through the intrauterine route can be regarded as a potential alternative to minimize the number of injections that dairy cows receive during synchronization protocols.
I dedicate this thesis to my wife Valeria and to my son Pedro.

I also dedicate this thesis to my parents Gabriel and Graciela and to my sisters Magdalena and Lucia.
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VITA


2005................................................................. D.V.M Catholic University of Córdoba, Cordoba, Argentina.

2009 to present.................................................... Graduate Teaching Associate, Department of Veterinary Preventive Medicine, The Ohio State University.
PUBLICATIONS

Peer Reviewed Publications


Abstracts

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CHAPTER 1

Introduction

Improving reproductive performance in dairy cattle is paramount to maximize dairy productivity and sustainability. Suboptimal reproductive performance leads to increased culling rates, decreased milk yield, and decrease genetic progress for traits of economic importance; thus negatively affecting economic returns (Plaizier et al., 1997). It is also well documented and accepted that poor reproductive performance increases the economic losses due to increased days open after the voluntary waiting period (Groenendaal et al., 2004; Meadows et al., 2005) and the value of a new pregnancy for lactating dairy cows (Stevenson, 2001; De Vries, 2006). Artificial insemination (AI) is the most common practice used to breed dairy cows in the US. According to the Animal Health Monitoring System (AHMS), approximately 78% of the services were performed through AI (USDA, 2009). Inadequacy in estrous detection and progressive decline in fertility in dairy herds led to the development of synchronization programs that allow the strategic and systematic breeding of animals with acceptable conception rates in dairy herds (Pursley et al., 1995). Since then, most research efforts have focused on the synchrony of follicular waves and timing of ovulation with respect to AI. Nevertheless,
implementation of these programs has been accompanied with increased number of times that animals need to be handled, increased use of hormones (e.g., GnRH, progesterone, and prostaglandin), and increased number of injections per animal (USDA, 2009). Despite the development of synchronization of ovulation protocols, which helped improve reproductive performance under controlled conditions (e.g., research herds), reproductive efficiency of lactating dairy cows continues to be challenged. Efficient detection of estrus, timing of AI, semen quality, and appropriate hygienic procedures are critical to achieve optimal fertility in dairy herds.

Furthermore, factors such as nutrition (Curtis et al., 1985), cow comfort (Huzzey et al., 2006), uterine diseases (LeBlanc et al., 2002; Sheldon et al., 2006; Dubuc et al., 2010), mastitis (Santos et al., 2004a; Ahmadzadeh et al., 2009), genetics (reviewed by Lucy, 2001), heat stress (reviewed by Wolfenson et al., 2000), compliance to synchronization protocols (Galvão et al., 2013), among others affect reproductive performance of dairy cattle. Accurate identification of the risk factors affecting reproductive performance is the first step to improve dairy herd productivity. Each individual herd must be assessed as a whole to effectively identify those areas for improvement in order to implement effective reproductive strategy.
CHAPTER 2

Review of Literature

2.1. Introduction

This review of literature focuses on basic and applied aspects of dairy cattle reproduction. Basic concepts and physiological processes that occur throughout the bovine estrous cycle are addressed. Additionally, a description of the gonadotropin releasing hormone functions, structure, receptors and applications in animal production systems will be discussed. Finally, the main factors (e.g. managerial, diseases, environmental) affecting reproductive performance in dairy herds will be covered in addition to alternative methods developed to aid in the identification of such factors. This review concludes with a problem statement and the rationale for conducting the research of this dissertation.

2.2. The Bovine Estrous Cycle

2.2.1. Hypothalamic-Pituitary-Ovarian Axis

Cattle are polyestrous animals that display estrous behavior approximately every 21 days with a normal range of 17 to 23 days (Ireland and Roche, 1983). The duration of estrus averages 7 to 10 hours (Walker et al., 1996; Dransfield et al., 1998) and ovulation of the dominant follicle (DF) occurs about 24-30 hours after the onset of behavioral
estrus (Walker et al., 1996; Bloch et al., 2006). Following ovulation, a corpus luteum (CL) develops and 16 to 18 days later, luteolysis occurs and a new cycle begins. All these changes that occur throughout the bovine estrous cycle are the result of the integrated action between the nervous and endocrine systems which are regulated by the hormones synthesized in the hypothalamus (GnRH), the pituitary gland (FSH and LH), the gonads (Progesterone, estradiol and inhibin) and the uterus (Prostaglandin F$_{2\alpha}$). Hormones secreted by the hypothalamic-pituitary-gonadal axis and the uterus act through a system of positive and negative feedback loops to regulate the events of the estrous cycle.

Gonadotropin releasing hormone is a decapeptide that was first isolated in the seventies (Amoss et al., 1971; Matsuo et al., 1971). This peptide is synthesized in neurons located in the medial basal hypothalamus and is released into the hypophyseal portal circulation where it will finally reach the gonadotropes localized in the anterior pituitary and stimulate the synthesis and release of LH and FSH (Conn et al., 1986; Fink, 1988; Page, 1988). The release of GnRH from hypothalamic neurons throughout the estrous cycle occurs in a pulsatile fashion (Clarke and Cummins, 1982); this pattern of GnRH secretion is critical for maintenance of normal pituitary function (Marshall and Kelch, 1986; Forde et al., 2011).

The frequency and amplitude of GnRH released from the hypothalamus varies according to the stage of the estrous cycle and is under the control of the ovarian steroids estradiol (E2) and progesterone (P4). During the luteal phase, when P4 concentrations are elevated, GnRH is secreted in high amplitude and low frequency pulses (Moenter et al., 1991; Clarke, 1995; Ferris and Shupnik, 2006). After luteal regression, when serum P4 concentrations fall and E2 levels rise, GnRH pulse frequency increases and amplitude
decreases (Moenter et al., 1991; Clarke, 1995; Ferris and Shupnik, 2006). Since membranes of GnRH secreting cells do not possess sex steroid receptors, its modulation by the ovarian steroids is mediated via intermediary neurons that possess steroid receptors (Herbison, 1998). Additionally, other hormones and peptides have been involved in GnRH regulation including leptin, insulin like growth factor I (IGF-I), gamma amino butyric acid (GABA), glutamate, neuropeptide Y, activin and inhibin (Clarke and Pompolo, 2005; Clarke, 2011). More recently, the discovery of the new peptide kisspeptin and its receptor (GPR54; Lee et al., 1999; Gottsch et al., 2006; Pompolo et al., 2006), and gonadotropin inhibitory hormone (GnIH; Tsutsui et al., 2000; Clarke et al., 2008), have emerge as key regulators of function of GnRH secreting neurons.

The gonadotropins LH and FSH are heterodimeric glycoproteins synthesized by the gonadotrope cells within the anterior pituitary (Baenziger and Green, 1988). Each glycoprotein consists of a common α-subunit (αGSU) and a unique β-subunit non-covalently associated. In all species, the αGSU is coded from a single gene and has an identical amino acid sequence. The unique β-subunit confers the specific properties to the hormone; it is coded from separate genes and has a specific amino acid sequence for each gonadotropin (Pierce and Parsons, 1981; Baenziger and Green, 1988). Synthesis and secretion of gonadotropins (LH and FSH) from the pituitary is controlled by GnRH secreted from the hypothalamus. Secretion of GnRH in high frequency pulses preferentially stimulates αGSU and LH β-subunit (LHβ) biosynthesis and secretion; a lower pulse frequency of GnRH stimulates FSH β-subunit (FSHβ) biosynthesis and secretion (Kaiser et al., 1997). Thus, high GnRH pulsatility preferentially stimulates LH
synthesis; while a lower pulse frequency of GnRH stimulates synthesis and secretion of FSH (Molter-Gérard et al., 1999; Ferris and Shupnik, 2006).

In heifers, during proestrus more than 80% of GnRH pulses had a corresponding LH pulse whereas during the luteal phase only 60% of GnRH pulses had a coincident LH pulse (Yoshioka et al., 2001). Coincidental release of LH and FSH from the gonadotropes can be observed during the preovulatory LH surge (Baird et al., 1981); however, during the remaining stages of the estrous cycle there is a clear difference in the pattern of gonadotropins secretion (Pawson and McNeilly, 2005). Walters and Schallenberger (1984a) observed differences in LH and FSH secretion throughout the estrous cycle of cows; during the early luteal phase 91% of LH pulses were associated with a pulse of FSH while 41% more pulses of FSH than LH were observed in the mid-luteal phase. While LH pulsatility is typically associated with pulses of GnRH, the pattern of FSH secretion is not tightly couple to secretion of GnRH and appears to be closely regulated by steroids, inhibin, activin and follistatin (Phillips, 2005).

In addition to GnRH, the ovarian steroids P4 and E2 are key regulators of the synthesis and secretion of gonadotropins from the anterior pituitary through feedback interactions at the central nervous system and pituitary level (Kesner et al., 1981). During the periovulatory period, the inhibitory effect of P4 on LH pulse frequency is removed leading to increased LH pulsatility. Using cultured ovine pituitary cells, it was demonstrated that E2 stimulated the insertion of GnRH receptors (GnRH-R) into the membranes of the gonadotrope cells (Gregg et al., 1990). Similar findings in sexually mature ewes revealed that estradiol increased concentrations of GnRH-R mRNA and the numbers of GnRH-R at the pituitary level (Turzillo et al., 1995). Additionally, prior to
ovulation the increasing E2 concentrations stimulate the release of GnRH from hypothalamic cells (Moenter et al., 1991). Thus, in a low P4 environment, pituitary sensitivity to GnRH is increased at the same time as GnRH secretion increases. This dramatic increase in GnRH secretion into the hypophyseal portal system is responsible for the preovulatory release of LH and consequently ovulation (Clarke, 1995).

In contrast to E2, the high circulating P4 concentrations observed during the luteal phase have been shown to inhibit the release of GnRH and LH pulse frequency (Walters et al., 1984b). Moreover, high P4 concentrations have been negatively associated with mRNA concentrations of GnRH-R and number of receptors for GnRH at a pituitary level; thus decreasing the release of LH (Laws et al., 1990; Turzillo et al., 1998). Ovariectomized cows treated with P4 showed reduced concentrations of GnRH-R in the pituitary gland when compared to animals that received E2 or a combination of P4 and E2 (Looper et al., 2003).

2.2.2. Luteal Phase

The luteal phase of the estrous cycle is characterized by the presence of a CL in the ovaries that actively secretes P4. This phase of the estrus cycle compromises the period of time from ovulation and subsequent development of a functional CL, until its regression in the non-pregnant cow approximately 16-18 days later (McCracken et al., 1999). Following ovulation, the follicular wall collapses into folds and the integrity of the basement membrane is disrupted allowing the migration of endothelial cells, fibroblasts, theca cells and capillaries into the previously avascular granulosa layer (Stocco et al., 2007). Subsequently, under the influence of growth factors (e.g., fibroblast growth factor,
vascular endothelial growth factor, insulin-like growth factor), vasoactive factors (endothelin-1, angiotensin II and nitric oxide), steroids (P4), prostaglandins and oxytocin, an active angiogenesis and remodeling process takes place to promote CL formation and P4 secretion (McCracken et al., 1999; Miyamoto et al., 2010). All these changes are initiated by the preovulatory LH/FSH release from the anterior pituitary; however, ovulation and subsequently luteinization are primarily attributed to the actions of LH that appears to be the major driver in CL formation and P4 production (Baird, 1992; Peters et al., 1994).

The CL is a heterogeneous transient ovarian gland composed of a mixture of cells such as small and large steroidogenic luteal cells, fibroblasts, endothelial, smooth muscle and immune cells and pericytes (Farin et al., 1986; O'Shea et al., 1989). The primary function of the CL is the secretion of P4, a requisite for establishment and maintenance of pregnancy in cattle. Following the preovulatory release of LH, theca interna and granulosa cells from the ovulatory follicle undergo a series of morphological and endocrine changes that will lead to its differentiation into large and small steroidogenic luteal cells (Miyamoto et al., 2010). Granulosa cells will increase in size (hypertrophy) and differentiate into large steroidogenic luteal cells, while theca cells number will increase (hyperplasia) and differentiate into small steroidogenic luteal cells (Farin et al., 1986; O'Shea, 1987; Smith et al., 1994). Small and large luteal cells differ in their ability to synthesize P4 and respond to LH (Smith et al., 1994). In vitro studies demonstrated that small steroidogenic luteal cells are responsible for the production of approximately 15% of the P4 by the CL, express LH receptors and respond to LH by increasing P4 production (Fitz et al., 1982). On the other hand, large luteal cells secrete approximately
85% of the P4 produced by the CL independently of LH stimulation (Fitz et al., 1982; Miyamoto et al., 2010). Following ovulation, the newly formed CL will show a rapid growth as the estrous cycle progresses. The weight of the bovine CL on day 3 of the estrous cycle averaged 0.64 g, and by the end of the luteal phase, on day 14 the weight averaged 5 g (Fields and Fields, 1996). This gradual increase in CL size is parallel to P4 production that showed a rapid increase from day 3 to 8 of the estrous cycle, reached maximum concentration by day 12 and remained constant until luteolysis on day 16-18 where P4 concentrations start to decrease.

In the absence of an embryo, maternal recognition of pregnancy does not occur and regression of the CL takes place on day 16-18 (McCracken et al., 1999). Functional luteolysis refers to the decline in P4 secretion from the CL, while structural luteolysis relates to the involution of the CL that will eventually become scar tissue in the ovary (Stocco et al., 2007). Uterine prostaglandin F2α (PGF2α) is the primary driver of luteal regression (Goding, 1974; Skarzynski and Okuda, 2010). Transport of PGF2α from the uterus to the ovary occurs via local countercurrent mechanism between the ovarian artery and the uterine vein minimizing the amount of PGF2α that enters the systemic circulation where it is enzymatically inactivated in the lungs (Hixon and Hansel, 1974; Davis et al., 1985).

Towards the end of the luteal phase, luteolysis occurs and P4 concentrations decline whereas circulating E2 concentrations (secreted by the DF) increase. This shift in steroid secretion leads to the upregulation of endometrial oxytocin and E2 receptors (Silvia et al., 1991; Goff, 2004). The expression of receptors is suppressed by high P4 concentrations during the early and midluteal phase (Wathes and Hamon, 1993; Ivell et
al., 2000). On the other hand, increased circulating E2 concentrations will activate their endometrial receptors and stimulate the synthesis of oxytocin endometrial receptors (Silvia et al., 1991). Furthermore, increased circulating E2 concentrations stimulate the release of oxytocin from the hypothalamus which will interact with its receptors in the endometrium; thus stimulating the synthesis and release of PGF$_{2\alpha}$ (McCracken et al., 1999). As previously described, PGF$_{2\alpha}$ will reach the ovary through a countercurrent mechanism where it will stimulate the release of oxytocin from the CL (McCracken et al., 1999). Oxytocin from ovarian origin interacts with its receptors in the uterus and further stimulates PGF$_{2\alpha}$ secretion establishing a positive feedback mechanism to regress the CL.

The actions of PGF$_{2\alpha}$ are mediated by growth factors (insulin like growth factor), vasoactive peptides (angiotensin II endothelin 1, nitric oxide), inflammatory mediators (tumor necrosis factor-$\alpha$ and interferon-$\gamma$, leukotrienes B$_4$ and C$_4$), inflammatory cells (macrophages, lymphocytes), and other factors (Berisha and Schams, 2005; Skarzynski and Okuda, 2010). These mediators induce the functional and structural luteolysis by decreasing P4 synthesis, blood flow, and increasing the rate of apoptosis within the CL (McCracken et al., 1999).

2.2.3. Follicular Phase

The follicular phase of the estrous cycle represents the period of time from luteal regression until ovulation of the DF. After regression of the CL, P4 concentrations decrease and GnRH pulse frequency increases. This increase in GnRH pulsatility leads to increased frequency of LH pulses (Moenter et al., 1991; Clarke, 1995; Yoshioka et al., 2001). In the cyclic cow, under the influence of low P4 concentrations, LH pulse
frequency is high (1 pulse/hour) and amplitude low, while under high P4 concentrations LH is secreted in high amplitude and low frequency pulses (1 pulse/3-4 h; Rahe et al., 1980).

The DF present in the ovary responds to the higher frequency of LH pulses by producing increasing concentrations of E2 and increasing follicular size (Allrich, 1994; Roche et al., 1998). As the follicle continues to grow, E2 concentrations start to increase and maximum levels are reached on the day prior to the onset of estrus. This increase in E2 will eventually lead to estrous behavior and will induce the preovulatory release of preovulatory release of GnRH/LH (Allrich, 1994). In the brain, E2 induces a sustained secretion of GnRH from the hypothalamus (Moenter et al., 1991) and influences behavioral centers that will lead to the onset of estrus behavior and sexual receptivity (Swanson and Hafs, 1971). At the pituitary level, E2 enhances the synthesis and insertion of GnRH-R into the pituitary gonadotropes membranes (Nett et al., 1984; Gregg et al., 1990). Altogether, as the DF continues to grow and produce E2, there is an increased sensitivity of the pituitary to GnRH followed by a dramatic increase in GnRH concentrations that leads to the preovulatory release of LH required for ovulation of the DF. It has been proposed that the exposure of the brain to the high concentrations of P4 from the preceding luteal phase is required for E2 actions during the follicular phase; thus, influencing the timing of the GnRH/LH surge (Clarke and Pompolo, 2005). Furthermore, it was reported that mRNA concentrations for GnRH-R increased prior to the rise in E2, suggesting that the decrease in P4 is responsible for the increased pituitary sensitivity to GnRH (Turzillo et al., 1998).
In the cow, the peak of E2 is observed 24 hours prior to the onset of estrus. This increase in E2 circulating concentrations will induce estrous behavior and the preovulatory release of GnRH/LH. The substantial amount of GnRH released from the hypothalamus reaches the anterior pituitary through the portal blood vasculature where it finally reaches its receptor in the gonadotrope cells. Upon binding to its receptor, GnRH initiates a cascade of events that leads to the preovulatory release of LH. The release of LH into the bloodstream eventually reaches the ovaries where it induces a cascade of events leading to ovulation of the DF.

2.2.4. Follicular Dynamics

Initial studies in heifers’ ovaries during 1960 suggested that the development of ovarian antral follicles throughout the estrous cycle occurred in two periods or waves of growth (Rajakoski, 1960). Controversy was observed in subsequent studies that either supported or refuted the proposed patterns of follicular development (Choudary et al., 1968; Swanson et al., 1972). The introduction of real time ultrasonography (US) made possible the understanding of ovarian follicular dynamics (Pierson and Ginther, 1984). Ultrasonographic studies confirmed that ovarian follicular growth is characterized by the sequential development of waves of follicular growth in cattle (Pierson and Ginther, 1988; Savio et al., 1988; Sirois and Fortune, 1988).

A follicular wave involves the synchronous development of a group of ~24 follicles initially recruited at a diameter of 3-4 mm followed by the development of a single large follicle (dominant) and regression of the remaining subordinates (Savio et al., 1988; Ginther et al., 1989a). More than 95% of estrous cycles are composed of two or
three follicular waves. While some studies reported a preponderance of two wave cycles (Ginther et al., 1989b; Ahmad et al., 1997), others have reported a predominance of three wave cycles (Sirois and Fortune, 1988). Furthermore, one wave of follicular development have been reported in mature cows right after calving and in heifers around the time of puberty (Savio et al., 1990; Evans et al., 1994) while presence of four waves have been reported in *Bos indicus* cattle (Rhodes et al., 1995).

Regardless of the patterns of follicular development (two or three waves), the first wave emerges on the day of ovulation (Day 0). The second wave, in two wave cycles, emerges on days 9-10 and on days 8-9 for three wave cycles, while emergence of the third wave (in three wave cycles) occurs on days 15-16 (Ginther et al., 1989b). In the presence of a functional CL, the DF undergoes atresia (Bergfelt et al., 1991). However, when luteolysis occurs and serum P4 concentrations fall, the DF present becomes the ovulatory follicle. Thus, in cows presenting two waves of follicular development, the ovulatory follicle is the DF from the second wave. In animals with three follicular waves, the ovulatory follicle is the DF form the third wave. In two wave cycles, luteolysis occurs earlier (day 16) than in three wave cycles (day 19), consequently the cycle length in cows with two waves averages 19-20 days while in cows presenting three waves averages 22-23 days (Ginther et al., 1989b; Adams et al., 2008). While some studies in cattle have reported increased fertility in animals with 3 waves of follicular development when compared to cows presenting two waves (Townson et al., 2002) others reported no improvement (Bleach et al., 2004). Furthermore, factors such as nutrition (Lucy et al., 1992), heat stress (Wolfenson et al., 1995), and breed (Bó et al., 2003) can affect the patterns of follicular development.
In cattle, follicular development can be subdivided in three phases, recruitment, selection and dominance. The recruitment of the cohort of follicles is temporally associated with a surge of FSH. Studies in heifers showed that a surge of FSH precedes the emergence of a follicular wave and it is responsible for the recruitment of a cohort of follicles (Adams et al., 1992; Ginther et al., 1996a). Circulating FSH concentrations start to increase two days prior to the emergence of a wave and reaches maximum levels one day prior to the onset of the wave; thus, two wave cycles have two FSH surges while three wave cycles have three FSH surges. Around the time of estrus, two surges of FSH have been demonstrated in cattle. The first FSH surge is coincidental with the preovulatory release of LH while the second one occurs 18-24 hours later and is associated with the emergence of the first follicular wave (Walters and Schallenberger, 1984a; Quirk and Fortune, 1986).

In monovular species like cattle a single follicle from the recruited cohort is selected, continues to grow and develops into the DF while the remaining follicles undergo atresia (Ginther et al., 1997). Deviation, the greatest difference in growth rates between the DF and the largest subordinate, begins approximately on day 3 when the DF averages 8.5 mm and the largest subordinate 7.2 mm (Ginther et al., 1997; Ginther et al., 2000). All antral follicles from the cohort have the potential of becoming DFs. In a study in heifers, it was demonstrated that any follicle with a diameter of 5mm is capable of becoming the DF when all the remaining follicles are ablated (Gibbons et al., 1997). Another study showed that treatment with FSH during the early stages of a follicular wave allowed more than one follicle to attain the diameter of the DF (Adams et al., 1993). Furthermore, it was demonstrated that after ablation of the largest follicle,
dominance can be achieved by the largest subordinate follicle rescued from regression (Ginther et al., 2001b).

Following the peak of FSH, that on average occurs 1 day prior to the emergence of a follicular wave, FSH concentrations begin to decrease and nadir concentrations are observed at the time of deviation (day 3) when one of the follicles from the cohort acquires a developmental advantage over the others and becomes the DF (reviewed by Ginther et al., 2001a). At this point in time, FSH is below the required concentrations for the subordinate follicles to keep growing, while the present DF adapts to the new hormonal milieu and continue to grow (reviewed by Ginther et al., 2001a). Initially, the growing follicles from a cohort produce increasing concentrations of E2 and inhibin which exerts a negative feedback on the release of FSH from the anterior pituitary (Adams et al., 1993). However, only the DF acquires a greater capacity to produce E2 than the subordinates and becomes the primary inhibitor of FSH secretion (reviewed by Fortune et al., 2001). Increased diameter and follicular fluid concentrations of E2 were observed in the future DF on day 2 of the first follicular wave of the estrus cycle (Evans and Fortune, 1997). Additionally, increased mRNA expression for key steroidogenic enzymes and gonadotropin receptors were observed in the DF (Evans and Fortune, 1997; Bao and Garverick, 1998).

One of the postulated mechanisms of deviation was related to the acquisition of LH receptors in the granulosa cells of the DF (reviewed by Ginther et al., 1996b). Follicles express FSH receptors in the granulosa cells and LH receptors in the theca cells, but only the granulosa cells from DF acquire receptors for LH (Xu et al., 1995; reviewed by Ginther et al., 1996a). Several experiments were performed in an attempt to evaluate
the temporal relationship between the presence of LH receptors in granulosa cells and the time of divergence. While the selection occurred without detectable levels of mRNA for LH receptor in the granulosa cells (Evans and Fortune, 1997), others showed increased LH receptor mRNA prior or at the time of deviation (Bodensteiner et al., 1996; Luo et al., 2011). This suggests that the acquisition of LH receptors by granulosa cells may not be critical for deviation, but rather important for continued growth and maintenance of dominance facilitating the shift in gonadotropin dependency from FSH to LH (reviewed by Ginther et al., 1996b; reviewed by Fortune et al., 2001).

Another mechanism implicated in ovarian follicular development and deviation involves the insulin-like growth factor (IGF) system. Components of the IGF system include the ligands IGF-I and IGF-II, two receptors (type I and type II), six isoforms of IGF binding proteins (IGFBP-1, -2, -3, -4, -5, -6) and proteases for IGFBPs (reviewed by Fortune et al., 2001; reviewed by Ginther et al., 2001a). The IFG system promotes E2 synthesis in ovarian antral follicles (reviewed by Fortune et al., 2001). In the future DF, FSH induces the synthesis of IGFBP-4/-5 protease which causes a reduction in IGFBP-4 and -5 (reviewed by Fortune et al., 2001). This reduction in IGFBP leads to an increase in free IGF that eventually leads to grow of the DF and increased E2 production with the subsequent reduction in circulating FSH concentrations (reviewed by Fortune et al., 2004). The exact mechanism of follicular deviation is not completely understood and many aspects involved in the process of deviation remain to be elucidated.
2.3. Use of GnRH in Animal Production Systems

2.3.1. GnRH Structure and Synthesis

The neuropeptide GnRH is a decapeptide with a molecular weight of 1.183 Daltons that controls the secretion of gonadotropins from the pituitary gland and plays a pivotal role in mammalian reproduction. This peptide was first characterized from the ovine and porcine hypothalamus in the early seventies (Amoss et al., 1971; Matsuo et al., 1971). Currently, 23 naturally occurring variants of GnRH have been identified across vertebrate species (reviewed by Millar et al., 2004; reviewed by Schneider et al., 2006). However, only two GnRH variants are expressed in most mammalian species.

The hypothalamic GnRH, also referred to as mammalian GnRH or GnRH-I has an amino acid sequence of pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂ (reviewed by Millar et al., 2004). The hypothalamic pituitary GnRH system of cattle is composed of the type I GnRH decapeptide hormone and the type I GnRH-R (Kakar, 1993). The second variant, GnRH-II, was first isolated from the chicken brain and differs from GnRH I by having the His⁵, Trp⁷ and Tyr⁸ residues (Sealfon et al., 1997; Millar, 2005). While the third form is referred to as salmon GnRH or GnRH-III. All the GnRH variants are 10 amino acid peptides with highly conserved amino acids in positions 1, 2, 4 (amino), 9, and 10 (carboxyl) and the most variable amino acids located on positions 5 through 8 (Millar, 2005). This review section will focus on the GnRH-I variants and it will be referred to simply as GnRH.

The gene encoding the GnRH molecule is comprised of four exons (1 through 4) and three introns (A through C). The 5’ untranslated region is encoded by exon 1, the
signal peptide, the GnRH molecule and the first 11 amino acids form the GnRH associated peptide (GAP) are encoded by exon 2, and exons 3 and 4 encode the remainder amino acids from of the GAP and the 3’ untraslated region (Herbison, 2006). Translation of the GnRH mRNA results in the production of a pre-pro hormone that undergoes further posttranslational modifications to maturation (Herbison, 2006). The GAP is co-secreted with GnRH from the nerve terminals into the portal system and it may be involved in the processing and packing of the decapptide (reviewed by Schneider et al., 2006).

Synthesis of the GnRH decapptide occurs in the neurosecretory GnRH cell bodies (Herbison, 2006). The GnRH cell bodies are distributed in paraventricular and preoptic area of the hypothalamus and their axons extend towards median eminence where they contact the portal capillary system (Herbison, 2006). A potential of action originated in the cell bodies of the GnRH secretory neuron will reach the synaptic terminal and will induce the release of the hormone into the portal system (Herbison, 2006). Once in the portal circulation, this decapptide will reach the gonadotrope cells at the anterior pituitary where it binds to its receptor and stimulate the release of gonadotropins (Jeong and Kaiser, 2006)

**2.3.2. GnRH Agonists**

The distinguishing characteristics of GnRH agonists should include the increased affinity for the GnRH-R and the longer half life in circulation (Karten and Rivier, 1986). The highly conserved NH$_2$ terminus (pGlu-His-Trp-Ser) and COOH terminus (Pro-Gly.NH2) amino acids suggest that these features are of critical importance for receptor
binding and activation (reviewed by Millar et al., 2004). Upon binding to its receptor the GnRH decapptide NH₂ and COOH terminal domains are closely opposed to each other as a consequence of a β-II bend involving residues 5-8 (Sealfon et al., 1997). Substitution of Gly⁶ with a D-amino acid increased the potency of GnRH by enhancing the β-II turn conformation and the binding affinity to its receptor (Karten and Rivier, 1986). Thus, substitution of the Gly⁶ residue with a D-amino acid will produce GnRH agonists more potent than the natural hormone (Karten and Rivier, 1986; Padula, 2005). Additionally, removal of the amino terminal Gly¹⁰ with alkyl amines produces nonapeptides that increases the affinity for the GnRH-R. Administration of potent GnRH agonists induces the release of large amounts of LH and FSH from the anterior pituitary, followed by a return to basal concentrations (Chenault et al., 1990). However, the continuous treatment with a GnRH agonist (Buserelin) leads to a reduced secretion of LH as a consequence of the down regulation of GnRH-R at the gonadotropes level and uncoupling of the second messenger pathways (Melson et al., 1986; Huckle and Conn, 1988; D'Occhio et al., 1996). Thus, the normal pulsatile pattern of GnRH secretion by hypothalamic neurons seems to be essential for the integrity of the hypothalamic pituitary axis.

2.3.3. GnRH Receptors

The GnRH receptors belong to the family of the G-protein coupled receptors (G₉/₁₁) that is characterized by the presence seven trans-membrane domain helices connected by three intracellular and extracellular loops, an extracellular amino terminal domain, an intracellular carboxyl terminal tail (reviewed by Schneider et al., 2006), and an intracellular C terminus linked by and extracellular loops. The extracellular domain is
involved in binding of the ligand, while the cytoplasmic domain interacts with the coupled G-protein (Stojilkovic et al., 1994).

The recognition of three different forms of GnRH in most vertebrate species suggested the existence of three GnRH receptors (GnRH-R) subtypes. The amino acid sequence of the GnRH-R was first identified from clones of a mouse pituitary αT3 cell line (Tsutsumi et al., 1992), and further phylogenetic analysis revealed the presence of three different GnRH-R subtypes: I, II and III (reviewed by Millar et al., 2004). The hypothalamic GnRH (type I) is considered the primary ligand for the pituitary GnRH-R type I in mammals (Millar, 2005).

Upon binding to its receptor, GnRH induces a conformational change that causes the substitution of GDP by GTP at the α-subunit causing it to dissociate from the βγ dimer. The α-GTP complex activates the enzyme phospholipase C that will in turn hydrolyze the membrane bound phosphatidylinositol 4-5 bisphosphate with the generation of the second messengers diacylglycerol (DG) and inositol 1,4,5 trisphosphate (IP3; Pawson and McNeilly, 2005). The cytosolic IP3 binds to the endoplasmic reticulum membrane and induces the release of calcium (Ca^{2+}) while DG activates the protein kinase C (PCK; reviewed by Burger et al., 2004). These intracellular signal transduction pathways induce the synthesis and release of LH and FSH from the anterior pituitary (Halvorson, 2000). In addition to increased IP3 and DG generation, it is now widely accepted that through coupling to G_{q/11} and PKC, GnRH is capable of increasing the concentration of mitogen-activated protein kinase (MAPK) in the gonadotropes (Sundaresan et al., 1996; Han and Conn, 1999; Roberson et al., 1999).
MAPKs transmit extracellular signals into the cell nucleus and activate a variety of transcription factors which in turn modulate gene expression (Roberson et al., 1995).

The density of GnRH-R in the membranes of the pituitary gonadotropes varies according to the stage of the estrous cycle (Rispoli and Nett, 2005). The increased circulating concentrations of E2 observed by the end of the estrous cycle are responsible for the increased pituitary sensitivity to GnRH by stimulating the synthesis and insertion of GnRH-R into the membranes of the gonadotropes (Schoenemann et al., 1985; Nett et al., 2002; Rispoli and Nett, 2005). In contrast, the high circulating concentrations of P4 observed during the luteal phase have a negative effect on the density of GnRH-R at the pituitary (Rispoli and Nett, 2005). Additionally, GnRH itself plays a critical role in regulating the number of GnRH-R at the pituitary as observed by the reduction in mRNA and GnRH-R numbers at the pituitary after the hypothalamic pituitary disconnection and treatment with GnRH antagonists (Brooks and McNeilly, 1994; Turzillo et al., 1995).

Many studies revealed the presence of extrapituitary GnRH-R in reproductive tissues including, oviducts, endometrium, gametes, gonads testes, placenta and embryos across a wide variety of mammalian species (reviewed by Ramakrishnappa et al., 2005). The presence of GnRH-R in these tissues has been involved in the autocrine or paracrine regulation of biological processes including steroidogenesis, luteinization, cellular proliferation and follicular atresia (reviewed by Ramakrishnappa et al., 2005; reviewed by Schneider et al., 2006). In the bovine GnRH-R mRNA has been identified in the ovarian follicles and the CL (Ramakrishnappa et al., 2003). Furthermore, during the follicular and luteal phases of the estrous cycle expression of GnRH-R mRNA and GnRH-R protein was demonstrated in bovine endometrial and oviductal cells (Singh et
More recently, an in-vitro study showed that GnRH was able to induce contraction of bovine uterine myoepithelial cells in vitro during the follicular phase (Giammarino et al., 2009).

It has been proposed that GnRH may play a role in the process of fertilization; addition of GnRH to in vitro fertilization media resulted in increased cleavage rate of bovine oocytes (Funston and Seidel, 1995). Moreover, in humans, GnRH increased the ability of sperm to bind to the oocyte’s zona pellucida (Morales, 1998). Similar findings were recently observed in a study where exposure of male and female bovine gametes to GnRH analogue showed an increased binding of spermatozoa to zona pellucida (Perera, 2010). Altogether, the presence of extrapituitary GnRH-R systems is suggests a direct regulatory role in the peripheral reproductive tissues, however, the specific function of GnRH and its receptor in peripheral tissues remains unclear.

2.3.4. LH Profile and Ovulation Following GnRH Administration

In cattle, the occurrence of estrus is associated with the presence of a DF that produces increasing amounts of E2 (Sirois and Fortune, 1988). Under no P4 influence, the E2 will exert a positive feedback at the hypothalamus and will induce the release of substantial amounts of GnRH that will subsequently lead to the preovulatory release of gonadotropins (LH and FSH) and ovulation. However, when concentrations of P4 are elevated the effects of the E2 on the hypothalamus are blocked and the release of GnRH is suppressed (Jeong and Kaiser, 2006).

The exogenous administration of GnRH induces the release of LH from the anterior pituitary followed by ovulation 24 to 32 hours later (Pursley et al., 1995). The
patterns of LH release following treatment with GnRH typically peaks by 2 hours after administration followed by a decrease to basal concentrations 4 to 5 hours later (Lucy and Stevenson, 1986; Chenault et al., 1990; Pursley et al., 1995). During the bovine estrous cycle, the naturally occurring preovulatory LH release reaches concentrations of approximately 9 ng/mL while release of LH following exogenous GnRH administration reaches concentrations of 17 ng/mL (Haughian and Wiltbank, 2002; Haughian et al., 2004). Additionally, differences in the duration of the peak of LH were observed, with exogenous GnRH induced LH surge lasting about 7 hours, while a duration of about 10 hours was observed following the endogenous peak; however, the area under the curve did not differ between treatments (Haughian and Wiltbank, 2002; Haughian et al., 2004). Furthermore, when the hour of ovulation was calculated from the onset of the preovulatory release of LH, no difference was observed between treated (GnRH) and untreated (Saline) animals (Haughian et al., 2004).

2.3.5. Applications of GnRH in Animal Production

Because of the central role of GnRH in the bovine reproductive cycle, GnRH and its analogues are widely used in animal production. In the US, GnRH use is labeled for the treatment of cystic ovaries. However, because of its role in the bovine estrous cycle GnRH is widely used to control fertility aspects of the bovine including follicular wave emergence and ovulation, prevention of early embryo mortality and induction of cyclicity in the post-partum period among others.
2.3.5.1. Treatment of Follicular Cysts

Follicular cysts (FC) can be defined as anovulatory ovarian structures with a diameter greater than 20-25 mm that can be present in one or both ovaries and that persist for a variable period of time in the absence of any active luteal tissue (reviewed by Garverick, 1997; reviewed by Vanholder et al., 2006). Additionally, knowledge of the circulating P4 concentrations facilitates the differentiation between follicular and luteal cysts (LC; reviewed by Garverick, 1997). While FC are thin walled structures (≤ 3 mm) that secrete little P4, LC have thicker walls (≥ 3 mm) and secrete greater amounts of P4 (reviewed by Silvia et al., 2002; reviewed by Vanholder et al., 2006). Follicular cysts are dynamic structures that can regress and be replaced by new follicles that will eventually ovulate or develop into new cysts (turnover), they can become a luteal cyst through luteinization or can recover by mechanisms that remain to be elucidated (Cook et al., 1990; reviewed by Garverick, 1997). Even in the presence of FC, waves of follicular development occur similar to those observed in normal cows; however, the interval between follicular waves has been reported to be longer and more variable (Hamilton et al., 1995)

Most evidence agrees that the underlying mechanism leading to the formation of cysts is related to the lack of an LH surge from the pituitary gland (reviewed by Silvia et al., 2002; reviewed by Wiltbank et al., 2002). This reduction in LH release does not seem to be a consequence of lower hypothalamic GnRH content or decreased LH reserves at the pituitary level (Cook et al., 1991). The physiological mechanism underlying the formation of FC appears to be related with a failure of E2 to stimulate the release of GnRH from the hypothalamus and the subsequent preovulatory release of LH from the
anterior pituitary (reviewed by Garverick, 1997; reviewed by Wiltbank et al., 2002). Typically, diagnosis of FC occurs during the first 60 days after parturition and its incidence in dairy cows has been reported to oscillate between 6 and 19% (reviewed by Garverick, 1997; reviewed by Vanholder et al., 2006). The presence of FC will alter the normal ovarian cycles causing irregular estrous cycles, anovulation, lack of estrous behavior or nymphomania and bull-like appearance of cows (Youngquist and Threlfall, 2007). Furthermore, FC have been associated increased days open and calving interval with the concomitant economic losses (reviewed by Silvia et al., 2002).

If left untreated, approximately 50% of the cysts that developed before the first ovulation in the post partum period and 20% of the cysts that developed after the first postpartum ovulation will spontaneously recover (Bierschwal et al., 1975; Kesler and Garverick, 1982). However, over the years with the gained knowledge on the pathophysiology of the cystic ovarian disease, alternative therapies to resolve this condition have been developed. Manual rupture of FC via rectal palpation is not recommended because of the increased risk of trauma and hemorrhage that can lead to adhesions with the resulting reduction in fertility (Bartolome et al., 2005). Early studies, reported that the treatment of FC with pituitary extracts containing gonadotropins was an effective therapy for FC (Casida et al., 1944). Later, it was demonstrated that human chorionic gonadotropin (hCG) and GnRH or its analogues (i.e. buserelin acetate) caused luteinization of the cystic structure (FC) and were implemented as treatment for cows with FC (Garverick et al., 1976; reviewed by Garverick, 1997).

The decapeptide GnRH is the most commonly used method to treat cows with FC and cure rates of about 80% have been reported (Brito and Palmer, 2004). Following
GnRH administration, a surge of LH (similar to the preovulatory) is observed and cyst luteinization occurs (Cantley et al., 1975). Ovulation of the cyst will not take place; however, if a DF is present it will be induced to ovulate (reviewed by Garverick, 1997). Following luteinization the steroidogenic synthetic pathway switches from E2 to P4 and the cyst becomes responsive to PGF$_{2\alpha}$ (reviewed by Garverick, 1997). Thus, initial treatment with GnRH (to induce cyst luteinization) followed 7 to 9 days later by exogenous PGF$_{2\alpha}$ seems to be an adequate strategy for treatment of FC (Kesler et al., 1978). More recently, the Ovsynch protocol (originally designed for timed AI) has been successfully used for the treatment of cystic ovarian disease (Brito and Palmer, 2004). Following treatment, the newly elevated levels of P4 will restore the hypothalamic positive feedback effect of E2, allowing the resumption of normal ovarian activity. An alternative treatment incorporates the administration of an exogenous P4 source that leads to decreased LH secretion, cyst regression and emergence of a new follicular wave (Garverick, 2007).

2.3.5.2. Regulation of the Bovine Estrous Cycle

Simultaneous studies by Lauderdale (1972), Rowson (1972) and Liehr (1972) reported that PGF$_{2\alpha}$ is a luteolytic agent capable of inducing the regression of the CL in cattle. The use of this arachidonic acid derivative was implemented for synchronization of estrous in cattle with the purpose of reducing the amount of time producers had to dedicate to estrus detection. The successful synchronization of estrus and ovulation in cattle treated with PGF$_{2\alpha}$ depends on the presence of a functional CL (reviewed by Macmillan, 2010). If administered during diestrus, exogenous PGF$_{2\alpha}$ administration will
induce expression of estrus behavior followed by ovulation 2 to 5 days later (Folman et al., 1990; reviewed by Macmillan, 2010). Unfortunately, estrus is not precisely synchronized because of differences in the developmental stage of the follicular population at the time luteal regression is induced (reviewed by Peters, 2005).

Early studies reported that GnRH was able to induce the release of LH from the bovine pituitary (Zolman et al., 1973; Kaltenbach et al., 1974). Later, in heifers, it was demonstrated that DFs ovulated in response to the exogenous administration of GnRH (Roche, 1975). One of the earliest reports suggesting that GnRH could be used in combination with PGF$_{2\alpha}$ for estrus synchronization programs was proposed by Thatcher et al. (1989). From this review it was concluded that the most adequate approach to synchronize cattle was to control the follicular dynamics and luteolysis and that this could be achieved by giving GnRH 1 week prior to PGF$_{2\alpha}$ administration (reviewed by Thatcher et al., 1989). Subsequently, a series of studies, (Twagiramungu et al. 1992ab) confirmed these results and showed that the combination of GnRH and PGF$_{2\alpha}$ was successful in synchronizing estrus behavior in beef cattle without affecting pregnancy and conception rates.

The benefit of using GnRH in breeding protocols was fully understand with the development of the Ovsynch protocol that allowed the artificial insemination of animals at a fixed time (Pursley et al., 1995). This synchronization program is based on 3 hormonal injections. The first GnRH injection has the purpose to induce the ovulation/luteinization of any DF present in the ovary and subsequent emergence of a new follicular wave. The luteolytic dose of PGF$_{2\alpha}$ administered 7 days later is intended to induce the regression of the CL and allow the maturation of the DF. Finally, 48 hours
later a second dose of GnRH is administered to induce ovulation of a DF within an 8 hours window (Pursley et al., 1995).

Since its introduction in 1995, the Ovsynch program has been widely accepted as a synchronization strategy by the dairy industry. Furthermore, several research studies have been carried out to optimize this timed artificial insemination (TAI) protocol. Additionally, this protocol served as the basis for the development of many other synchronization programs for both, dairy and beef cattle including: COsynch (Geary and Whittier, 1998), resynch (Fricke et al., 2003), Ovsynch+CIDR (Stevenson et al., 2006), G6G (Bello et al., 2006), Double Ovsynch (Souza et al., 2008) and 5 day COsynch + CIDR (Bridges et al., 2008).

2.3.5.3. Pregnancy Losses

In an attempt to properly classify bovine reproductive related terms, the period of time encompassed from fertilization to completion of organogenesis (approximately on day 42 of gestation) was defined as the embryonic period, while the fetal period was characterized as the period of time elapsed from completion of organogenesis to completion of the second stage of parturition (Committee on Bovine Reproductive Nomenclature, 1972). When taking into consideration the lifespan of the CL and P4 concentrations, pregnancy losses can be categorized into those that occur prior to day 24, referred to as early embryonic losses; those occurring between days 24 and 42-50 indicating late embryonic losses and losses occurring later than day 50 of gestation that are termed fetal losses (reviewed by Kastelic et al., 1991; Humblot, 2001).
In early studies in heifers, and moderate producing dairy cows fertilization rates of 90-100% were reported when high fertility semen was used (reviewed by Diskin et al., 2011). More recently, fertilization rates in high producing dairy cows appears to be closer to 83% (reviewed by Sartori et al., 2009). Summarized data from several studies evidenced that fertilization rates in lactating and non-lactating dairy cattle averaged 76.2% (range: 55.3% to 87.8%) and 78.1% (range: 58% and 98%) respectively, while in heifers fertilization rates of 100% were observed (reviewed by Santos et al., 2004b). In lactating beef cattle, fertilization rates averaged 75% (range: 60% to 100%), while in non-lactating beef cattle the average rate of fertilization was 98.6% (range: 94% and 100%; reviewed by Santos et al., 2004b) and in beef heifers the average fertilization rate reported was 88% (range: 75-100%). Furthermore, of the fertilized oocytes 79% were considered viable in beef cattle while 70% were viable in dairy cows (reviewed by Santos et al., 2004b). Similarly, in a study by Boyd et al. (1969) embryo survival rates of 60% were reported by 26 days post breeding; while in more recent studies involving high producing dairy cows less than 50% of the embryos recovered by day 7 post AI were viable (reviewed by Sartori et al., 2009).

Evaluation of embryonic loses from Holstein cows in 44 dairy herds in France showed that early and late embryonic losses in dairy cows were 31.6 and 14.7% respectively (reviewed by Humblot, 2001). Similarly, a recent review showed that embryo viability by day 5-6 post AI was approximately 50 and 57% for lactating dairy and beef cows respectively and that late embryonic loses averaged 12.8% (reviewed by Santos et al., 2004b). Diskin et al., (2006) estimated that under pastoral conditions approximately 43% of the embryonic losses occurred during the first 20 days of gestation.
(early embryonic losses) and that late embryonic and fetal loss were estimated to be 7%. Altogether, this suggests that embryonic losses during the first 20 days post breeding account for up to 75 to 80% of the total losses, while late embryonic losses account for 10 to 12% (reviewed by Inskeep and Dailey, 2005). Fetal losses are less prevalent than embryonic losses and have been estimated to be less than 11% when pregnancy diagnosis was performed between days 25 and 58 of gestation (reviewed by Santos et al., 2004b). However, when pregnancy diagnosis is performed earlier than 35 days of gestation fetal losses are inflated because some of the late embryonic losses accounted for (reviewed by Santos et al., 2004b). When pregnancy diagnoses was performed 40 days post-AI, the estimated fetal losses for beef and dairy cattle averaged 3% or less (reviewed by Inskeep and Dailey, 2005).

Overall, total losses from fertilization to birth are estimated to be around 60% with a final conception rate of 28% (reviewed by Santos et al., 2004b). Many factors have been implicated in embryo or fetal losses including: genetics, P4 during the cycle immediately before and post AI, oocyte quality, nutritional factors and energy balance, health disorders at parturition (i.e., dystocia, retained fetal membranes, milk fever), uterine diseases (i.e., metritis, endometritis, pyometra), heat stress, infectious diseases (i.e. leptospirosis, trichomoniasis, bovine viral diarrhea, infectious bovine rhinotracheitis, campylobacteriosis) and sire among others (Sartori et al., 2002; reviewed by Santos et al., 2004b; reviewed by BonDurant, 2005; reviewed by Grooms and Bolin, 2005; reviewed by Inskeep and Dailey, 2005; reviewed by Diskin et al., 2011).

Adequate P4 concentrations are crucial for the establishment and the maintenance of a pregnancy. Progesterone is essential for maintaining the histotrophic uterine
environment necessary for embryo growth and development as well as for implantation and placentation (Bazer, 1975; reviewed by Spencer et al., 2006). Additionally, P4 plays a critical role in modulating the immune function at the uterine level and also affects the capability of embryos to produce the luteolysis inhibitor interferon-tau (IFN-τ; reviewed by Morris and Diskin, 2008). Induction of ovulation at an appropriate time relative to AI and the ability to form an accessory CL with the potential increase in plasma P4 concentrations is the rationale for GnRH treatment at the time of AI or during the early embryonic period (reviewed by Peters, 2005). In addition to stimulating P4 secretion, these changes also result in decreased E2 concentration which in turn could lead to the inhibition of oxytocin receptors and the subsequent inhibition of PGF$_{2\alpha}$ secretion and luteolysis (reviewed by Peters, 2005).

The effect of GnRH administration at the time of AI during first and third or greater (repeat breeders) services showed an overall improvement of 6 and 7% points respectively in PAI when compared to untreated controls (Mee et al., 1990; Stevenson et al., 1990). Similar results were observed in a meta-analysis were the use of GnRH or its analogues at the time of AI increased the risk of pregnancy by 12.5% points with greater effects in repeat breeder cows (Morgan and Lean, 1993). However, results from these studies are not consistent and varied according to study, type and concentration of GnRH used and number of services. Similarly, numerous studies have evaluated the effect of GnRH during the early embryonic period (days 5 to 14 post AI) in an attempt to increase P4 levels at the time of maternal recognition of pregnancy. Although GnRH clearly increased the number of CL and P4 concentrations, considerable variation was observed with respect to pregnancy outcomes (reviewed by Peters, 2005). While some studies
reported increased PAI and reduced embryonic losses, others showed no benefit in the application of GnRH or its analogues after AI (reviewed by Peters, 2005). Using meta-analysis to evaluate the use of GnRH and its analogues given 11 and 14 days post AI, showed that only 6 out of 14 studies significantly improved pregnancy when GnRH was administered (Peters et al., 2000).

### 2.3.5.4. Other Applications of GnRH in Animal Production

Resumption of post partum cyclicity is crucial for the establishment of a new pregnancy and from an economic standpoint is highly desirable that ovarian cycles begin early in the post partum period. While early resumption of ovarian activity has been associated with increased probability of pregnancy at first AI in dairy cattle (Shrestha et al., 2004), anovular cows during this period are at higher risk of being culled from the herd (Thatcher and Wilcox, 1973). Recent studies have shown that between 20 and 30% of the dairy cows are anovular at 60 to 75 DIM (Silva et al., 2007; Walsh et al., 2007; Stevenson et al., 2008) with a range of 5 to 45% (Walsh et al., 2007).

The substantial percentage of anovular cows by the end of the voluntary waiting period encouraged the development of strategies to stimulate cyclicity in anovular cows. In non-cycling cows ovulation can be induced using GnRH or its analogues (reviewed by Rhodes et al., 2003). In a meta-analysis, Beckett and Lean (1997) reported that a single dose of GnRH during the post partum period did not affect the time or the risk of pregnancy at first service. Another approach has been the use of the Ovsynch protocol known to be successful in synchronizing lactating dairy cows (Pursley et al., 1995; Gümen et al., 2003); however, reduced fertility to the TAI protocol is observed in
anovular when compared to cyclic animals (Gümen et al., 2003). Another study showed that the use of Ovsynch in anestrous cows resulted in earlier conception but no improvement in final pregnancy rate was observed (McDougall, 2010). Incorporation of P4 to an Ovsynch program resulted in increased fertility in anovular lactating and beef cattle (Lamb et al., 2001; Rhodes et al., 2003; Stevenson et al., 2006; Stevenson et al., 2008). Despite anovulatory cows may respond to the Ovsynch synchronization protocol, incorporation of P4 will result in higher pregnancies per AI and more cows with normal subsequent luteal-phase lengths (Gümen et al., 2003; Rhodes et al., 2003; McDougall, 2010).

2.3.6. Routes of Delivery for GnRH

The most commonly used routes to deliver reproductive hormones in dairy and beef cattle production systems are the intramuscular (i.m.), subcutaneous (s.c.), intravaginal, and oral (p.o.). Administration of GnRH or its analogues (i.e., buserelin, deslorelin, fertilin acetate), PGF$_2$α or its analogues (i.e. cloprostenol), estradiol salts (cypionate, valerate, benzoate) and hCG is most commonly performed through the i.m. route. Delivery of P4, can be performed through the intravaginal route (CIDR, PRID), subcutaneously (e.g. Crestar) or p.o (MGA).

Administration of GnRH or its agonists through the i.m route induces the release of LH from the anterior pituitary with peak concentrations reached within 2 hours of hormone administration and return to basal levels by 5 hours (Chenault et al., 1990; Rajamahendran et al., 1998; Haughian et al., 2004; Souza et al., 2009). The magnitude of the release of LH is affected by the dose of GnRH administered and the potency of the
analogue used (Chenault et al., 1990; Souza et al., 2009; Dias et al., 2010; Giordano et al., 2012b). Chenault et al. (1990) evaluated the release of LH following the administration of varying doses of GnRH (gonadorelin), fertilin acetate or buserelin and observed that fertilin acetate and buserelin were 10 and 50 times more potent than GnRH respectively as measured by LH and FSH released. Additionally, differences in the release of LH following the administration of higher GnRH concentrations have been observed. While most studies reported enhanced release of LH (Colazo et al., 2009; Souza et al., 2009; Dias et al., 2010; Giordano et al., 2012b) others reported no change (Yamada et al., 2002; Rantala et al., 2009).

Because of its susceptibility to gastrointestinal peptidase degradation GnRH agonists bioavailability is greatly reduced making the p.o. an unsuitable route of delivery (reviewed by Conn and Crowley, 1991; reviewed by Padula, 2005). Administration of GnRH and its analogues is usually performed intramuscularly; however, because of their low molecular weight (10 or less amino acids) they can also be absorbed subcutaneously or across mucous membranes layers (Raehs et al., 1988; Donnez et al., 1989). Alternative routes for GnRH administration have been evaluated in the bovine and other species. When GnRH is administered subcutaneously rather than intravenously, higher doses are required to achieve the same effects; additionally, s.c administration results in delayed release of LH (reviewed by Conn et al., 1986). Troxel et al. (1983) studied the effects of i.m. GnRH injections and s.c. implants in suckled beef cows and observed similar LH surges among treatments; however, LH release was delayed in the cows that received GnRH subcutaneously in a gelatin capsule. In rats, evaluation of the vaginal absorption of a potent GnRH analogue (leuprolide) in combination with organic acids to enhance
absorption, resulted in 20% bioavailability (Okada et al., 1983). Addition of buserelin acetate to the semen dose for induction of ovulation was evaluated in rabbit does (Quintela et al., 2004; Viudes-de-Castro et al., 2007). Results from these studies showed that similar pregnancies can be achieved with the addition of buserelin to semen and subsequent vaginal delivery when compared to i.m. administration of the hormone (Quintela et al., 2004; Viudes-de-Castro et al., 2007).

In Brown Swiss and Holstein heifers the intrauterine (i.u.) administration of 1 and 10 mg of LHRH induced a release of LH similar to that resulting after the IV administration of LHRH (Dermody et al., 1976a). In mature dairy cows (unknown stage of lactation), i.u. administration (in saline or semen diluents) of 50 µg of a potent GnRH analogue (D-leu6-des-gly NH210 pro-ethylamide9) increased serum concentrations of LH when compared to 100 µg of a synthetic native GnRH analogue (Manns et al., 1980). Collectively, this data suggests that the i.u. route can be regarded as an alternative mean of delivering GnRH in cattle. In addition, i.u. GnRH administration could present further reproductive benefits if added to the semen extender. Receptors for GnRH have been identified in the bovine uterus and oviducts and a recent study reported that GnRH was able to induce contraction of the bovine uterine myoepithelial cells in vitro during the follicular phase (Singh et al., 2008; Giammarino et al., 2009). Furthermore, it has been proposed that GnRH may play a role in the process of fertilization. Addition of GnRH to in vitro fertilization media resulted in increased cleavage rate of bovine oocytes (Funston and Seidel, 1995). Moreover, in humans, GnRH increased the ability of sperm to bind to the oocyte zona pellucida (Morales, 1998). Similar findings were recently observed in a
study where exposure of male and female bovine gametes to GnRH analogue showed an increased binding of spermatozoa to zona pellucida (Perera, 2010).

2.4. Factors Affecting Reproductive Performance of Dairy Cattle

2.4.1. Transition Diseases and Metabolic Disorders

The transition period, can be defined as the period of time encompassed between the 3 weeks prior to parturition and the 3 weeks postpartum. This period represents a challenging time for the dairy cow because it is characterized by marked nutritional, metabolic and hormonal changes that may contribute to the development of infections and metabolic diseases (reviewed by Goff and Horst, 1997).

2.4.1.1. Metabolic Disorders

In the bovine, a decline in feed intake (FI) as parturition approaches is commonly observed (reviewed by Hayirli et al., 2002; Grummer et al., 2004). Dry matter intake (DMI) starts to decrease at 2 to 3 weeks prior to parturition with the lowest levels observed around calving (reviewed by Ingvartsen and Andersen, 2000; reviewed by Grummer et al., 2004). After delivery, DMI rapidly increases within the first 3 weeks that follows parturition; however, the nutritional requirements exceed the FI potential (reviewed by Goff and Horst, 1997; reviewed by Ingvartsen and Andersen, 2000).

Because of the imbalance between the energy requirements and the amount of energy obtained from the diet, animals will enter a state of negative energy balance (NEB; reviewed by Goff and Horst, 1997; reviewed by Grummer, 2007). As a result,
blood glucose levels are not adequate to meet the energy requirements and animals will rely on fat mobilization as a source of energy (Bell, 1995; Goff and Horst, 1997; Busato et al., 2002; Accorsi et al., 2005). Lipids are released as nonesterified fatty acids (NEFA) from the adipose tissue to be used as an energy source during periods of NEB (reviewed by Grummer et al., 2004). Circulating NEFA can be completely oxidized to carbon dioxide or partially oxidized to ketone bodies (acetone, acetoacetate and β-hydroxybutyrate –BHBA) to provide an alternative source of energy, or can undergo reesterification to triglycerides (reviewed by Drackley, 1999; reviewed by Grummer et al., 2004; reviewed by Roche et al., 2009). When adaptation mechanisms fail to compensate the NEB, the supply of NEFA to the liver exceeds its ability to completely oxidize them and ketone bodies accumulate in blood milk and urine with the resulting development of ketosis (reviewed by Goff and Horst, 1997; reviewed by Herdt, 2000).

Ketosis can be defined as a metabolic disease characterized by the presence of high circulating levels of ketone bodies (serum BHBA concentrations >2600μmol/L) that affects dairy cows during the postpartum period (Herdt and Gerloff, 1999). Subclinical ketosis (serum BHBA concentrations >1000 μmol/L) can be defined as a metabolic disease characterized by the presence of excess levels of ketone bodies in the absence of clinical sings (reviewed by Andersson, 1988; Duffield, 2000). The clinical form of the disease (serum BHBA concentrations >27 mg/dL) is characterized by reduced appetite, dry feces, dullness, reduced milk production and occasionally nervous sings (reviewed by Baird, 1982; Duffield, 2000). The prevalence of subclinical ketosis was estimated to range between 8.9 and 34% during the first 2 months of lactation and the reported
incidence varied between 2 to 15% with a peak incidence of about 30% occurring in the first week post calving (Baird, 1982; Dohoo and Martin, 1984; Duffield, 2000).

An early study by Dohoo and Martin (1984) described the association between increased levels of ketone bodies and metritis. More recently, levels of BHBA greater than 1200 μmol/L were associated with a greater risk of metritis in lactating dairy cows (Duffield et al., 2009). Similarly, cows with metritis and subclinical endometritis had increased BHBA when compared to normal cows at 1-4 weeks postpartum (Hammon et al., 2006). A potential explanation for the development of uterine diseases can be related to the negative effects of hyperketonemia on immune cell function (reviewed by Suriyasathaporn et al., 2000; Grinberg et al., 2008). Hoeben et al. (1997) reported that exposure of polymorphonuclear cells to elevated levels of BHBA reduced respiratory burst. Collectively these findings suggest that hyperketonemia increase the susceptibility to infections during the postpartum period. Additionally, ketosis has been associated with the development of ovarian cysts (Dohoo and Martin, 1984; Andersson et al., 1991). Ketosis has also been associated with reduced reproductive performance. Cows with BHBA milk concentrations >100 μmol/L were 1.5 times more likely to be anovular by 9 weeks post calving (Walsh et al., 2007). Furthermore, animals experiencing ketosis within the first 2 weeks of lactation were less likely to conceive at first service and more likely to be opened by 130 DIM (Walsh et al., 2007).

Another metabolic disorder that affects dairy cows during the postpartum period is hypocalcemia (reviewed by DeGaris and Lean, 2008; reviewed by Walsh et al., 2011). With the onset of lactation the demands of calcium are dramatically increased and the animal is challenged to maintain calcium homeostasis (reviewed by DeGaris and Lean,
While the bovine fetus requires 10.3 gr of calcium by the end of gestation, after calving for the production of 10 kg of colostrum the calcium requirements ascend to 23 gr (reviewed by Goff and Horst, 1997). Therefore, due to the increased calcium demand most cows will likely develop some degree of hypocalcemia (reviewed by Goff and Horst, 1997). Across several studies form North America, Europe and Australasia, the reported incidence of milk fever averaged 3.45, 6.17 and 3.5% respectively, with a range between 0 and 10% (reviewed by DeGaris and Lean, 2008). Similar results were observed in US dairy herds where the reported incidence of milk fever was estimated to be 6% while the subclinical form of the disease was of 50% (reviewed by Goff, 2008; Reinhardt et al., 2011). Typically, 75% of the cases of milk fever are observed within 24 hours of calving, an additional 12% of the cases occur between 24 to 48 hours after delivery, and the remainder cases occur at the time of delivery (6%), prior to parturition (3%) and 48 hours after calving (4%; Oetzel, 1988; Oetzel and Goff, 1999).

Clinical (milk fever) and subclinical hypocalcemia cause a progressive neuromuscular dysfunction that reduces the smooth and skeletal muscle contraction ability leading to a flaccid paralysis (Oetzel, 1988). Clinical signs of milk fever may be observed when calcium blood levels fall below 4 mg/dL (Goff and Horst 1997; Goff, 1999). More recently it was reported that milk fever and subclinical hypocalcemia intensify the immunosuppression observed during the periparturient period (Kiamura, 2006). Hypocalcemia seems to affect activation and function of immune cells (Kehrli Jr and Goff, 1989; Gilbert et al., 1993). Additionally, it was observed that cows with the
subclinical form of the disease had increased plasma cortisol levels, a recognized immune suppressive hormone (Horst and Jorgensen, 1982).

The lack of adequate muscular contraction and the compromised immune function can lead to the development of diseases of the reproductive tract and other body systems (reviewed by DeGaris and Lean, 2008). Some studies reported that cows with hypocalcemia were between 2.5 and 6 times more likely to experience dystocia when compared to cows that did not experience low circulating calcium concentrations (Curtis et al., 1983; Erb et al., 1985; Correa et al., 1993). In addition, following parturition and due to the lack of muscle contraction cows experiencing hypocalcemia are more likely to experience uterine prolapse (Risco et al., 1984). Furthermore, cows diagnosed with clinical hypocalcemia were 3.2 times more likely to experience retained placenta than cows that did not have clinical hypocalcemia (Curtis et al., 1983). Whiteford and Sheldon (2005) reported that cows with milk fever had greater diameter of the gravid uterine horn by 45 days following parturition indicating a slower uterine involution process. Hypocalcemia has been associated with reduced number of CL and fewer ovulatory follicles in affected dairy cattle (Kamgarpour et al., 1999; Whiteford and Sheldon, 2005). In addition, hypocalcemia was associated with extended calving to first service and calving to conception intervals and increased number of services per conception (Borsberry and Dobson, 1989).

In conclusion, metabolic diseases, including ketosis and hypocalcemia, are commonly observed in cows experiencing severe NEB after calving. These conditions have shown to affect reproductive performance in dairy cows. Thus, an optimal transition
management is required to minimize the detrimental effects of these condition in future health and performance of dairy cows.

2.4.1.2. Uterine Diseases

Following parturition the physical barriers of the vulva, vagina and cervix are compromised facilitating bacterial colonization of the reproductive tract (reviewed by Sheldon and Dobson, 2004). Moreover, the immune function of dairy cows is suppressed in the parturient cow (Kehrli et al., 1989ab; Mallard et al., 1998; Hammon et al., 2006). This suppression of the immune system is exacerbated by high estradiol and cortisol levels around calving, the decreased FI and NEB coupled with increased levels of nonesterified fatty acids, ketone bodies and transient hypocalcemia that follow parturition (reviewed by Goff and Horst, 1997; Zerbe et al., 2000; reviewed by Ingvartsen, 2006). Consequently, after delivery of a calf, the uterus of most dairy cows will have some degree of bacterial contamination and will be at risk of developing reproductive tract diseases such as metritis (reviewed by Sheldon and Dobson, 2004; reviewed by LeBlanc et al., 2011). While most cows are able to control bacterial contamination; at least 20% of the animals may develop metritis (Walsh, 2011).

Bacteria isolated from the uterine lumen can be categorized according to their pathogenicity as recognized, potential and opportunistic uterine pathogens (Sheldon et al., 2002). The recognized uterine pathogens (Arcanobacterium pyogenes, Escherichia coli, Fusobacterium necrophorum and Prevotella melaninogenicus) are associated with clinical disease and the most prevalent isolated uterine pathogens in dairy cows with uterine disease are A. pyogenes and E. coli (Williams et al., 2005; Brick et al., 2012). It
has been demonstrated that *A. pyogenes, F. necrophorum and P. melaninogenicus* cause damage of the endometrial tissue and act synergistically to enhance the likelihood and severity of uterine disease (reviewed by Sheldon et al., 2009b).

In addition to endometrial integrity, ovarian function is also disrupted. In sheep it was demonstrated that the *E. Coli* lipopolysaccharide (LPS) blocked release of GnRH and LH from the hypothalamus and pituitary affecting the ability of the DF to ovulate (Battaglia et al., 2000; Williams et al., 2001). Furthermore, LPS decreases aromatase activity in granulosa cells; thus, reducing follicular estradiol secretion (Herath et al., 2007). Luteolysis is also altered in cows with endometritis because there is a shift in the type of prostaglandin produced (from the F to the E series), and as a consequence luteolysis is delayed (Herath et al., 2006; Herath et al., 2009). Altogether, bacterial infections not only disrupt the uterine integrity, but also affect ovarian function directly and by affecting the control centers in the hypothalamus and pituitary (reviewed by Sheldon and Dobson, 2004; reviewed by Sheldon et al., 2008).

Retained fetal membranes or retained placenta (RP) can be defined as the failure to expel the placenta within 24 hours of parturition (Kelton et al., 1998). Based on 50 studies from 1979 to 1995, the lactational incidence of RP was estimated to be 8.6% (Kelton et al., 1998). Presence of RP has been associated with decreased milk production and the development of metritis, endometritis and with the subsequent impaired reproductive performance (reviewed by Fourichon et al., 2000; LeBlanc, 2008). It has been estimated that between 25-50% of cows with RP will develop metritis, and that cows with retained placenta had 14% lower conception rate than cows without RP (Gröhn and Rajala-Schultz, 2000; reviewed by LeBlanc, 2008).
Puerperal metritis can be defined as a severe inflammatory process that affects all layers of the uterus (endometrium, submucosa, muscularis and serosa) within 21 days after parturition (reviewed by Bondurant, 1999; reviewed by Sheldon et al., 2006). This condition is characterized by the presence of an abnormally enlarged uterus with foul smelling red-brown watery discharge, pyrexia (> 39.5 °C), and systemic signs of illness including decreased milk production, inappetence or anorexia, elevated heart rate and dehydration (reviewed by Sheldon et al., 2006). Risk factors for puerperal metritis include dystocia, stillbirth and RP (reviewed by Sheldon et al., 2006; Dubuc et al., 2010). Puerperal metritis is usually observed within the first week following parturition (Sheldon and Dobson, 2004). The term clinical metritis is used to classify animals with delayed uterine involution and foul smelling purulent uterine discharge in the absence of pyrexia or systemic signs of illness within 21 days postpartum (reviewed by Sheldon et al., 2008). The lactational prevalence of metritis averages 20% with a range between herds of 8 to 40% (Markusfeld, 1984; Curtis et al., 1985; Goshen and Shpigel, 2006; Hammon et al., 2006; Galvão et al., 2009b; Dubuc et al., 2011b). This condition was associated with the development of clinical and subclinical endometritis and a reduction conception rates compared to animals without metritis (Fourichon et al., 2000; Gröhn and Rajala-Schultz, 2000; Galvão et al., 2009b)

Clinical endometritis (CE) is defined as the inflammation of the endometrium and characterized by the presence of purulent (> 50% pus) or mucupurulent (50% pus, 50% mucus) uterine discharge without systemic signs of illness later than 3 weeks postpartum (reviewed by Bondurant, 1999; reviewed by Sheldon et al., 2006). Because of the absence of clinical signs this condition is usually diagnosed through vaginoscopy,
ultrasonography, the metriceheck device or a gloved hand (reviewed by LeBlanc et al., 2002; Williams et al., 2005; McDougall et al., 2007; LeBlanc, 2008; Brick et al., 2012). The prevalence of CE averages 17% with a range between herds of 5 to more than 30% (Kelton et al., 1998; LeBlanc et al., 2002; McDougall et al., 2007; reviewed by LeBlanc, 2008; Galvão et al., 2009b; Brick et al., 2012; Leutert et al., 2012). Risk factors for CE included dystocia, RP, stillbirth, abortion, metritis, vulvar conformation and ketosis (Gröhn and Rajala-Schultz, 2000; Galvão et al., 2009b; Dubuc et al., 2010; Potter et al., 2010). CE is associated with reproductive failure as reflected by decreased pregnancy rate, increased calving to first insemination or conception interval and increased likelihood of being culled due reproductive failure (LeBlanc et al., 2002; reviewed by Sheldon et al., 2008; Dubuc et al., 2011a; Brick et al., 2012; Dubuc et al., 2012).

Subclinical endometritis (SCE) can be defined as the inflammation of the endometrium diagnosed by cytology (reviewed by Sheldon et al., 2006). Typically, the diagnosis of SCE is performed by assessing the proportion of neutrophils present in a sample collected using a cytology brush or the low volume uterine lavage technique (Kasimanickam et al., 2004; Gilbert et al., 2005; Kasimanickam et al., 2005). It has been reported that SCE affects between 40 to 50% of lactating dairy cows being the most prevalent uterine disease (Kasimanickam et al., 2004; Gilbert et al., 2005; Kasimanickam et al., 2005; Hammon et al., 2006; Galvão et al., 2009a). Similar to CE, SCE is associated with reduced fertility characterized by increased days to conception, increased proportion of animals that fail to become pregnant and with more cows culled for reproductive reasons (reviewed by LeBlanc, 2008; Galvão et al., 2009a; reviewed by Sheldon et al., 2009a).
Diseases of the reproductive tract including RP, metritis, CE and SCE are common conditions that affect normal reproductive function of dairy cows. The resulting decreased fertility is associated with delayed ovulation after parturition, persistence of the CL and reduction in conception rates. Best management practices during the transition period in addition to accurate diagnoses and effective treatment are critical to mitigate the detrimental effects of uterine diseases.

2.4.1.3. Mastitis

Mastitis is the inflammation of the mammary gland (International Dairy Federation, 1987). In dairy cattle, mastitis can have an infectious or non-infectious etiology; however, the majority of bovine mastitis cases are caused primarily by bacterial infection. Clinical mastitis is defined as the inflammation of the mammary gland that is accompanied by with physical, chemical and microbiological changes of milk. Clinical sings include inflammation (i.e. redness, swelling, heat and pain) of the mammary gland, decreased milk production and altered composition and appearance of the milk. Subclinical mastitis indicates inflammation but not necessarily infection of the udder (International Dairy Federation, 1987). Clinical mastitis is one of the most common and a costly disease affecting dairy herds (reviewed by DeGraves and Fetrow, 1993). Economic losses have been associated with reduced milk production and quality and increased milk discarded, culling rates and treatment costs (reviewed by DeGraves and Fetrow, 1993; Kossaibati and Esslemont, 1997; reviewed by Seegers et al., 2003). In addition the costs associated with poor reproductive efficiency associated with the occurrence of mastitis have to be accounted for.
One of the first studies to propose a relationship between mastitis and altered reproductive function in dairy cattle was performed by Moore et al. (1991). In this preliminary work authors observed altered interestrus interval (less than 18 days or more than 24 days) in animals with clinical mastitis when compared to healthy herdmates (Moore et al., 1991). Barker et al. (1998) evaluated the relationship between mastitis and reproductive performance in Jersey dairy cows and observed that the number of days to first AI and AI per conception were greater for animals with clinical mastitis when compared to healthy animals or those developing mastitis after pregnancy diagnosis (Barker et al., 1998). Similar results were observed in 758 lactating Jersey cows where animals with clinical or subclinical mastitis before the first AI had increased days to first service, days open, and services per conception when compared with healthy controls (Schrick et al., 2001). In a retrospective study, Santos et al. (2004a), observed detrimental effects of mastitis on reproductive and lactational performance of dairy cows. Infected cows had increased DIM at first AI, increased culling rates, higher incidence of abortions and lower conception rates at first service (Santos et al., 2004a). A more recent study, it was observed that reproductive efficiency was decreased by the presence of clinical mastitis alone; however, in cows that experienced mastitis and other diseases the negative effects were further aggravated (Ahmadzadeh et al., 2009). Collectively, results from these studies suggested that mastitis is associated with anovulation at estrus, fertilization failure or embryo mortality.

Several mechanisms have been postulated to explain the negative effects of mastitis on fertility. One possible mechanism is that the hyperthermia (pyrexia) developed as a consequence of mastitis can affect ooeyte and early embryonic
development, thus reducing embryo quality (Edwards and Hansen, 1997; Al-Katanani et al., 2002; Sartori et al., 2002) Additionally, in response to the inflammation of the mammary gland a wide variety of inflammatory mediators are released including: interleukins (IL), tumor necrosis factor-α (TNF-α), nitric oxide (NO) and PGF2α. These inflammatory molecules can disrupt oocyte maturation and embryo development, can cause changes in the uterine function and can also disrupt the hypothalamic-pituitary-ovarian axis (Hockett et al., 2000; reviewed by McCann et al., 2000; Soto et al., 2003ab; reviewed by Hansen et al., 2004; Lavon et al., 2010; Lavon et al., 2011ab).

Mastitis is one of the most common diseases affecting lactating dairy cows with major economic losses due to antibiotic treatments, reduced quality of milk and milk production and increased culling rates. Nevertheless, the negative effects of mastitis exceed the milk losses and can ultimately affect reproductive efficiency of dairy hers. Therefore, the implementation of prevention and control strategies for mastitis should be a priority in any dairy operation.

2.4.1.4. Diseases of the Hoof

In addition to mastitis and poor reproductive performance, lameness is one of the main reasons to permanently remove cows from dairy herds (USDA, 2007). Because lameness is a particularly painful condition, it has been proposed as an indicator of the welfare in dairy cattle modern production systems (Whay et al., 1998; Whay et al., 2003). The prevalence of lameness in dairy cattle has been reported to oscillate between 2 and 20% (Clarkson et al., 1996; Bergsten, 2001; USDA, 2007). Economic losses related with lesions of the hoof have been associated to decreased milk yield (Rajala-Schultz et al.,
1999; Warnick et al., 2001; Green et al., 2002), impaired fertility (Hernandez et al., 2001; Melendez et al., 2003; Bicalho et al., 2007), increased risk of culling (Esslemont and Kossaibati, 1997; Booth et al., 2004; Bicalho et al., 2007) and treatment costs (Kossaibati and Esslemont, 1997; Cha et al., 2010).

Numerous studies have demonstrated an antagonic effect of hoof diseases on dairy cattle reproductive performance. It was postulated that lameness lead to poor reproductive performance because animals spend more time lying down, are reluctant to move and therefore less likely to demonstrate estrus behavior (Weaver, 1985). Using lameness as a chronic stressor model, Walker et al. (2008a), observed that lame cows had reduced P4 concentrations prior to estrus and a reduced intensity of behavioral estrous. A subsequent study by the same author concluded that lame cows spend more time lying and consequently less time standing, walking and expressing estrous behavior (Walker et al., 2008b). Lameness has also been associated with extended calving to first service and calving to conception intervals as well as reduced conception rates (Lucey et al., 1986; Collick et al., 1989; Lee et al., 1989; Sprecher et al., 1997; Hernandez et al., 2001). Furthermore, based on the severity of lameness observed a linear association between the degree of lameness and the time to conception was observed; non-lame cows became pregnant sooner than cows classified as lame and moderately lame cows became pregnant sooner that lame cows (Hernandez et al., 2005).

Using historical records from a 3000 Holstein cows herd, Melendez et al. (2003) evaluated the association between lameness, ovarian cystic condition and fertility. Results from this study revealed that cows that become lame within the first 30 days of lactation had increased incidence of ovarian cysts and were less likely to conceive when compared
to non-lame control cows (Melendez et al., 2003). More recently, it has been reported that cows diagnosed with lameness in the first 70 postpartum were 25% less likely to become pregnant compared with non-lame cows (Bicalho et al., 2007). Garbarino et al. (2004) examined the relationship between lameness and postpartum ovarian cyclicity and concluded that affected cows had delayed resumption of cyclicity based on weekly P4 concentrations. Similarly, based on circulating P4 levels, Petersson et al (2006), demonstrated that lame cows had increased interval from calving to commencement of luteal activity, reduced luteal activity during the first 60 days after calving and extended interval from calving to first ovulatory estrus. More recently, Morris et al (2011), evaluated the mechanisms by which chronically lame cows are less fertile than healthy herdmates and concluded that failure to express estrus or ovulate was associated with lameness altered LH pulse frequency and estradiol concentrations.

Free stall housing leads to increased exposure of the hoof to concrete which is a known risk factor for the development of lameness in dairy cows (reviewed by Cook and Nordlund, 2009). Poor facility design and management strategies can aggravate overall hoof health (reviewed by Cook and Nordlund, 2009). Diseases of the hoof translate in poor animal welfare, decreased milk yield and poor reproductive performance and increase risk of culling (Green et al., 2002; Bicalho et al., 2007). In addition, lameness is a particularly painful condition and the rate of new cases should be reduced by adequate stall design, access to feed and water, good hygiene, stocking density, among others (reviewed by Cook and Nordlund, 2009).
2.4.2. Environment and Management

2.4.2.1. Heat Stress

Cows maintain their temperature by continuously exchanging heat with the environment through convection, conduction, evaporation and radiation mechanisms. The thermo neutral zone in lactating dairy cows fluctuates between -0.5 to 26 °C, and under this optimal temperature zone no additional energy is required to heat or cool the body (West, 2003). An index that combines ambient temperature with relative humidity is frequently used to measure the degree of thermal stress. For dairy cows, this temperature humidity index (THI) is considered stressful when it exceeds 72 (Igono et al., 1992).

Environmental heat stress (HS) is a worldwide problem that affects approximately 60% of the cattle population (reviewed by Wolfenson et al., 2000). Annual economic losses associated with HS in the dairy industry have been estimated around $879 million (St-Pierre et al., 2003). Decreased milk production (Fuquay, 1981; West, 2003) and impaired reproductive performance (Thatcher, 1974; Fuquay, 1981; Cavestany et al., 1985; Santos et al., 2004b) account for the majority of the economic losses associated with HS. Comparing data from the summer and winter in Florida, De Vires (2004) estimated a reduction in milk production of 15% and a reduction in conception rates of 53% during the summer months. Data from Israel showed a 7% reduction in milk yield and a 51% reduction in conception rates during the summer months (Flamenbaum and Galon, 2010). In addition, dairy cows under HS conditions have increased somatic cell counts and clinical cases of mastitis (Igono et al., 1988), increased incidence of RP and other diseases (DuBois and Williams, 1980; Kadzere et al., 2002), higher mortality rates
(Hahn, 1985), decreased feed intake (De Rensis and Scaramuzzi, 2003) and length and intensity of estrus (Nebel et al., 1997; Hansen and Aréchiga, 1999; White et al., 2002).

Environmental HS negatively affects reproductive performance of dairy cattle. Pregnancy rate (a measurement of the probability that open cows become pregnant per unit of time) is the most widely accepted measurement of reproductive performance in dairy cattle, dramatically decreases during the summer months (de Vries and Risco, 2005; de Vries et al., 2005). Failure of cows to establish and maintain a pregnancy under HS conditions can be directly related to the negative influences of elevated temperatures on the normal reproductive physiological process (e.g. dysfunction of the hypothalamic-pituitary axis, diminished expression of estrus, modified steroidogenesis in ovarian follicles and CL, alterations of oocyte quality) and early embryo development. Proposed mechanisms mediating altered reproductive function include: modified follicular dynamics (Badinga et al., 1993; Badinga et al., 1994; Wolfenson et al., 1995), reduced steroidogenic capacity of follicles (Wolfenson et al., 1993; Wolfenson et al., 1997; Roth et al., 2001), inadequate luteal function (Howell et al., 1994), possible dysfunction of the hypothalamic–pituitary axis (reviewed by Wolfenson et al., 2000) and altered oocyte quality, embryo development and uterine function (Ealy et al., 1993; Ryan et al., 1993; Edwards and Hansen, 1997; reviewed by Wolfenson et al., 2000; Edwards et al., 2005).

2.4.2.2. Cow Comfort and Facilities

Cow welfare could be defined when an animal “is healthy, comfortable, well nourished, safe, able to express innate behavior, and if it is not suffering from unpleasant states such as pain, fear, and distress” (World Organization for Animal Health, 2008).
The definition of animal welfare differs between animals, people's perception of welfare and how welfare is measured (Roche et al., 2009). Nevertheless, the concept of animal welfare should typically focus on the performance of the animal, how the animal is feeling and longevity or to live a reasonably natural life (Fraser et al., 1997).

Numerous factors including facilities design (i.e., design of stalls, feed bunk space, type of bedding used, and ventilation systems) and management practices (i.e., stocking density, delivery of feed, grouping) contribute to cow comfort. Inadequate comfort of cows has been associated with decreased quality of milk, increased hoof problems, increased aggressive interaction between animals, increased stress, reduced feed intake and decreased reproductive performance among others (Grant and Albright, 2001; Bach et al., 2008; Cook and Nordlund, 2009; Schefers et al., 2010; van Gastelen et al., 2011).

Overstocking of dairy cattle occurs when the number of cows exceeds the number of available stalls or the recommended linear feeding space per cow. Unfortunately, overstocking has become a strategy employed by dairy producers to expand their herd size without investing in new facilities (Bewley et al., 2001). Overstocking has been associated with decreased milk production and the development of diseases. In a recent study, Bach et al (2008) observed that the number of free-stalls per lactating cow was among the main non-dietary factors affecting milk yield. Additionally, it has been well documented that cows housed in overcrowded pens spend less time lying and ruminating (Cooper et al., 2007; Fregonesi et al., 2007), had difficult access to feed (DeVries et al., 2004; Huzzey et al., 2006) and showed increased aggressive interactions with herdmates (Olofsson, 1999; DeVries et al., 2004). Furthermore, as a consequence of overcrowding
and reduced lying time animals can experience health related disorders. Cows that are standing for extended periods of time have increased risk of developing hoof lesions (Cook and Nordlund, 2009) and increased stress as indicated by elevated cortisol levels that is associated with suppression of immune function and increase susceptibility of infections (Munksgaard and Simonsen, 1996).

Reproductive performance is also affected by inadequate design of facilities and management practices. When stocking density is too high the expression of estrous behavior is reduced, making detection of animal in heat more difficult (Diskin and Sreenan, 2000). Inadequate design of facilities (i.e., stalls designs, flooring) and high animal density contribute to hoof lesions with the consequent reproductive performance failure (Lucey et al., 1986; Faull et al., 1996; Vanegas et al., 2006). Poor cow comfort and frequent rearrangement of animals in groups is associated with increased aggressive interaction and stress which interferes with normal reproductive function and fertility in dairy cattle (Dobson and Smith, 2000).

Recent research has increased our knowledge of animal behaviour and their needs in terms of facility design. In addition, variation in management strategies and economic and environmental conditions should be taken into consideration when designing new facilities or improving previously existing ones. Adequate housing and management of dairy cows will be translated into improved welfare, herd health, productivity and reproductive outcomes.
2.4.2.3. Artificial Insemination

Artificial insemination (AI) is the most common practice employed to breed dairy cows in the US (Thibier and Wagner, 2002; Vishwanath, 2003; Caraviello et al., 2006b). According to the USDA 78.3% of the first services and 77.8% of second and greater services in dairy cattle were performed through AI (USDA, 2009). Efficient detection of estrus, timing of AI, optimal semen quality and site of semen deposition, appropriate hygienic procedures and healthy uterine environment are key components for excellent fertility in dairy cows bred to AI (reviewed by Dalton et al., 2010). Most of the efforts aimed to improve reproductive efficiency in dairy cattle have focused in the development of new synchronization strategies or modifications of previously existing synchronization programs resulting extra handling of animals and administration of extra hormonal treatments (i.e., GnRH, prostaglandin, hCG, progesterone). However, the AI procedure is often time overlooked.

Prior to the development of the AI technique that allowed the cervical fixation per rectum and deposition of semen into the body of the uterus, semen was deposited intracervically or in the vagina with the aid of a vaginal speculum (Foote, 2002). During natural service, the bull deposits billons of spermatozoa in the vagina of the cow with only a small percentage reaching the uterine body while the majority of sperm are lost from the reproductive tract. Intrauterine AI bypasses the cervix; thus, allowing the use of relatively low number of sperm cells while achieving acceptable reproductive outcomes when compared to natural service.

Studies in cows showed that up to 60% of spermatozoa deposited into the uterus are lost by retrograde transport within 12 hours of AI (Dobrowolski and Hafez, 1970;
Mitchell et al., 1985). Gallagher and Senger (1989) evaluated retrograde loss of spermatozoa following cornual, uterine body and cervical insemination. Results from this study showed no difference in retrograde transport of spermatozoa between cornual and uterine body inseminations, while retrograde spermatozoa loss of cervically deposited semen doubled uterine inseminations losses (Gallagher and Senger, 1989). Altogether this suggests the importance of accurately depositing semen into the uterine body. Nevertheless, Peters et al. (1984) demonstrated that only 39% of insemination catheter placement attempts were positioned in the desired anatomical location (uterine body) with cervical inseminations accounting for 25% of attempted uterine body inseminations.

The effect of inseminator skills on fertility was evaluated in 2820 first services (Senger et al., 1984). Based on pregnancy diagnosis by rectal palpation 35 to 45 days after AI, marked difference between inseminators were observed with conception rates ranging between 63% and 39% (Senger et al., 1984). More recently a field trial was conducted to assess conception rates following AI by professional or farm personnel inseminators (Dalton et al., 2004). Conception rates greatly differed between professional inseminators (45%) when compared to farm personnel inseminators (27%; Dalton et al., 2004). Thus, it seems that marked improvements on fertility following AI could potentially be achieved by continuously monitoring and retraining those individuals performing the breeding in dairy herds.

Macpherson et al. (1968) and Moller et al. (1972) concluded that deposition of semen into the uterus resulted in increased fertility when compared with cervical deposition. Consequently, in order to maximize PAI, individuals performing AI must develop adequate skills to transverse the cervix and deposit semen into the uterine body.
It was speculated that deposition of semen deeper into the reproductive tract would improve fertility by minimizing sperm losses following AI. Thus, several studies were conducted to evaluate the effects of alternative sites of semen deposition on fertility. While some studies reported increased fertility following semen deposition into the uterine horns (Senger et al., 1988; López-Gatius, 1996; Pursley, 2004); others (Hawk and Tanabe, 1986; Williams et al., 1988a; McKenna et al., 1990) observed no difference.

To further increase the efficiency of the AI technique Senger et al. (1976) evaluated motility and acrosomal integrity in bovine semen thawed at 5 °C and 35 °C followed by exposure to various post-thaw temperatures. The best results in terms of motility and acrosome integrity were observed when semen was thawed at 35 °C and maintained at post-thawing temperatures between 20 °C and 37 °C. A subsequent study by Barth and Bowman (1988) was designed to determine the best practical method for thawing semen straws. Results from this study indicated that semen straws should be thawed at 35 °C for a minimum of 30 seconds and that once thawed, semen should be maintained in the water bath at the same temperature for no more than 15 minutes before AI (Barth and Bowman, 1988). Dalton et al. (2004) evaluated the effect of simultaneously thawing multiple semen straws and the subsequent sequence of inseminations (1st, 2nd, 3rd, or 4th) on conception rate. Results from this study revealed that the sequence of AI (from 1st to 4th) and the time elapsed from the initial thawing process to the 4th AI did not affect conception rate (Dalton et al., 2004). Collectively, it can be concluded that thawing of semen straws should be performed in a water bath maintained between 35 and 37 °C for 30 seconds and that straws in the same water bath can be maintained for no more than 15 minutes prior to AI. Additionally, simultaneous
thawing of 4 straws may not compromise fertility when semen is deposited into the uterine body within 10 minutes after thawing.

The culminating process required to get a cow pregnant is AI. Therefore, appropriate semen handling, site of semen deposition, hygiene, timing of semen deposition relative to ovulation and training of individuals performing AI is critical in order to attain optimal reproductive outcomes.

**2.4.2.4. Nutritional Factors**

Following parturition there is an abrupt increase in energy requirements due to the rapid increase in milk production. However, the cow is not able to meet these nutritional demands because the rate of energy intake is lower than the energy output for milk production; consequently, high yielding cows will experience some degree of NEB (reviewed by Goff and Horst, 1997; reviewed by Butler, 2003). Under this post-partum period of NEB, dairy cattle will prioritize the use of metabolizable energy towards milk production and regaining body condition, while energy designated to reproductive function is negligible (reviewed by Wade and Jones, 2004). Changes in the endocrine and metabolic milieu associated with NEB include reduced glucose, insulin and IGF-1 that lead to uncoupling of the growth hormone-IGF-1 system (reviewed by Butler et al., 2003). Plasmatic concentrations of E2 and LH pulsatility are correlated with plasma IGF-1 levels (reviewed by Butler, 2000; reviewed by Lucy, 2000); consequently, follicular development, oocyte competence, resumption of ovarian cycles and embryo viability are compromised (reviewed by Butler, 2000; Velazquez et al., 2009).
Inadequate nutrition related managerial practices including feed preparation, delivery, and availability can have deleterious effects on dairy cattle fertility and productivity by extending the length of the NEB. A study performed 47 dairy herds (approximately 3,129 lactating cows) that received the same diet revealed that dairies that pushed-up feed produced 4 kg/day extra of milk when compared to those that did not (Bach et al., 2008). This suggests that feed push-up increases the availability of feed to cows which maybe beneficial to recover from the state of NEB that follows parturition. Adequate BCS during the dry period affects performance in the subsequent lactation. Overconditioned cows at calving exhibit decreased dry matter intake and may develop a severe NEB compared to cows with moderate BCS; consequently, overconditioned animals have increased body fat mobilization which can lead to the development of fatty liver and the subsequent increase in NEFAS and BHBA (Rukkwamsuk et al., 1999). Furthermore, increased liver triglycerides have been linked with extended intervals to first ovulation and reduced fertility (Rukkwamsuk et al., 1999; Jorritsma et al., 2000).

Additionally, composition of the diet can affect reproductive function of dairy cows. Adequate amounts of protein in the diet are beneficial for embryo development, immune function and milk production. However, excess protein in the feed delivered, has been associated with a declined uterine pH and uterine environment alterations which can affect embryo survival (reviewed by Butler, 1998; Ocon and Hansen, 2003). Similarly, in addition to improving energy intake, adding of supplemental fat to the diet (especially omega 3 and 6) modulates uterine secretion of PGF$_{2\alpha}$ affecting ovarian dynamics and improving embryo survival (Santos et al., 2010; Thatcher et al., 2011).
2.5. Risk Factors Affecting Reproductive Performance in Dairy Herds

The decline in fertility of dairy cows is multi-factorial and managerial in nature. Factors such as nutrition (Curtis et al., 1985), cow comfort (Huzzey et al., 2006), uterine diseases (LeBlanc et al., 2002; Sheldon et al., 2006; Dubuc et al., 2010), genetics (reviewed by Lucy, 2001) mastitis (Santos et al., 2004a; Ahmadzadeh et al., 2009), heat stress (reviewed by Wolfenson et al., 2000), AI technique (Peters et al., 1984; Senger et al., 1984) among others have been reported to negatively impact fertility. Improvement in reproductive performance should translate into increased milk yield and profitability. However, the risk factors associated with poor reproductive performance must be identified to be able to develop and implement the appropriate corrective strategies.

One method developed to identify factors affecting fertility in dairy herds was through the use of microcomputer expert systems (Domecq et al., 1991). This system performs an initial evaluation and identifies the reproductive parameter (i.e., days to first service, estrous detection and conception rate) with the largest negative influence on days open. Once an area has been selected the expert system uses farm, dairy herd improvement (DHI) records and a questionnaire to provide recommendations for the identified problems (Domecq et al., 1991). Another approach used by Caraviello et al., (2006a) consisted in the use of alternating decision tree machine learning algorithm to identify factors affecting first service conception rates and pregnancy status at 150 DIM in high producing dairy cows. A transition cow index that uses Dairy Herd Improvement Association (DHIA) records has been developed to objectively evaluate the transition cow management at herd level (Nordlund, 2006). This instrument uses farm records.
(e.g., SCC, milk yield, fat content) to monitor the herd performance over time and each cow within the herd serve as their own control (Nordlund, 2006).

2.6. Statement of the Problem

Reproductive failure is one of the top reasons (in addition to mastitis and lameness) for removal of cows from dairy herds and is associated with severe economic losses to dairy producers through decreased milk production, extended days open, and increased replacement costs (Rajala-Schultz and Gröhn, 1999ab; Groenendaal et al., 2004; Meadows et al., 2005; De Vries, 2006). These factors, in addition to nutrition and herd management, greatly influence profitability and thus sustainability of dairy production systems. Due to its multi-factorial nature, the identification of risk factors affecting reproductive performance can be challenging. Thus, accurate on-farm identification of areas for improvement is critical for the sustainability and success of any dairy operation. Identification of the risk factors affecting reproductive performance is the first step to improve dairy herd productivity and each individual herd must be assessed as a whole to effectively identify the areas for improvement prior to implementation of any reproductive strategy. The development of user-friendly tools to facilitate the identification of risk factors affecting reproductive performance within herds is critical to enhance the overall herd performance and sustainability.

The present dissertation because it addresses managerial aspects of the farm (e.g., facilities, reproduction, animal health) through the development of an instrument to aid in the identification of factors affecting reproductive performance of dairy herds, while implementing two reproductive strategies (intrauterine hormone delivery and
insemination technique) designed to improve reproductive outcomes in lactating dairy cows without the need of additional handling (e.g., hormone administration). The overall objectives of this dissertation were to generate research-based information on the implementation of two management strategies related to dairy cattle reproductive performance and to develop an instrument to facilitate the identification of areas for improvement in dairy herds. We propose to achieve our overall objectives by pursuing the following specific aims:

I. Evaluate the effectiveness of using AI cover sheaths at the time of AI on fertility of lactating dairy cows: The working hypothesis is that the use of cover sheaths (clean AI technique) will minimize vaginal contamination of the AI catheter and improve pregnancies PAI in lactating dairy cows.

II. Evaluate the release of LH and ovulatory response following the intrauterine administration of GnRH in lactating dairy cows: The working hypothesis is that the release of LH and ovulatory response will be similar in cows receiving the intrauterine dose of the GnRH analogue when compared to cows receiving GnRH by the intramuscular route.
CHAPTER 3

The Use of Plastic Cover Sheaths at the Time of AI Improved Fertility of Lactating Dairy Cows

Bas S., A. Hoet, P. Rajala-Schultz, D. Sanders, and G.M. Schuenemann

Department of Veterinary Preventive Medicine, The Ohio State University

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Abstract

An adequate and clean artificial insemination (AI) technique is recommended to improve reproductive outcomes in dairy cattle. The objective of this study was to evaluate the effectiveness of using protective plastic sheaths (PS) to minimize contamination of the AI catheter (AIC) on pregnancies per AI (PAI) in lactating dairy cattle. Lactating cows housed in free-stall barns on a commercial dairy farm were presynchronized with 2 injections of PGF$_{2\alpha}$ given 14 days apart (starting at 26 ± 3 d postpartum) followed by Ovsynch [OV, GnRH-7 d-PGF$_{2\alpha}$-56 h-GnRH-16 h-timed-AI (TAI)] 12 days later. Cows presenting signs of standing heat any time during the protocol received AI, whereas the remaining animals were subjected to TAI 16 h after second OV GnRH. At the time of AI (one AI technician), 996 services from 773 lactating dairy cows were randomly assigned to 1 of the 2 groups; with (TRT, n = 487) or without (CON, n = 509) the use of disposable PS. In the TRT group, the AIC protected with a PS was introduced into the vagina; once in the cranial portion of the vagina adjacent to the cervical os, the PS was pulled back and only the AIC was manipulated through the cervix into the uterine body for semen deposition. In the CON group, cows were inseminated without the use of PS. Sterile cotton swab samples were taken from the tip of the AIC (n = 51) after AI from both treatment groups. Pregnancy diagnosis was determined by ultrasonography 39 ± 3 d after AI. Cultured swab samples revealed that the use of PS was effective in minimizing contamination of the AIC (positive bacterial growth; TRT = 61.53% vs. CON = 100%; $P = 0.0002$). Overall, the proportion of cows pregnant was greater ($P = 0.03$) for cows in TRT (42.7) compared with CON group (36.1). For first
services postpartum, PAI did not differ ($P = 0.87$) between CON (43.01%, $n = 194$) and TRT (43.8%, $n = 182$) groups. However, PAI for second or greater services were greater ($P = 0.007$) in TRT (43.8%, $n = 305$) than in CON cows (32.3%, $n = 315$). Results from this study provided evidence that the use of PS during AI improved PAI for second or greater services in lactating dairy cows. Performing a clean AI technique through the use of PS may be an effective strategy to improve reproductive outcomes in dairy cattle.

**Key Words**: AI technique, vaginal contaminant, dairy cow fertility

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**Introduction**

Artificial insemination (AI) is the most common breeding practice for dairy cattle in the United States (USDA, 2009; Caraviello et al., 2006b). Efficient detection of estrus, timing of AI, optimal semen quality, appropriate hygienic procedures, and healthy uterine environment are key components for excellent fertility in dairy herds. This study focuses on the use of disposable protective plastic sheaths (PS) at the time of AI as a way to minimize vaginal contamination of the AI catheter (AIC) and its potential impact on fertility. The relative importance of vaginal contaminants that may be introduced into the uterus of lactating dairy cows at the time of AI and the subsequent fertility remains unclear.

It has been shown that the presence of semen in the uterine lumen triggers a local immune response characterized by a neutrophil influx to the female reproductive tract (Schuberth et al., 2008; Troedsson, 2006; Zerbe et al., 2003). This physiological immune
reaction has been described in a wide variety of species (i.e., sows, mares and mice) and has been referred to as post-mating inflammatory response (Schuberth et al., 2008). In mares, this inflammatory response rises until it peaks between 6 and 12 h and under normal conditions the inflammatory products are cleared out of the uterus within 48 h (Katila et al., 1996). However, during AI not only semen but also bacteria and debris may be introduced into the lumen of the uterus. Consequently, this inflammatory response of the female reproductive tract may lead to a persistent post-breeding endometritis with major negative consequences in the mare’s ability to conceive (Maischberger et al., 2008). In the bovine, this physiological response appears to be weak when compared to other species (Zerbe et al., 2006). In a recent study, uterine cytology samples from lactating dairy cows were collected 4 h post-AI for determination of polymorphonuclear cells (PMN) as an indicator of post-breeding subclinical endometritis (SE; Kaufmann et al., 2009). This study showed that lactating dairy cows with a higher proportion of uterine PMN 4 h post-AI resulted in lower conception rates (Kaufmann et al., 2009).

A clean and strict protocol-driven AI technique is recommended to maximize reproductive outcomes (King et al., 1984). A possible approach for a clean AI technique may be the use of protective PS to avoid vaginal contamination of the AIC during the AI procedure. Previous studies reported no improvement on conception to first services in dairy cattle (King et al., 1984; Richards et al., 1984) when using a protective PS to avoid vaginal contamination of the AIC as opposed to no protection. Although these studies evaluated conception to first services, no data is available on fertility to second or greater services. Additionally, the potential detrimental effects of introduced bacteria (from vaginal origin) at the time of AI and the subsequent reproductive performance on dairy
cattle need further investigation. Therefore, the objectives of this study were to: 1) evaluate the effectiveness of using disposable protective PS to minimize vaginal contamination of the AIC at the time of AI, and 2) assess pregnancies per AI (PAI) in lactating dairy cows inseminated with or without the use of disposable protective PS. We hypothesize that the use of protective PS (clean AI technique) would: 1) minimize vaginal contamination of the AIC and 2) improve PAI in lactating dairy cows.

Materials and Methods

Animals, Facilities and Feeding

A total of 1014 services (all performed by one AI technician) from 791 lactating Holstein dairy cows (601 primiparous and 190 multiparous) in one commercial dairy farm were enrolled in a field trial to investigate the effectiveness of disposable protective PS (Continental Plastic Inc., Delavan, WI) at the time of AI. Briefly, cows were housed in free-stall barns and milked thrice daily at approximately 8 h intervals. The average herd rolling milk yield was 10,262 kg and the reported voluntary waiting period (VWP) was 60 d. Cows were fed twice daily, in the morning and afternoon, with a TMR formulated to meet or exceed dietary nutritional requirements for lactating dairy cows (NRC, 2001). This study was conducted from April to June 2009. The procedures described below were approved by The Ohio State University Veterinary Teaching Hospital Clinical Research Advisory Committee.

Breeding Management and Treatments
For first postpartum services, cows were presynchronized with 2 injections of PGF$_{2\alpha}$ (25 mg; Lutalyse, Pfizer Animal Health, New York, NY) given 14 days apart at 26±3 and 40±3 d postpartum. Twelve days after the second injection of PGF$_{2\alpha}$, all cows initiated Ovsynch (OV; Brusveen et al., 2008; Pursley et al., 1995). The initial GnRH (100 μg; Cystorelin, Merial, Duluth, GA) of OV was followed 7 days later by an injection of PGF$_{2\alpha}$ and fifty-six hours (56 h) later cows received the second dose of GnRH followed by timed artificial insemination (TAI; 72 h after the PGF$_{2\alpha}$ injection). Following the first GnRH of OV, estrus was detected by visual observation plus tail chalking once daily and all animals presenting visual signs of standing estrous behavior (cows staying still when mounted or presenting rubbed off tail chalking) received AI. Animals that did not display estrous behavior during the synchronization protocol were subjected to TAI 72 h after the PGF$_{2\alpha}$ injection of OV. Cows that were previously inseminated, but showed visual signs of estrous behavior before the pregnancy diagnosis were re-inseminated. Additionally, open cows at the time of pregnancy diagnosis (39±3 d post-AI) were re-enrolled in an OV program as previously described. Reconfirmation of pregnancy was performed approximately 30 days after first pregnancy diagnosis (69±3 d post-AI).

Visual observation of estrous behavior (i.e., reading of tail chalk) and AI were performed by the same AI technician. At the time of AI, 1014 services from lactating dairy cows were randomly assigned to 1 of the 2 treatment groups (alternating the use of PS on every other cow), with (TRT, n = 493) or without (CON, n = 521) the use of protective PS as previously described. The PS device (30 cm in length x 0.7 cm in diameter) is a rigid PVC tube that was developed to help prevent vaginal contamination of the AI gun at the time of AI (King et al., 1984). On one end, where the insemination
catheter is introduced has a funnel shape, while the other end is sealed with a scored rubber cap that can be easily perforated when pressure is applied. For all services performed in the TRT group, the AIC protected with a PS, was introduced in the vagina; once in the cranial portion of the vagina adjacent to the cervical os, the PS was pulled back exposing the AIC that was manipulated through the cervix into the uterine body for semen deposition. In the CON group, the AIC was introduced to the vagina without the use of PS and was manipulated through the cervix for deposition of the seminal dose in the uterine lumen.

Bacterial Growth and Identification

To assess the effectiveness of protective PS to minimize vaginal contamination of the AIC at the time of AI, sterile cotton swab samples (BD, Franklin Lakes, NJ) from the tip of the AIC were collected from a subset of cows (n = 51) immediately after AI. At the beginning of the experiment (5-week period), cows were stratified by parity and randomly selected for collection of swab samples. It was estimated that the use of protective PS at the time of AI (TRT) would reduce positive bacterial cultures by 40 percentage points (e.g., 100% to 60%) as opposed to AI without PS (CON); thus, 25 swabs samples from the tip of the AIC per treatment group would be needed to detect statistical significance (α=0.05 and β=0.10). Briefly, the AIC used in CON group (n = 25), were carefully pulled out of the reproductive tract (i.e., uterine body, cervix, vagina and vulva) and sterile swab samples were collected (one swab per insemination). Prior to removal of AIC from the vagina, the lips of vulva were opened to avoid contact of the AIC with the outside portion of the vulvar skin; therefore, direct contamination with
manure and/or skin surface was avoided. Once outside, a sterile premoistened (in sterile saline solution) cotton swab sample was collected from the tip and distal third portion of the AI sheath covering the AIC. In the TRT group \(n = 26\), after semen deposition, the AIC was reintroduced into the PS before leaving the cervix; therefore, the AIC was covered with the PS on its transit out of the vagina (The AIC was only exposed to the cervix and uterine environment). Once outside, the PS was pulled back exposing the AIC and sterile premoistened cotton swab samples were collected as previously described for CON group.

Swab samples were immediately transported to the laboratory in Stuart transport media (BD, Franklin Lakes, NJ) at ambient temperature for further bacteriologic analysis. Once in the laboratory, swab samples were cultured onto blood (Tryptic Soy Agar with 5% Sheep Blood) and MacConkey agar plates for 24 h at 37 °C under aerobic conditions. When colony growth was visually observed on blood agar and MacConkey plates after the incubation period, each unique colony was selected based on morphology, pigmentation and hemolytic pattern and further purified in individual blood agar plates for genus identification. Pure isolates were then stored at -20 °C until final genus identification at the Diagnostic and Research Laboratory on Infectious Diseases (Columbus, OH).

The growth density of bacteria immediately after culture was assessed semiquantitatively based on the number of bacterial colonies that grew in blood agar and MacConkey plates. Growth density was assessed on swab samples collected on week 2-5. Briefly, the proportion of growth density in each culture plates (from TRT and CON groups) was classified using the following semiquantitative scale: 1) Light or sparse
growth (1-20 visible colonies located at the inoculation site); 2) Moderate growth (21-100 visible colonies); and 3) Heavy growth (over 100 visible colonies per plate or not possible to count due to heavy growth).

Statistical Analyses

Swab samples, from a subset of cows (\(n = 51\)), were collected from the tip of the AIC immediately after AI to assess the effectiveness of using disposable protective PS to minimize vaginal contamination of the AIC. The proportion of visible colonies after culture and proportion of bacteria isolated (from the total number of identified isolates) in the two treatment groups (CON or TRT) were analyzed by Proc Freq (SAS, 2009). A \(P < 0.05\) was considered statistically significant.

Prior to data analysis, enrolled lactating dairy cows that met the exclusion criteria (i.e., cows that were AI but died before the pregnancy diagnosis, cows with history of abortion prior to AI, dead and sold animals) were removed from the analysis. Data were arranged in a complete randomized design. Following AI, the proportion of cows that conceived (PAI) at first and second or greater services in the two treatment groups (CON, \(n = 487\) or TRT, \(n = 509\)) were evaluated. Data pertaining to PAI were analyzed using generalized linear mixed models (Proc Glimmix; SAS, 2009). A model procedure that included treatment (CON or TRT), parity (primiparous or multiparous), DIM at the time of AI, sire, and SCC at the closest DHIA test relative to service was used to compare differences in PAI between treatments. Non-significant variables were eliminated from the logistic model one at a time using the Wald statistic backward selection criterion (\(P > 0.15\)). Week of the experimental period was included as a random effect. The estimates
(proportions of PAI) from the final model were reported as least square means (Bar et al., 2008; Tsousis et al., 2009; Pinedo and De Vries, 2010). The differences between least square means were calculated by including the PDIF option in the LSMEANS statement (Tsousis et al., 2009). Differences in individual least squares means were adjusted by using Tukey-Kramer method. A $P < 0.05$ was considered statistically significant.

**Results**

Initially, a total of 1014 services were performed by one AI technician, of which 18 cows were removed from the analysis (7 animals died, 8 had an abortion before AI, and 3 cows were sold). Therefore, 996 services from 773 lactating Holstein dairy cows (590 primiparous and 183 multiparous) were available for the final analysis. The distribution of cows receiving the first post-partum services and those receiving the second or greater services was stratified with respect to parity (primiparous vs. multiparous), DIM, number of services, milk production, and SCC at the closest DHIA test relative to AI (Table 3.1).

**Bacterial Growth and Identification**

The proportion of positive bacterial growth (from the 51 swabs samples collected) was greater in CON (100%) than TRT group (61.53%, $P = 0.0002$; Table 3.2). Additionally, 10 (38.47%) of the 26 swab samples collected from the AIC in TRT group were negative and yielded no bacterial growth. Conversely, all swab samples from the AIC in CON group were positive for bacterial growth (100 %). Regarding the bacterial
density growth, the majority (66.67%; Table 3.2) of the samples from the TRT group had light or sparse bacterial growth compared with the CON group in which the majority of the samples showed heavy colony growth (71.43%; Table 3.2). Since bacterial growth density was measured from swabs samples collected on week 2-5, the growth density from week 1 were missing (4 from CON and 1 from TRT groups; Table 3.2). From a total of 176 bacterial isolates obtained (CON and TRT combined), 67.04% (118) were identified and reported in the present study (Table 3.3). Swab samples collected from AIC without the use of protective PS (92 isolates) resulted in more bacteria isolated with greater proportion of E. coli compared to TRT group (26 isolates; Table 3.3).

Effect of PS on PAI

Sire ($P = 0.0002$) and DIM at service ($P = 0.0005$) were significantly associated with PAI and stayed in the final model. Overall, the proportion of cows pregnant was greater ($P < 0.03$) for cows in the TRT (42.7%) compared to cows in CON (36.1%; Table 3.4) group. No differences were observed between parities. For first post-partum services, PAI did not differ ($P < 0.87$) between CON (43.01%) and TRT (43.8%; Table 3.4) groups. However, PAI for second and greater services were greater ($P < 0.007$) in TRT (43.8%) than in CON group (32.3%; Table 3.4). Additionally, no differences ($P = 0.44$) were observed in pregnancy losses between pregnancy diagnosis (39 ± 3 d post-AI) and reconfirmation of pregnancy (69 ± 3 d post-AI) for cows in the TRT (8.98%) and CON (11.57%) groups.
Discussion

The primary findings of the present study are: 1) the use of protective PS was effective in minimizing contamination of the AIC at the time of AI and 2) increased PAI were observed for the second or greater services in lactating dairy cows inseminated with the use of protective PS.

The effectiveness of using protective PS to minimize vaginal contamination of the AIC at the time of AI was assessed in this study. Rigid PS has been developed to protect the AIC at the time of AI from vaginal contaminants (clean AI technique) with the aim of improving reproductive outcomes in dairy cattle (Richards et al., 1984). In this study, swab samples collected from the AIC immediately after AI revealed that the use of PS was effective in minimizing contamination of the AIC compared to unprotected AIC. The use of PS reduced nearly 40 percentage points the positive bacterial cultures (clean AI technique) as well as the growth density (heavy colony growth) per plate. Regarding the bacteria isolated, swab samples from the AIC in CON cows had a greater proportion of recognized, potential, and opportunistic uterine pathogens compared to the AIC from TRT cows. Additionally, the most common bacteria isolated from the AIC immediately after AI was \textit{E. coli}, which was identified in nearly 49% of the isolates from CON cows as opposed to 35% in TRT cows. Although the potential detrimental effects of introduced bacteria into the bovine uterus at the time of AI has not been reported yet, the bacteria may colonize the uterine lining and trigger an inflammatory response (post-breeding SE) as shown in mares (Katila et al., 1996; Maischberger et al., 2008) that may prevent the events leading to maternal recognition of pregnancy. Recognized uterine pathogens (A.
pyogenes, Prevotella melaninogenica, E. coli, Fusubacterium necrophorum and Proteus spp), potential uterine pathogens (Bacillus spp and Pasteurella spp) and opportunistic uterine contaminants (Streptococcus spp, Providencia spp, Klebsiella spp and coagulase negative Staphylococcus, Corynebacterium spp.) have been associated with uterine diseases (e.g., metritis, endometritis) and decreased fertility in dairy cattle (Sheldon et al., 2002; Williams et al., 2005; 2007). Furthermore, it has been shown that the presence of E. coli and A. pyogenes in the bovine uterus was associated with ovarian dysfunction (smaller follicle diameter and corpora lutea with lower plasma estradiol and progesterone; Williams et al., 2007). The prevalence of anaerobic bacteria (e.g., Prevotella spp, F. necrophorum, F. nucleatum; Sheldon et al., 2009a), mycoplasmas and ureaplasmas (Doig et al., 1979; Mulira et al., 1992) were not investigated in this study; however, there is a real risk that these bacteria may reduce fertility in dairy cattle. The potential detrimental effects of introduced bacteria (e.g., from vaginal origin) into the uterine lumen at the time of AI need further investigation.

Reproductive outcomes (PAI) following the use of protective PS at the time of AI were assessed in this study. In the present study the proportion of PAI for first services were comparable to a similar reproductive protocol (Silva et al., 2009), and no difference was observed between cows inseminated with or without the use of PS. Further analysis revealed that PAI increased for the second or greater services in lactating dairy cows inseminated with the use of PS. Previous studies reported no improvement on conception to first services in dairy cattle (King et al., 1984; Richards et al., 1984) when using the same PS at the time of AI. These studies were carried out more than 20 years ago, using several AI technicians, and only non-return rates to first service were evaluated. In our
study, all the AI were carried out by one AI technician following the same reproductive program within one commercial dairy farm and similar distribution of services for both treatment groups (TRT and CON) with respect to parity (multiparous and primiparous), DIM, milk yield, and SCC at the closest DHIA test relative to AI.

It is well documented that fertility in high-producing dairy cows has decreased over the last decade (Lucy, 2001; Thatcher et al., 2006). Furthermore, lactating dairy cows have higher fertility to the first postpartum services as opposed to second or greater services (Silva et al., 2009). Multiple events such as difficult calving (Lombard et al., 2007), uterine diseases (e.g., metritis and endometritis; LeBlanc et al., 2002), and anovular cows at the time of the AI (Santos et al., 2009) have been associated with lower reproductive performance in dairy cattle. During AI, not only semen but also bacteria and debris can be introduced into the uterine lumen and lead to chronic inflammation and decreased fertility in mares (Maischberger et al., 2008). This physiological immune reaction, described as post-mating inflammatory response (Zerbe, 2006; Schuberth et al., 2008), under normal conditions is cleared within 48 hours (Katila et al., 1996). In lactating dairy cows, higher proportion of polymorphonuclear cells (PMN; >15%) immediately before (Williams et al., 1988b) and 4 h post-AI were associated with poor reproductive performance (Kaufmann et al., 2009). Estrogen has been shown to regulate leucocyte migration into the cervix, the uterus, and the extracellular matrix of the reproductive tract (Ramos et al., 2000; Stygar et al., 2006). Although hormone profile was not assessed in this study, reduced level of estradiol at the time of AI may lead to poor immunological response in the uterus (Lacetera et al., 2004; van Knegsel et al., 2007) and to reduce fertility (Leroy et al., 2004). Therefore, lactating dairy cows may
benefit from the use of protective PS at the time of AI which can prevent the introduction of vaginal contaminants into the uterus, resulting in optimal uterine environment and improved PAI as observed for cows in the TRT group.

In conclusion, results from this study suggested that the use of PS at the time of AI (clean AI technique) reduced contamination of the AIC and improved PAI for second or greater services in lactating dairy cows. Further investigation is needed under various reproductive management conditions to confirm and determine the underlying mechanisms for these findings, especially whether a correlation exists between the introduction of vaginal contaminants into the uterine lumen and the subsequent reproductive performance of lactating dairy cows (e.g., repeat breeders). Performing a clean AI technique through the use of PS may be an effective strategy to improve reproductive outcomes in dairy cattle.

Acknowledgements

The authors thank the collaborating dairy farm and the staff for providing the animals used in this experiment. Also, the authors thank Coba/Select Sires Inc. (Columbus, OH) for the donation of the protective AI plastic sheaths.
<table>
<thead>
<tr>
<th>Item</th>
<th>First Services</th>
<th>Second or Greater Services</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRT (n = 182)</td>
<td>CON (n = 194)</td>
</tr>
<tr>
<td>Parity</td>
<td>1.22</td>
<td>1.29</td>
</tr>
<tr>
<td>DIM</td>
<td>62.73</td>
<td>65.23</td>
</tr>
<tr>
<td>Number of Services</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Milk Yield (Kg)</td>
<td>34.5</td>
<td>34.7</td>
</tr>
<tr>
<td>SCC (x10³)</td>
<td>136.8</td>
<td>207.56</td>
</tr>
</tbody>
</table>

Table 3.1. Distribution of services (first and second or greater services) with respect to parity (primiparous or multiparous), DIM, milk yield (Kg), and SCC in lactating dairy cows.

At the time of AI, 996 services from lactating dairy cows were randomly assigned to 1 of the 2 treatment groups, with (TRT, n = 487) and without (CON, n = 509) the use of protective plastic sheath (PS).
<table>
<thead>
<tr>
<th></th>
<th>Number of Positive Cultures† and Growth Density‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRT</td>
</tr>
<tr>
<td>Positive Bacterial Growth, %</td>
<td>61.53 (16/26)</td>
</tr>
<tr>
<td>(n/n)</td>
<td></td>
</tr>
<tr>
<td>Light colony growth, %</td>
<td>66.66 (10/15)</td>
</tr>
<tr>
<td>(n/n)¹</td>
<td></td>
</tr>
<tr>
<td>Moderate colony growth,%</td>
<td>20 (3/15)</td>
</tr>
<tr>
<td>(n/n)²</td>
<td></td>
</tr>
<tr>
<td>Heavy colony growth % (n/n)³</td>
<td>13.33 (2/15)</td>
</tr>
</tbody>
</table>

Table 3.2. Proportion of positive bacterial growth and density from the AIC of dairy cows inseminated with (TRT) and without (CON) the use of protective plastic sheath (PS) in blood agar and MacConkey plates.

†Swab samples were collected from the tip of the AI catheter (IAC) immediately after AI for further identification of bacteria from 51 lactating dairy cows, inseminated with (TRT, n = 26) and without (CON, n = 25) the use of PS.

‡Growth density = Bacterial growth density (Light, moderate and heavy) immediately after culture was assessed semiquantitatively based on the number of bacterial colonies visible that grew in blood agar and MacConkey plates. In CON group, growth density from 4 plates was not recorded. In TRT group, growth density from 1 plate was not recorded.

¹Identification of 1-20 visible colonies at the inoculation site.

²Identification of 21-100 visible colonies.

³Identification of over 100 visible colonies per plate or not possible to count due to heavy growth.
<table>
<thead>
<tr>
<th>Species</th>
<th>TRT(^2)</th>
<th>CON</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter spp.</em></td>
<td>-</td>
<td>1.09 (1/92)</td>
</tr>
<tr>
<td><em>Actinobacillus spp.</em></td>
<td>-</td>
<td>3.26 (3/92)</td>
</tr>
<tr>
<td><em>Bacillus spp.</em></td>
<td>7.69 (2/26)</td>
<td>9.78 (9/92)</td>
</tr>
<tr>
<td><em>Staphylococcus spp.</em></td>
<td>26.92 (7/26)</td>
<td>7.61 (7/92)</td>
</tr>
<tr>
<td><em>Corynebacterium spp.</em></td>
<td>23.08 (6/26)</td>
<td>4.35 (4/92)</td>
</tr>
<tr>
<td><em>E. coli spp.</em></td>
<td>34.62 (9/26)</td>
<td>48.91 (45/92)</td>
</tr>
<tr>
<td><em>Enterobacter spp.</em></td>
<td>-</td>
<td>2.17 (2/92)</td>
</tr>
<tr>
<td><em>Klebsiella spp.</em></td>
<td>-</td>
<td>1.09 (1/92)</td>
</tr>
<tr>
<td><em>Pasteurella spp.</em></td>
<td>7.69 (2/26)</td>
<td>435 (4/92)</td>
</tr>
<tr>
<td><em>Providencia spp.</em></td>
<td>-</td>
<td>1.09 (1/92)</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>-</td>
<td>2.17 (2/92)</td>
</tr>
<tr>
<td><em>Streptococcus spp.</em></td>
<td>-</td>
<td>14.13 (13/92)</td>
</tr>
</tbody>
</table>

Table 3.3. Proportion of bacteria isolated from the AI catheter (AIC) immediately after AI in lactating dairy cows inseminated with (TRT) and without (CON) the use of protective plastic sheath (PS).

\(^1\)From a total of 176 bacterial isolates obtained, 118 isolates (67.04%) were identified and reported as a proportion (%) over the total (n) number of isolates of one particular genus from the total (n) number of identified isolates obtained in TRT (26) and CON (92) groups.

\(^2\)Ten swabs samples from TRT group yielded no bacteria growth (negative culture).
Table 3.4. Pregnancies per AI (PAI) from lactating dairy cows that were inseminated with (TRT) and without (CON) the use of protective plastic sheath (PS).

At the time of AI, 996 services (performed by the same AI technician) from lactating dairy cows were randomly assigned to 1 of the 2 treatment groups, with (TRT, \( n = 487 \)) and without (CON, \( n = 509 \)) the use of protective PS.

\(^{a,b}\) Different superscript letter within a row differ significantly at \( P < 0.05 \).

<table>
<thead>
<tr>
<th>Item</th>
<th>TRT</th>
<th>CON</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall, %</td>
<td>42.7(^a)</td>
<td>36.1(^b)</td>
<td>0.03</td>
</tr>
<tr>
<td>First services, %</td>
<td>43.8(^a)</td>
<td>43.01(^a)</td>
<td>0.87</td>
</tr>
<tr>
<td>Second or greater services, %</td>
<td>43.8(^a)</td>
<td>32.3(^b)</td>
<td>0.007</td>
</tr>
</tbody>
</table>
CHAPTER 4

Effect of Using Plastic Cover Sheaths at the Time of AI on Reproductive Performance and Economics of Lactating Dairy Cows
Abstract

The objectives of the present study were to evaluate the effectiveness of using protective plastic sheaths (PS) to minimize contamination of the AI catheter (AIC) on pregnancies per AI (PAI) and to estimate the economic benefits of using PS in lactating dairy cattle. Services (n = 2,995) from lactating Holstein cows (1,149 primiparous and 917 multiparous) from 2 commercial dairy herds were included. Animals were presynchronized with 2 injections of PGF$_{2\alpha}$ given 14 d apart (starting at 26±3 DIM) followed by Ovsynch (OV; GnRH-7 d-PGF$_{2\alpha}$-56 h-GnRH-16 h-TAI) or Cosynch (COS; GnRH- 7d- PGF$_{2\alpha}$-72 h-GnRH+TAI) 12 - 14 d later. Cows presenting signs of standing estrus at any time during the protocol were AI while the remaining animals were subjected to TAI. At the time of AI, services were randomly allocated to 1 of 2 groups: with (TRT = 1,492) or without (CON = 1,503) the use of PS. In TRT, the AIC protected with a PS was introduced into the vagina, once in adjacent to the cervix, the PS was pulled back and only the AIC was manipulated through the cervix into the uterine body for semen deposition, while in CON cows were AI without the PS. Swab samples were collected from AIC after AI of TRT (n = 52) and CON (n = 50) groups for bacteriology. Pregnancy was diagnosed by ultrasonography 35-45 d post AI. Data analyses were performed using the FREQ (culture) and GLIMMIX (PAI) procedures of SAS. Cultured swab samples revealed that PS was effective in minimizing contamination of the AIC in TRT (53.85%) compared to CON (98%; P < 0.05). Overall, PAI was greater (P = 0.04) for cows in TRT (36.8%) than in CON cows (31.3%). PAI did not differ (P = 0.8) for first post-partum services. However, for second and greater post-partum services PAI
were greater (P = 0.02) for animals in TRT (37%) compared to CON (29.2%). The use of PS provided an additional $4 cow/yr (or $4,000/yr for a 1,000-cow herd) for each point increase in CR. These findings showed that the use of PS increased profitability at a minimal cost by improving PAI for lactating dairy cows. Performing a clean AI technique through the use of PS resulted in a cost-effective strategy to improve reproductive performance in dairy herds.

**Introduction**

Artificial insemination (AI) is the most common practice employed to breed dairy cows in the US (Thibier and Wagner, 2002; Vishwanath, 2003; Caraviello et al., 2006b). According to a report from the National Animal Health Monitoring System, 78.3% of first services and 77.8% of second or greater services in dairy cattle were performed through AI (USDA, 2009). In dairy herds that use AI, accurate animal identification, estrus detection, timing of AI with respect to ovulation, optimal semen quality and handling, site of semen deposition, and hygiene of the AI procedure are key components to enhance reproductive performance (Barth and Bowman 1988; Dalton et al., 2004; Dalton et al., 2010; Bas et al., 2011). However, the AI procedure is often overlooked. Although improved reproductive efficiency has been achieved by the development of new synchronization strategies (Pursley et al., 1995; Brusveen et al., 2008) or modifications of previously existing protocols in dairy cattle (Portaluppi et al., 2005; Sterry et al., 2007; Giordano et al., 2012a), these strategies increased the risk for non-
compliance under field conditions due to additional handling of animals or timing of hormonal injections.

It was shown that accurate delivery of semen into the uterine body was critical to minimize the retrograde losses of spermatozoa and to improve reproductive performance of cattle (Gallagher and Senger, 1989). Nevertheless, only 39% of insemination catheter placement attempts were positioned in the uterine body and cervical inseminations accounted for 25% of attempted uterine body inseminations (Peters et al., 1984). Additionally, marked differences on the inseminator skills were observed with conception rates (CR) ranging between 63 and 39% (Senger et al., 1984). A field trial conducted to assess CR following AI by professional or herdsmen inseminators showed that CR greatly differed between professional inseminators (45%) when compared to herd inseminators (27%; Dalton et al., 2004). In contrast, simultaneously thawing multiple semen straws (up to 4), the subsequent sequence of AI (from 1st to 4th), and the time elapsed from the initial thawing process to the 4th AI (7 to 10 min) did not affect CR (Dalton et al., 2004). Therefore, improved fertility following AI could potentially be achieved by continuously monitoring and retraining those individuals performing the breeding tasks in dairy herds.

A clean and strict protocol-driven AI technique is recommended to maximize reproductive outcomes (King et al., 1984; Dalton et al., 2004; Bas et al., 2011). A practical approach for a clean AI technique may be through the use of protective plastic sheaths (PS). Protective PS, are rigid PVC tubes developed to prevent vaginal contamination of the AI catheter (AIC) at the time of AI with the aim of improving reproductive outcomes. Early studies reported no benefits of using PS on conception rates
to first services in dairy or beef cattle (King et al., 1984; Richards et al., 1984). However, a recent study revealed that using single use PS not only reduced contamination of the AIC at the time of AI, but also increased pregnancies per AI (PAI; Bas et al., 2011). Therefore, the objectives of the present study were to: 1) assess the effectiveness of using protective PS on vaginal contamination of the AIC at the time of AI, 2) evaluate PAI in lactating dairy cows inseminated with or without the use of protective PS, and 3) estimate the economic benefits of using PS in dairy herds. It was hypothesized that the use of disposable protective PS (clean AI technique) would improve PAI in dairy cattle by reducing contamination of the AIC, resulting in improved reproductive efficiency and profitability of the herd.

Materials and Methods

Animals, Facilities, and Feeding Management

The present study was conducted on two commercial dairy herds (approximately 2,100 and 4,200 cows each) located in Ohio between April 2009 and December 2010. A total of 3,221 services from 2,284 lactating Holstein cows were enrolled to assess the effect of using disposable PS (Continental Plastic Inc., Delavan, WI). Data from a previous study (Bas et al., 2011) were included in the analyses. The rolling herd averages ranged from 9,715 kg to 10,236 kg with a reported voluntary waiting period of 55-60 DIM. Cows were housed in free-stall barns and were milked three times daily at approximately 8 h intervals. Animals were fed twice daily, in the morning and afternoon,
with a TMR formulated to meet or exceed dietary nutritional requirements for high-producing lactating dairy cows (NRC, 2001).

**Breeding Management and Treatments**

Weekly, a cohort of cows at 26±3 DIM were presynchronized with 2 injections of PGF$_{2\alpha}$ (25 mg; Lutalyse, Pfizer Animal Health, NY, USA) given 14 d apart. Twelve to 14 d after the second PGF$_{2\alpha}$ injection, all cows were initiated in either an Ovsynch (OV; GnRH-7 d- PGF$_{2\alpha}$-56 h-GnRH-16 h-Timed AI [TAI]; Brusveen et al., 2008) or Cosynch (COS; GnRH-7 d-PGF$_{2\alpha}$-72 h-GnRH+TAI; Portaluppi et al., 2005) synchronization programs. Following the initial GnRH (100 μg; Cystorelin, Merial, Duluth, GA, USA) of OV or COS, estrus was detected either by visual observation plus tail chalking once daily or using automated estrous detection system (AfiFarm™ 3.05, Kibbutz Afikim, Israel). After the initial GnRH administration, all animals detected in estrous (i.e. cows staying still when mounted, presenting worn off tail chalking or increased activity) were AI. Animals that did not display estrus behavior during the synchronization program were subjected to TAI 72 h after the PGF$_{2\alpha}$ injection of OV or COS. Cows that where already bred, but showed visual signs of estrus behavior before the pregnancy diagnosis were also AI. All AI were performed by 3 technicians using frozen semen (25 bulls) as part of the routine breeding management of the herds. Pregnancy was diagnosed by transrectal ultrasonography (Easi-Scan, BCF Technology Ltd., Livingston, UK) using a 7.5-MHz linear-array transducer at 35 to 45 d after AI by the herd veterinarian. At the time of pregnancy diagnosis (PD), non-pregnant cows were re-enrolled in an OV or COS synchronization program as described earlier. Reconfirmation of pregnancy was
performed by transrectal ultrasonography 30 d after the first PD. The proportion of pregnancy losses was determined between first and the second PD.

At the time of AI, services (n = 3,221) from 2,484 lactating dairy cows were assigned to 1 of 2 treatment groups: with (TRT, n = 1,603) or without (CON, n = 1,618) the use of PS as described previously (Bas et al., 2011). A systematic randomization was performed with the first cow randomly allocated to one of the two treatment groups; later the use of PS was alternated on every other cow. In the TRT group the AIC protected with a PS was introduced into the vagina; once in the cranial portion of the vagina adjacent to the cervical os, the PS was pulled back and only the insemination catheter was manipulated through the cervix into the uterine body for semen deposition. While in the CON group, cows were AI without the use of PS. Body condition score (BCS) was recorded in a subset of cows (n = 1,019) using the 5-point scoring system (Ferguson et al., 1994). Individual cow records including DIM, milk yield, lactation number, service number, health events (retained fetal membranes, metritis, mastitis) were obtained from on-farm records (DairyComp 305; Valley Agricultural Software, Tulare, CA). Lactation number (primiparous or multiparous), service number (first service and second or greater services), health status of cows (Yes = one or more health events or No = no health event) and BCS (≤2.75 or ≥3.0) were dichotomized for further analysis.

Culture and Identification of Bacteria

To assess the effectiveness of using PS to minimize vaginal contamination of the AIC at the time of AI, a subset of cows (n = 102) were blocked by parity and randomly selected for collection of swab samples. Immediately following AI, sterile cotton swab
samples (BD, Franklin Lakes, NJ, USA) from the tip of the AIC were collected as previously described (Bas et al., 2011). Briefly, in the TRT group (n = 52), after semen deposition in the uterine body the AIC was reintroduced into the PS before leaving the cervix. Consequently, the AIC was protected by the PS on its transit out of the vagina and was only exposed to the cervix and uterine body environments. Once outside, the PS was pulled back exposing the tip of the AIC and a sterile premoistened cotton swab sample was collected. In the CON group (n = 50), once semen was deposited into the uterine lumen, the vulva was cleaned with paper towels, the vulvar lips opened to prevent contact of the AIC with the vulvar skin, the AIC were carefully pulled out of the reproductive tract and a sterile premoistened swab sample was collected.

Within 6 h of collection, samples were transported in Stuart transport media (BD, Franklin Lakes, NJ, USA) at ambient temperature to the laboratory for further bacteriology analysis. Once in the laboratory, samples were cultured onto blood (Tryptic Soy Agar with 5% Sheep Blood) and MacConkey agar plates for 24 h at 37°C under aerobic conditions. After the incubation period, selection of each unique colony from MacConkey and blood agar plates that presented colony growth was performed based on morphology, pigmentation and hemolytic patterns. Selected unique colonies were further purified in individual blood agar plates for genus identification. Pure isolates were then stored at -20°C until final genus identification.

The growth density of bacteria in culture plates from TRT and CON groups was assessed immediately after culture based on the number of colonies that grew in MacConkey and blood agar plates. Culture plates were classified using the following semiquantitative scale (Bas et al., 2011): 1 = light colony growth (0-20 visible colonies);
2 = moderate colony growth (21-100 visible colonies); 3 = heavy colony growth (more than 100 visible).

Computational of Economical Benefits

The economic benefit of 5.5 percentage points increase in PAI (Table 4.4) by using protective PS at the time of AI was assessed using an individual-based stochastic model to simulate reproductive performance of a dairy herd (Galvão et al., 2013). The proportion of pregnant cows from TRT (36.8%) and CON (31.3%) groups were used in the model as the conception rate (CR) to estimate the economic benefits. For the simulation, lactating dairy cows were enrolled in an OV for first AI followed by estrus detection (ED) and resynchronization of cows diagnosed open 32 d after AI using OV. Herd size was maintained at 1,000 cows. Mortality was set at 6%, abortions at 13%, and stillbirth at 6.5%. Cows not AI after 365 DIM and open cows were culled after 450 DIM. Culled cows were immediately replaced with pregnant heifers. The dry and VWP were set to 60 d. Compliance to each injection of the synchronization program was 95% and ED was set to 60% with an accuracy of 95%. Simulation was performed at CR of 31.3% or 36.8% for 3,000 d until steady-state was reached, then the subsequent 2000 d were used to calculate the new economic values ($/cow/yr). Mean values from 20 runs were used. Profit from the net daily value was calculated by subtracting the costs of replacements ($1,600/heifer), feeding costs ($0.25/kg of lactating cow diet; $0.15/kg of dry cow diet), breeding costs ($0.15/cow/d for ED; $2.65/dose PF2α; $2.4/dose GnRH; $0.25/injection administration; $3 per pregnancy diagnosis; semen ($10 per straw); IA technician fee ($5 per service) and other costs ($2.5/d) from the daily income [milk sales
($0.44/kg milk), cow sales ($1.65/kg live weight), and calf sales ($200/calf)]. The costs of the PS ($0.20 each) were only added to the breeding costs of TRT cows.

Statistical Analyses

The effectiveness of using disposable PS to reduce contamination of the AIC was assessed after AI. For both treatment groups, the proportion of bacteria isolated from the AIC was analyzed using the FREQ procedure of SAS (SAS, 2009). A $P < 0.05$ was considered statistically significant. The density of bacterial growth and bacteria isolated from cultured samples were reported as a proportion (%) of the total positive cultures and the total number of identified isolates respectively.

Prior to data analysis, animals that met the exclusion criteria (culled or died before the end of the study period, had aborted before AI or presented vaginal discharge at AI, and animals with incomplete or missing records) were removed from the analysis. Data were arranged in a complete randomized design. The distribution cows by lactation number, DIM, BCS, number of services, and milk yield (Kg) from the closest DHIA record across treatment groups (TRT or CON) were analyzed using the MIXED procedure of SAS (SAS, 2009). Least-square mans ($\pm$ SEM) were reported.

Dichotomous variables such as PAI (overall, first and second or greater services) and pregnancy losses were analyzed using generalized linear mixed models (GLIMMIX) procedure of SAS (SAS, 2009). Non-significant variables and interactions were eliminated from the model using the Wald statistic backward selection criterion ($P > 0.15$). Herd and sire were treated as a random effect. For second or greater services, a model procedure that included treatment (CON or TRT), health status (Yes or No),
breeding method (ED or TAI), and SCC at the closest DHIA test relative to service was used to compare differences in PAI between treatments. For first services, a model procedure that included treatment (TRT or CON), breeding method (ED or TAI), and health status (Yes or No) was used to compared differences in PAI between treatments. For pregnancy losses, a model procedure that included the effect of treatment (TRT or CON) was used to compare differences in between treatments. The estimates (proportions of PAI and pregnancy loss) from the final models were reported as least squares means (Pinedo and De Vries, 2010; Bas et al., 2011). The differences between least squares means were calculated by including the PDIFF option in the LSMEANS statement (Tsousis et al., 2009). Differences in individual least squares means were adjusted by using Tukey-Kramer method. Least squares means (± SEM) were reported. A P < 0.05 was considered statistically significant.

Results

From 3,221 services of 2,284 cows that were originally enrolled in the present study, 226 services (TRT, n = 111; CON, n = 115) from 218 cows were not included because they were culled or died before the end of the study period (n = 94), had aborted before AI or presented vaginal discharge at AI (n = 68), or had incomplete or missing records (n = 56). Therefore, a total of 2,995 services from 2,066 lactating Holstein cows (1,149 primiparous and 917 multiparous) were available for the final analyses. Distribution of lactation number, DIM, BCS, number of services, and milk yield at the
time of AI with respect to parity (primiparous or multiparous) did not differ (P > 0.05) between treatments (Table 4.1).

Culture and Identification of Bacteria

The proportion of samples yielding positive bacterial growth was reduced for TRT compared to CON groups (P < 0.05; Table 4.2). Furthermore, the majority of the swab samples from the TRT group yielded light or moderate colony growth (Table 4.2). Conversely, bacterial cultures from CON group had greater proportion of heavy colony growth compared to TRT (Table 4.2). Overall, a total of 303 bacterial isolates were obtained from TRT and CON swab samples, and 194 isolates (64.0%; TRT, n = 50 vs. CON, n = 144) were identified and reported (Table 4.3). Furthermore, genus identification revealed that E. coli was the most prevalent bacteria isolated from CON cows (Table 4.3).

Effect of PS on PAI, Pregnancy Losses, and Economics

Overall, services performed with the use of protective PS (TRT) had greater PAI (P = 0.04) compared to CON group (Table 4.4). For first postpartum services, PAI did not differ (P = 0.8) between TRT and CON groups (Table 4.4). However, PAI were greater (P = 0.02) for second or greater services in TRT compared to CON cows (Table 4.4). Furthermore, pregnancy losses did not differ (P = 0.55) between groups (Table 4.4). According to the economic model, the use of PS provided an additional $4 cow/yr (or $4,000/yr for a 1,000-cow herd) for each percentage point increase in CR (Table 4.5).
Discussion

Accurate estrous detection, compliance to synchronization protocols, and an appropriate AI technique are required to achieve optimal reproductive performance over time. The primary findings of the present study were that: 1) PS was effective in reducing contamination of the AIC at the time of AI, 2) overall and second or greater services PAI were improved, and 3) the use of PS increased herd profitability by improving PAI at a minimal cost.

Samples collected from the AIC immediately after AI revealed that the use of PS was effective in minimizing vaginal contamination of the AIC. Using disposable PS to protect the AIC not only prevents contamination with perineal or vaginal contaminants, but may also help to reduce contamination with other fomites (e.g., AI gun warmer) once the semen straw is loaded into the insemination catheter. In the present study, the proportion of AIC with bacterial growth was reduced by almost 45 percentage points when inseminations were performed with the use of disposable PS. Furthermore, cultures revealed that the density of bacterial growth was minimized when PS were used to cover the AIC as reflected by the reduced number of visible colonies that grew in the cultures. Similar findings were observed in a previous study, where PS effectively reduced bacterial contamination of the insemination catheter (from 100 to 61%) along with reduced density of bacterial growth obtained (Bas et al., 2011).

Williams et al. (2005) categorized bacteria according to their pathogenic potential in the uterus as 1) recognized uterine pathogens (A. pyogenes, Prevotella melaninogenicus, E. coli, Fusubacterium necrophorum), 2) potential pathogens (i.e.
Bacillus spp., Pasteurella spp., Staphylococcus aureus) and 3) opportunistic contaminants (i.e. Streptococcus spp., Providencia spp., Klebsiella spp., Staphylococcus spp.). In the present study, the most prevalent bacteria isolated from AIC within the CON group were E. coli (approximately 43% of the isolates) while the most commonly isolated bacteria in TRT cows were Staphylococcus spp., which can be classified as an opportunistic contaminant not associated with endometritis (Sheldon et al., 2002; Williams et al., 2005; Williams et al., 2007). In sheep, it was shown that E. coli lipopolysaccharide (LPS) blocked the release of GnRH and LH form the hypothalamus and pituitary affecting the ability of the dominant follicle to ovulate (Battaglia et al., 2000; Williams et al., 2001). Similarly, E. coli endotoxin infusions into the uterine lumen of heifers prevented the preovulatory LH surge and ovulation (Peter et al., 1989). Furthermore, in vitro exposure of granulosa cells to LPS resulted in decreased aromatase activity within the ovary; thus, reducing follicular estradiol secretion (Herath et al., 2007). Corpus luteum regression is also compromised in cows suffering E. coli uterine infections due to a shift in the type of prostaglandin synthesized by the endometrium (from the F series to the E series), and consequently luteolysis is delayed (Herath et al., 2006; Herath et al., 2009). Altogether, these findings suggested that E. coli (in synergism with other recognized uterine pathogens) can affect ovarian functions directly (Herath et al., 2009) or indirectly by affecting the control centers located at the hypothalamus and pituitary (Sheldon and Dobson, 2004; Sheldon et al., 2008).

In addition to ovarian functions, the endometrial integrity and uterine environment are negatively affected the presence of pathogens. Presence of bacterial products such as LPS and inflammatory mediators (e.g., chemokines and cytokines)
within the uterine lumen trigger an inflammatory response with the influx of neutrophils which can disrupt the integrity of the endometrium and lead to a suboptimal uterine environment (Sheldon et al., 2009a; Gilbert, 2011). These changes have been associated with impaired oocyte maturation (Soto et al., 2003a) and compromised gamete transport or inadequate signaling for the establishment of pregnancy (Hill and Gilbert, 2008; Sheldon et al., 2009b). In mares, it has been demonstrated that bacteria and other debris introduced into the uterus during breeding may colonize the uterine lining and trigger a post-breeding inflammatory reaction that could lead to persistent uterine inflammation and reduced fertility (Causey, 2006). In cattle, the potential detrimental effect of introduced bacteria into the uterus at the time of AI has not been fully investigated. A recent study investigated the effect of AI on polymorphonuclear cells (PMN) and CR in lactating dairy cows (Kaufmann et al., 2009). Results from this study revealed that cows with higher proportion of uterine PMN 4 h post-AI had lower CR (Kaufmann et al., 2009). The potential reproductive benefit observed in the present study through the use of protective PS may be related to a reduction in the amount of pathogens such as *E. coli* introduced into the uterine lumen following AI.

The overall PAI was significantly increased by 5.5 percentage points in TRT compared to CON cows. The beneficial effects of using PS during AI were maximal for second or greater services, where PAI was increased by almost 8 percentage points (from 29.2 to 37.0%). Results from this study are in agreement with previous findings that reported increased overall and second or subsequent services PAI following inseminations with the use of PS (Bas et al., 2011). Conversely, earlier studies reported no improvement in reproductive outcomes when services were performed with the use of
PS in dairy or beef cattle (King et al., 1984; Richards et al., 1984). However, these studies were carried out more than 20 years ago, using several AI technicians, and non-return rates to first services were evaluated. In addition, cows were allocated to inseminations with or without PS on alternated days or weeks (King et al., 1984; Richards et al., 1984). In the present study, all AI were carried out by three AI technicians, alternating the use of PS every other cow within herd, using similar reproductive program, and cows were evenly distributed with respect to parity, DIM, BCS, service number, and milk yield for both treatment groups (TRT and CON).

The economic benefit of 5.5 percentage point increase PAI by using PS was assessed using an individual-based stochastic model (Galvão et al., 2013). According to the simulation model, the use of PS provided an additional $22/cow per yr ($4/cow/yr for each increase in CR) compared to CON cows. For each 1 percentage point increment in CR, farm income increased between $8 and $4/cow per yr by using 100% TAI over ED programs (Giordano et al., 2011). Compliance with the AI procedure (semen handling, accuracy of ED, hygiene, site of semen deposition) affects the bottom line of dairy herds and should not be compromised for convenience. Therefore, investing in educational training for professional AI technicians should be a top priority to achieve consistent reproductive outcomes over time.

It has been shown that the rate of clearance of steroid hormones is increased in high producing dairy cows (Sangsritavong et al., 2002). A positive correlation between the level of milk production and dry matter intake was observed (Harrison et al., 1990), which has been associated with increased liver blood flow and hepatic clearance of steroid hormones (Sangsritavong et al., 2002; Wiltbank et al., 2006). The clearance of
steroid hormones by hepatic enzymes can lead to reduced circulating concentrations of estrogen (E2) and progesterone (P4). These steroid hormones have been associated with uterine immune functions. Under high E2 concentrations, there is an increased blood flow to the uterus, increased mucus production and intensified activity of leucocytes (Hawk et al., 1960). Furthermore, E2 regulates leukocyte migration into the cervix, the uterine lumen, and the extracellular matrix of the reproductive tract (Ramos et al., 2000; Stygar et al., 2006). Although the hormonal profiles were not assessed in the present study, reduced concentrations of estradiol may lead to poor immunological response in the uterus (ability to clear post-breeding bacteria or debris) with the subsequent altered mechanisms to maintain pregnancy. A possible explanation for the lower PAI observed in the CON group is that high-producing dairy cows may have a suboptimal uterine environment with weak host defenses, concomitant with altered hormone profiles, which may interfered with maternal recognition of pregnancy. Therefore, lactating dairy cows may benefit from the use of protective PS at the time of AI by preventing the introduction of vaginal contaminants into the uterus, resulting in optimal uterine environment and improved PAI as observed for cows in the TRT group. The mechanisms underlying the benefit of using PS remain to be elucidated.

In conclusion, results from this study showed that using disposable PS at the time of AI (clean AI technique) was effective in reducing contamination of the AIC and improved overall and second or greater services PAI in lactating dairy cows. The improved reproductive performance obtained with the use of PS translated into an additional profit of $4 cow/yr (or $4,000/yr for a 1,000-cow herd) for each percentage point increase in CR. Performing a clean AI technique through the use of PS was a cost-
effective strategy to improve reproductive outcomes in dairy cattle. The present study demonstrated the importance of reducing bacterial contamination at the time of AI, which should be emphasized to professional AI technicians and on-farm breeders in order to achieve consistent reproductive performance over time.
<table>
<thead>
<tr>
<th>Items</th>
<th>TRT (n = 770)</th>
<th>CON (n = 788)</th>
<th>P</th>
<th>TRT (n = 722)</th>
<th>CON (n = 715)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parity</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>2.9 ± 0.04</td>
<td>2.8 ± 0.04</td>
<td>0.81</td>
</tr>
<tr>
<td>DIM (d)‡</td>
<td>137.3 ± 3.6</td>
<td>133.7 ± 3.6</td>
<td>0.48</td>
<td>136.3 ± 2.9</td>
<td>132.6 ± 2.9</td>
<td>0.37</td>
</tr>
<tr>
<td>BCS‡</td>
<td>2.9 ± 0.02</td>
<td>2.9 ± 0.02</td>
<td>0.76</td>
<td>2.9 ± 0.02</td>
<td>2.8 ± 0.02</td>
<td>0.18</td>
</tr>
<tr>
<td>Number of services</td>
<td>3.0 ± 0.08</td>
<td>2.8 ± 0.08</td>
<td>0.18</td>
<td>2.9 ± 0.08</td>
<td>2.8 ± 0.08</td>
<td>0.62</td>
</tr>
<tr>
<td>Milk yield (Kg)‡</td>
<td>72.3 ± 0.6</td>
<td>71.1 ± 0.6</td>
<td>0.18</td>
<td>91.5 ± 0.8</td>
<td>92.5 ± 0.8</td>
<td>0.38</td>
</tr>
</tbody>
</table>

Table 4.1. Distribution (LSM ± SEM) of parity, DIM, BCS, number of services, and milk yield (kg) at the time of AI with respect to parity (primiparous or multiparous) between treatment groups in lactating dairy cows.

1At the time of AI 2,995 services from 2,066 lactating dairy cows were randomly allocated to 1 of 2 treatment groups: with (TRT, n = 1,492) and without (CON, n = 1,503) the use of protective PS.

‡At the time of AI, a subset of cows (n = 1,019) had their BCS recorded using a 5-point scoring system (Ferguson et al., 1994).

‡Milk yield (kg) and DIM (d) were recorded at the closest DHIA test relative to service.
Table 4.2. Proportion of positive bacterial growth and density from the AI catheter (AIC) of dairy cows inseminated with (TRT) and without (CON) the use of protective plastic sheath (PS) on blood agar and MacConkey plates.

†Swab samples were collected from the tip of the AI catheter (AIC) immediately after AI for further identification of bacteria from 102 lactating dairy cows, inseminated with (TRT, n = 52) and without (CON, n = 50) the use of protective PS.

Bacterial growth density (Light, moderate and heavy) was assessed semiquantitatively based on the number of bacterial colonies visible that grew on blood agar and MacConkey plates.

1Identification of 1-20 visible colonies.

2Identification of 21-100 visible colonies.

3Identification of over 100 visible colonies per plate or not possible to count due to heavy growth.
<table>
<thead>
<tr>
<th>Species</th>
<th>TRT</th>
<th>CON</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli spp.</td>
<td>18 (9/50)</td>
<td>43.06 (62/144)</td>
</tr>
<tr>
<td>Staphylococcus spp.</td>
<td>36 (18/50)</td>
<td>13.19 (19/144)</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>22 (11/50)</td>
<td>6.94 (10/144)</td>
</tr>
<tr>
<td>Streptococcus spp.</td>
<td>8 (4/50)</td>
<td>11.11 (16/144)</td>
</tr>
<tr>
<td>Bacillus spp.</td>
<td>4 (2/50)</td>
<td>6.94 (10/144)</td>
</tr>
<tr>
<td>Pasteurella spp.</td>
<td>4 (2/50)</td>
<td>6.25 (9/144)</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>4 (2/50)</td>
<td>2.78 (4/144)</td>
</tr>
<tr>
<td>Arcanobacterium spp.</td>
<td>2 (1/50)</td>
<td>1.39 (2/144)</td>
</tr>
<tr>
<td>Actinobacillus spp.</td>
<td>-</td>
<td>2.08 (3/144)</td>
</tr>
<tr>
<td>Moraxella spp.</td>
<td>2 (1/50)</td>
<td>-</td>
</tr>
<tr>
<td>Acinetobacter spp.</td>
<td>-</td>
<td>0.69 (1/144)</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>-</td>
<td>1.39 (2/144)</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>-</td>
<td>1.39 (2/144)</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>-</td>
<td>1.39 (2/144)</td>
</tr>
<tr>
<td>Klebsiella spp.</td>
<td>-</td>
<td>0.69 (1/144)</td>
</tr>
<tr>
<td>Providencia spp.</td>
<td>-</td>
<td>0.69 (1/144)</td>
</tr>
</tbody>
</table>

Table 4.3. Distribution of bacterial isolates from the AI catheter (AIC) immediately after AI in lactating dairy cows inseminated with (TRT) and without (CON) the use of protective plastic sheath (PS).

1Following culture, a total of 303 bacterial isolates were obtained from which 194 isolates (64.02 %) were identified. Bacterial isolates were reported as a proportion (%) over the total (n) number of isolates of one particular genus from the total (n) number of identified isolates obtained in TRT (n = 52) and CON (n = 50) groups.
<table>
<thead>
<tr>
<th>Item</th>
<th>TRT</th>
<th>CON</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall PAI (%)</td>
<td>36.8 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.3 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04</td>
</tr>
<tr>
<td>First service PAI (%)</td>
<td>33.8 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.1 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.80</td>
</tr>
<tr>
<td>(n = 504)</td>
<td>(n = 534)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second or greater services PAI (%)</td>
<td>37.0 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.2 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.02</td>
</tr>
<tr>
<td>(n = 988)</td>
<td>(n = 969)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall pregnancy losses (%)</td>
<td>10.1 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Table 4.4. Pregnancies per AI (PAI) and pregnancy losses from lactating dairy cows that were inseminated with (TRT) and without (CON) the use of protective plastic sheath (PS).

At the time of AI 2,995 services (performed by 3 AI technicians) from lactating dairy cows were randomly assigned to 1 of 2 treatment groups: with (TRT, n = 1,492) and without (CON, n = 1,503) the use of protective PS.

<sup>1</sup>Least squares means (LSM± SEM) were reported.

<sup>ab</sup>Different superscript letters within a row differ significantly at P < 0.05.
Table 4.5. Economic benefit for lactating dairy cows that were inseminated with (TRT) and without (CON) the use of protective plastic sheath (PS)\(^1\).

\(^1\)The economic benefit of 5.5 percentage point increase in CR by using protective PS at the time of AI (TRT, 36.8%) compared to CON cows (31.3%) was assessed using an individual-based stochastic model to simulate reproductive performance of a dairy herd (Galvão et al., 2013). Herd size was maintained at 1,000 cows.

\(^\dagger\)The proportion of PAI obtained for TRT (36.8%) and CON (31.3%) cows were used in the model as the conception rate (CR) to estimate the economic benefits.

\(^\ddagger\)Breeding costs included the hormones ($0.15/cow/d for ED; $2.65/dose PF\(_{2\alpha}\); $2.4/dose GnRH; $0.25/injection administration), $3 per pregnancy diagnosis, $10 per straw of semen, and IA technician fee ($5 per AI). The costs of PS ($0.20 each) were only included for TRT cows.

\(^\S\)Profit ($/cow/yr) was calculated by subtracting the costs of replacements, feeding, breeding, and other costs from the herd incomes (milk, cow, and calf sales).

\(\ast\)The difference between TRT and CON values was calculated as: TRT – CON = Value.

<table>
<thead>
<tr>
<th>Items</th>
<th>TRT</th>
<th>CON</th>
<th>Difference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR (%)(^\dagger)</td>
<td>36.8</td>
<td>31.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Breeding costs ($/cow/yr)(^\ddagger)</td>
<td>107.5</td>
<td>114.5</td>
<td>-7</td>
</tr>
<tr>
<td>Profit ($/cow/yr)(^\S)</td>
<td>1,806</td>
<td>1,784</td>
<td>22</td>
</tr>
</tbody>
</table>
CHAPTER 5

Effect of Intrauterine Administration of Gonadotropin Releasing Hormone on Serum LH Concentrations in Lactating Dairy Cows

S. Bas\textsuperscript{a}, C.G. Pinto\textsuperscript{a}, M.L. Day\textsuperscript{b}, G.M. Schuenemann\textsuperscript{a,*}

\textsuperscript{a}Department of Veterinary Preventive Medicine, College of Veterinary Preventive Medicine, The Ohio State University, Columbus, OH 43210
\textsuperscript{b}Department of Animal Sciences, College of Agriculture and Natural Resources, The Ohio State University, Columbus, OH 43210

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Abstract

The objectives were to compare: 1) preovulatory serum LH concentrations, and 2) synchronization of ovulation, following i.m. or intrauterine (i.u.) administration of the second GnRH treatment of Ovsynch in lactating dairy cows. Lactating cows (n = 23) were presynchronized with two injections of PGF$_{2\alpha}$ given 14 d apart (starting 34 ± 3 DIM), followed by Ovsynch (GnRH-7 d-PGF$_{2\alpha}$-56 h-GnRH) 12 d later. At the time of the second GnRH (GnRH2) of Ovsynch (Hour 0), cows were blocked by parity and randomly assigned to 1 of 3 groups: 1) control group (CON; n = 7) were given 2 mL sterile water i.m.; 2) intramuscular group (IM; n = 8) received 100 µg of GnRH i.m.; and 3) intrauterine group (IU; n = 8) had 100 µg GnRH infused in the uterus (2 mL). Blood samples for serum LH concentrations were collected at Hours 0, 0.5, 1, 1.5, 2, 3, and 4. Furthermore, ultrasonography was performed twice daily (12 h intervals) from Hours 0 to 60 to confirm ovulation. The LH concentrations were greater (P < 0.05) in the IM than IU and CON groups at Hours 0, 0.5, 1, 1.5, 2, 3, and 4. Although LH concentrations were numerically higher in the IU group, LH concentrations within the IU and CON groups did not change over time. More cows ovulated in the IM (8/8) and IU (7/8) groups within 60 h after GnRH2 administration compared with the CON (2/7) group. In summary, serum LH concentrations were lower in the IU versus IM group, but the proportion of cows that ovulated within 60 h was similar between these two groups. Therefore, i.u. administration of GnRH may be an alternative route of delivery to synchronize ovulation in beef and dairy cattle.
Introduction

Gonadotropin releasing hormone (GnRH), a decapeptide hormone synthesized by hypothalamic neurons, has a central role in mammalian reproduction (Fink, 1988). Hypothalamic neurons release GnRH into the hypothalamus-hypophysial portal circulation, stimulating the release of LH and FSH by gonadotropes in the pituitary gland (Fink, 1988; Page, 1988). Subsequently, LH and FSH control steroidogenesis and gametogenesis in the gonads (Schneider, 2006). Because of the central role in the bovine reproductive cycle, products containing GnRH and its analogues are used in protocols to synchronize ovulation in dairy and beef cattle (Pursley et al., 1995; Haughian, et al., 2004; Peters, 2005; Herbert and Trigg, 2005). Furthermore, GnRH in combination with products containing PGF$_{2\alpha}$ are widely used in estrus and ovulation synchronization programs to coordinate ovarian follicular development, luteolysis, and ovulation (Peters, 2005; Herbert and Trigg, 2005). Giving GnRH i.m. induces an LH release from the anterior pituitary; blood concentrations peaked within 2 h and returned to basal concentrations by 4 h (Haughian, et al., 2004). If a dominant follicle is present at the time of GnRH administration, ovulation is expected to occur within 24-32 h (Pursley et al., 1995).

The i.m. route of administration of GnRH is the most common and is routinely used in synchronization of ovulation protocols. In a recent report on injection practices on dairy operations in the USA (USDA, 2009), dairy cows received an average of 14 injections (ranging from 5 to 24) per year, and administration of reproductive hormones accounted for approximately 27% of total injections. Development of effective
reproductive protocols such as Ovsynch (Pursley et al., 1995; Brusveen et al., 2009) and Presynch (Vasconcelos et al., 1999), followed 12 d later by Ovsynch, has provided clear alternatives to improve reproductive performance of lactating dairy cows. However, these protocols have increased the number of i.m. injections per animal. It is noteworthy that intrauterine (i.u.) administration of GnRH has been investigated in cattle and other species (Dermody et al., 1976ab; Manns et al., 1980). In that regard, the i.u. route (via semen extender) may be appropriate to administer GnRH for synchronization of ovulation while concurrently reducing i.m. injections in cattle.

In addition to synchronization of ovulation, perhaps i.u. administration of GnRH would provide additional reproductive benefits. Receptors for GnRH have been identified in the bovine uterus and oviducts (Singh et al., 2008; Giammarino et al., 2009) and GnRH receptor mRNA was demonstrated in bovine ovarian follicular and luteal tissues (Ramkrishnappa et al., 2003). Additionally, it was recently reported that GnRH induced contraction of bovine uterine myoepithelial cells in vitro during the follicular phase (Giammarino et al., 2009). Furthermore, GnRH may play a role in the process of fertilization; in that regard, the addition of GnRH to in vitro fertilization media increased cleavage rate of bovine oocytes (Funston and Seidel, 1995). Moreover, in humans, GnRH increased the ability of sperm to bind to the zona pellucida (Morales, 1998). Similarly, it was recently reported that exposure of male and female bovine gametes to a GnRH analogue increased binding of sperm to the zona pellucida (Perera, 2010).

In the current study, we hypothesized that administering GnRH through i.u. route in lactating dairy cows would induce a preovulatory release of LH similar to that following i.m. administration. Consequently, the objectives of the present study were to
Materials and Methods

Animals, Facilities, and Feeding

Lactating Holstein dairy cows (n = 39), from one dairy herd located at the Ohio Department of Rehabilitation and Correction, London, OH, USA, were used. These cows were housed in free-stall barns and were milked twice daily at approximately 12-h intervals. Cows had *ad libitum* access to water and were fed twice daily, in the morning and afternoon, with a TMR formulated to meet or exceed dietary nutritional requirements for lactating dairy cows (NRC, 2001). The study was conducted from November 2009 to February 2010. All procedures used in the present study were reviewed and approved by Institutional Animal Care and Use Committee at The Ohio State University, Columbus, OH, USA.

Enrollment of Cows and Treatment Groups

Cows were presynchronized (Presynch) with two injections of PGF$_{2\alpha}$ (25 mg; Lutalyse® sterile solution, Pfizer Animal Health, NY, USA) given 14 d apart starting at 34 ± 3 days in milk (DIM; Fig. 5.1). Twelve days after the second PGF$_{2\alpha}$ treatment, the Ovsynch program was initiated (Brusveen et al., 2009). Just prior to the first GnRH (100 µg GnRH diacetate tetrahydrate; Cystorelin®, Merial, Duluth, GA; USA) administration
of Ovsynch (GnRH1; 60 ± 3 DIM), the ovaries of all cows were screened by transrectal ultrasonography (Fig. 5.1) using a 5 MHz linear transducer (Aloka 500; Tokyo, Japan). Cows with a CL ≥ 15 mm and at least one ovarian follicle ≥ 10 mm in diameter were included in the study. Sixteen cows that did not meet these inclusion criteria were excluded. The remaining cows (n = 23; 8 primiparous and 15 multiparous) were given PGF$_{2\alpha}$ 7 d later to induce regression of the CL (Fig. 5.1). Fifty six hours (56 h) later, at the time of the second GnRH administration (GnRH2) of Ovsynch, cows were blocked by parity and randomly assigned to 1 of 3 treatment groups (Fig. 5.1): 1) control group (CON; n = 7) received an i.m. injection of 2 mL of sterile water (Vedco Inc., Saint Joseph, MO, USA); 2) intramuscular group (IM; n = 8) received an i.m. injection of 100 µg of GnRH (2 mL), and 3) intrauterine group (IU; n = 8) where 100 µg GnRH were infused in the uterus (2 mL). Intrauterine infusions were performed using individually wrapped single-use infusion pipettes (Continental Plastic, Delavan, WI, USA) and GnRH was deposited into the uterine body. The i.m. injections were administered in the semitendinosus muscle using 3.75 cm x 18 gauge needles. The time of GnRH2 administration was defined as Hour 0. Body condition score (BCS) was recorded using a five-point scoring system (Ferguson et al., 1994) at the time of GnRH1 (60 ± 3 DIM; Fig. 5.1).

Collection of Blood Samples and Hormone Assays

Blood samples for determination of serum progesterone concentrations (P4) were collected by coccygeal vessels (BD Vacutainer®, Franklin Lakes, NJ, USA) at the time of each hormonal administration of Ovsynch (GnRH1, PGF$_{2\alpha}$, and GnRH2) and 7 d after
GnRH2 administration (Fig. 5.1). Similarly, blood samples for circulating LH concentrations were collected from the coccygeal vessels at Hours 0, 0.5, 1, 1.5, 2, 3, and 4 (Fig. 5.1). Within 5 h after collection, blood samples were centrifuged (2,785 x g for 20 min) and serum was stored at –20 ºC until assayed for P4 and LH.

Serum P4 concentrations were determined in duplicates in a single assay using a modified commercially available RIA kit (Coat-a-Count®, Siemens Medical Solution Diagnostics, Los Angeles, CA, USA) as described previously (Burke et al., 2003). The intra-assay CV was 6.9% and the sensitivity of the assay was 0.10 ng/mL. Serum concentrations of LH were determined in duplicate by using a double-antibody RIA, as described elsewhere (Anderson et al., 1996). The intra- and inter-assay CVs were 7.3 and 14.3%, respectively. The sensitivity of the assay was 0.11 ng/mL.

Assessment of Ovarian Structures and Measurements

Using a pre-designed paper sheet, the diameter and location of all follicles ≥ 5 mm and CL were recorded for all cows. Measurements and location of ovarian structures (CL and follicles) were determined with transrectal ultrasonography and recorded at all injections of the Ovsynch protocol (GnRH1, PGF2α, and GnRH2), and 7 d after GnRH2 administration (Fig. 5.1). Follicular and CL diameters were calculated by averaging the perpendicular measurements of the largest cross sectional diameters of each structure (Martins et al., 2011). If a CL had a fluid-filled cavity, the final diameter was calculated by subtracting the cavity diameter from the CL diameter (Martins et al., 2011). After GnRH2, cows were subjected to transrectal ultrasonography twice daily (approximately every 12 h) for 60 h for detection of ovulation (Fig. 5.1). Ovulation was defined as
disappearance of the largest follicle (ovulatory follicle) previously recorded and was confirmed 7 d later by visualization of a CL on the same ovary that previously contained the dominant follicle (Martins et al., 2011; Souza et al., 2009).

Statistical Analyses

The distribution of cows by lactation number, DIM, milk yield (kg) and BCS at the time of GnRH1 across treatment groups was analyzed using the MIXED procedure of SAS (SAS, 2009). Data were arranged in a randomized complete block design. For all continuous data, a test for normality of residuals was performed using the UNIVARIATE procedure of SAS (SAS, 2009). Data transformation (log base 10) was performed for LH and P4 concentrations, diameters of CL and follicles when the Shapiro-Wilk test was P < 0.90. Concentrations of LH, area under the curve (AUC) for LH, concentrations of P4, mean time to ovulation (h), and diameters of ovarian structures (CL and follicles) were analyzed using the MIXED procedure of SAS (SAS, 2009). Non-significant variables and interactions were eliminated from the model one at a time using the Wald statistic backward selection criterion (P > 0.15). Concentrations of LH and P4 over time were analyzed as repeated measures. Cows were treated as a random effect. For analysis of LH concentrations, variables contained in the final model included the effect of treatment, hour of sample collection, and their interactions. The final model for P4 included the effects of treatment, date, lactation number and treatment x date interaction. Least squares means (LSM) ± SEM were reported. The AUC for LH was calculated by using the trapezoidal rule (Souza et al., 2009). For analysis of the AUC for LH, variables contained in the final model included the effect of treatment and milk yield at Hour 0. For
analysis of the time to ovulation (h), the final model included the effect of treatment at GnRH2. For analysis of diameters of ovarian structures (CL and follicles), the model included the effect of treatment. For all analyses, $P < 0.05$ was considered significant.

**Results**

A total of 23 animals that met the inclusion criteria (CL ≥15 mm and follicle ≥10 mm) at GnRH1 were enrolled in the present study (Table 5.1). Distribution of animals with respect to lactation number, DIM, milk yield, and BCS at GnRH1 did not differ ($P > 0.05$) among treatment groups (Table 5.1).

*Circulating Concentrations of LH*

There was a significant treatment x hour interaction for LH concentrations. In the IM group, mean LH concentrations increased ($P < 0.05$) by Hour 0.5, were maximal by Hour 1.5, and had decreased ($P < 0.05$) from the peak by Hour 4 (Fig. 5.2). Although concentrations of LH within the IU and CON groups did not change over time ($P > 0.05$), in the IU group, there was a slight numerical increase between Hours 2 and 3 (Fig. 5.2). Cows in the CON group had the lowest serum LH concentrations, with no apparent increase in circulating LH concentrations throughout the sampling period (Fig. 5.2).

Among treatments, concentrations did not differ at Hour 0, but were greater ($P < 0.05$) thereafter in the IM than IU and CON groups. Serum LH concentrations did not differ ($P > 0.05$) between IU and CON groups throughout the sampling period (Fig. 5.2). The AUC for LH differed among treatments ($P < 0.05$); there was a greater AUC for IM
cows (1648) compared to IU (523.3) or CON (231.9) cows, whereas the AUC for LH did not differ (P > 0.05) between IU and CON cows.

**Circulating Concentrations of Progesterone**

For serum P4 concentrations, there were no differences (P > 0.05) among treatment groups at GnRH1, PGF$_{2\alpha}$, or GnRH2 of Ovsynch (Table 5.2). As anticipated, P4 concentrations were lower (< 1 ng/mL) in all treatment groups between PGF$_{2\alpha}$ and GnRH2 (Table 5.2). At the time of ovulation confirmation, 7 d after GnRH2, P4 concentrations were not different (P > 0.05) among treatments (Table 5.2).

**Measurement of Ovarian Structures**

Follicle diameter did not differ (P > 0.05) among treatment groups at GnRH1, PGF$_{2\alpha}$, or GnRH2 (Table 5.3). Similarly, diameter of the CL was not different (P > 0.05) among treatment groups at GnRH1, PGF$_{2\alpha}$, or GnRH2 (Table 5.3). All animals (8/8) in the IM group ovulated within 60 h (42.5 ± 6.4 h) after GnRH2 administration, whereas 7 of 8 cows ovulated in the IU group after the infusion of GnRH2 (51.8 ± 6.8 h; Table 5.3). For CON cows, ovulation within 60 h after GnRH2 occurred in only 2 of 7 cows (Table 5.3), although ovulation was detected in three additional cows within 7 d after GnRH2 administration.

**Discussion**
The primary findings of the present study were that: 1) IM cows had higher LH concentrations compared to IU or CON cows; 2) all IM cows ovulated within 60 h after GnRH2; and 3) although LH concentrations were lower than IM cows, ovulation was detected in 7 of 8 cows in the IU group. The objectives of the present study were to compare the preovulatory LH surge and synchronization of ovulation between IM and IU administration of the second GnRH of Ovsynch in lactating dairy cows. The proportion of cows that ovulated and timing of ovulation was similar between the IU and IM groups. However, serum LH concentration after GnRH2 was significantly greater in the IM compared to IU cows. Previous studies (Dermody et al., 1976a; Manns et al., 1980) evaluated the effect of i.u. administration of GnRH on LH release in Holstein and Brown Swiss heifers following estrus detection and in mature Holstein cows (of unknown lactation stage) after PGF$_{2\alpha}$ administration. The present study not only evaluated the efficacy of a synthetic commercially available GnRH product to induce LH release, but also assessed the ovarian structures and time to ovulation relative to the GnRH2 administration of Ovsynch in lactating Holstein cows.

In the current study, LH increased immediately, peaked at Hour 1.5, and subsequently decreased in cows receiving i.m. GnRH. This response was consistent with previous studies where LH reached maximum concentrations within 2 h after GnRH administration and gradually decreased to basal concentrations 4 h later (Chenault et al., 1990; McDougall et al., 1995; Martinez et al., 2003; Souza et al., 2009). Contrary to what was originally hypothesized in the present study, IU administration of GnRH did not result in a surge release of LH as observed in the IM group. Rather, LH concentrations in the IU group appeared to slightly increase 1 h after GnRH infusion, with maximum
concentrations at Hour 3, whereas LH concentrations remained basal in the CON group. Furthermore, the AUC for LH was greater in IM cows than IU or CON cows. Although non-significant, it was interesting to note that IU cows had a twofold increase in the AUC for LH compared to CON cows.

In Brown Swiss and Holstein heifers, i.u. administration of 1 or 10 mg of GnRH induced a release of LH similar to that resulting after i.v. administration of the same dose of GnRH (Dermody et al., 1976a). In mature dairy cows (at unknown stages of lactation), i.u. administration (in saline or semen diluents) of 50 µg of a potent GnRH analogue (Leuprolide acetate; D-Leu⁶-des-Gly NH₂¹⁰, Pro-ethylamide⁹) increased serum LH concentrations when compared to 100 µg of a synthetic native GnRH analogue (Manns et al., 1980). It was speculated that the reduction in circulating LH concentrations in the present study may have been due to reduced absorption of the GnRH through the uterine wall. Since 7 of 8 animals in IU group ovulated within 60 h after GnRH administration, a putative explanation is that the 4-h bleeding scheme used in the present study failed to detect the peak of the LH response (the LH peak may actually occurred after the 4 h period). Although the absorption of GnRH may have been affected by an interaction with the uterine environment, the potential capability of the reproductive tract mucosa for absorption of GnRH warrants further investigation. Additionally, degradation of GnRH by several proteolytic enzymes in the uterine lumen and during passage through the uterine wall directly affects the bioavailability of the drug following i.u. administration (Hoffman et al., 1995). These non-specific enzymes may have degraded GnRH in the uterine environment, with the subsequent reduction in serum LH concentrations as observed in the IU cows.
Serum concentrations of P4 were not different among treatment groups at GnRH1, PGF$_{2\alpha}$, and GnRH2. Similarly, there were no significant differences among treatment groups in follicle and CL diameters. At the time of GnRH2, at least one dominant follicle > 8.5 mm (Ginther et al., 1996b) that is responsive to an LH surge is expected. In the present study, all animals had a dominant follicle at GnRH2 that was similar in diameter to previous reports (Galvão et al., 2010; Bello et al., 2006). As expected, P4 concentrations < 1 ng/mL at GnRH2 were detected in all cows in the present experiment and elsewhere (Pursley et al., 1995). Previous reports indicated that high concentrations of P4 negatively affected release of LH from the anterior pituitary and ovulation of the dominant follicle in dairy cattle (Roche and Ireland, 1981; Kesner et al., 1982). In the present study, all cows responded to the luteolytic dose of PGF$_{2\alpha}$ of Ovsynch and underwent luteolysis, as evidenced by the reduction in serum P4 concentrations between PGF$_{2\alpha}$ (circulating P4 concentrations were > 3 ng/mL for all groups) and GnRH2 (all groups had P4 < 1 ng/mL). Furthermore, circulating serum P4 concentrations and diameter of CL at GnRH2 were similar among treatment groups.

Ovulation of the dominant follicle is expected to occur within 24 to 32 h after GnRH2 (Pursley et al., 1995), with reported ovulatory responses ranging from 75 to 92% (Galvão et al., 2010; Bello et al., 2006). In the present study, all animals in the IM group ovulated within 60 h after GnRH2. Despite the lesser concentrations of LH in the IU group, it was interesting to note that ovulation occurred within 60 h in 7 of 8 cows. As expected, ovulation occurred in only a small proportion (2/7) of cows in CON group within 60 h post saline administration, probably as a result of a spontaneous LH surge that occurred after the 4 h sampling period. A recent study reported a mean time to
ovulation of 103 h after PGF$_{2\alpha}$ administration in lactating Holstein dairy cows that did not receive GnRH2 in Ovsynch (Martins et al., 2011). When ovulation was re-assessed 7 d after GnRH2, three additional cows in the CON group spontaneously ovulated after the 60 h ultrasonography period. Further investigation is needed, administering various GnRH doses or analogues in the uterus of cows to optimize the release of LH and the ovulatory response. Also, the potential benefits of adding GnRH into semen extender and its impact on fertility of lactating Holstein cows or heifers warrant investigation.

Several synchronization protocols have been developed (Macmillan, 2010), many of them requiring a series of i.m. injections, with increased risk for non-compliance under field conditions. Reproductive strategies based on estrus detection or synchronization of ovulation (e.g., Cosynch) would allow the assessment of reproductive performance in beef and dairy cattle following delivery of GnRH via the semen extender. The effect on compliance rate for synchronization protocols as well as timing to ovulation with respect to AI and their subsequent reproductive performance need further investigation. It is important to note, however, that in the USA, administration of GnRH is approved only for the treatment of ovarian follicular cysts in dairy cattle. Therefore, dairy producers and veterinarians must be aware that GnRH is not currently approved to synchronize ovulation in dairy cattle.

In summary, i.m. administration of 100 µg of GnRH resulted in higher serum LH concentrations compared to cows receiving the same dose of GnRH via the i.u. route. Despite the lesser concentrations of LH in the IU group, ovulation occurred within 60 h in 7 of 8 cows. The IU administration of GnRH via semen extender may provide an alternative route of delivery to synchronize ovulation in beef and dairy cattle.
Acknowledgements

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Figure 5.1. Scheme of the experimental design. Cows were presynchronized (Presynch) with two injections of PGF$_{2\alpha}$ given 14 d apart (starting at 34±3 DIM), followed 12 d later by Ovsynch. At the time of the first GnRH administration of Ovsynch (GnRH1; 60±3 DIM), the ovaries of all cows were screened by transrectal ultrasonography (US). At the time of the second GnRH administration of Ovsynch (GnRH2), cows were blocked by parity and randomly assigned to 1 of 3 treatment groups: 1) control group (CON; n = 7) received an i.m. injection of 2 mL of sterile water, 2) intramuscular group (IM; n = 8) received an i.m. injection of 100 µg of GnRH (2 mL), and 3) intrauterine group (IU; n = 8) where 100 µg GnRH were infused in the uterus (2 mL). Blood samples (BS) for determination of serum P4 concentrations were collected at the time of each hormonal administration of Ovsynch and 7 d after GnRH2. Additionally, BS for determination of circulating LH concentrations were serially collected at Hours 0, 0.5, 1, 1.5, 2, 3, and 4. Ovarian structures (CL and follicles) were recorded by US at the time of each hormonal administration of Ovsynch, twice a day for 60 h after GnRH2 administration, and at 76±3 DIM.
Figure 5.2. Effect of route of GnRH administration on mean (± SEM) serum LH concentrations in lactating dairy cows. At the time of the second GnRH of Ovsynch (GnRH2), cows were blocked by parity and randomly assigned to receive 1 of 3 treatment groups: 1) i.m. injection (2 mL) of sterile water (CON, n=7), 2) 100 µg of GnRH given i.m. (IM, n=8), or 3) 100 µg GnRH infused in the uterus (IU, n=8). The GnRH2 administration was defined as the Hour 0 of the study. Blood samples for determination of circulating LH concentrations were serially collected at Hours 0, 0.5, 1, 1.5, 2, 3, and 4. Mean values with an asterisk differed (P < 0.05).
Table 5.1. Distribution (LSM ± SEM) of lactation number, DIM, milk yield (kg), and BCS across treatment groups.

*Distribution of Holstein lactating dairy cows with respect to lactation number (n), days in milk (DIM), milk yield (kg), and body condition scores (BCS) at the time of first GnRH (GnRH1) of Ovsynch.

‡Milk yield (kg) was recorded at the closest DHI test relative to GnRH1 of Ovsynch.

†Body condition score (BCS) was recorded using a 5-point scoring system (Ferguson et al., 1994) at GnRH1 of Ovsynch.

<table>
<thead>
<tr>
<th>Treatment groups *</th>
<th>CON (n=7)</th>
<th>IM (n=8)</th>
<th>IU (n=8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactation number (n)</td>
<td>2 ± 0.5</td>
<td>1.9 ± 0.5</td>
<td>2.4 ± 0.5</td>
<td>0.73</td>
</tr>
<tr>
<td>DIM (d)</td>
<td>62.2 ± 1</td>
<td>62 ± 0.9</td>
<td>59 ± 0.9</td>
<td>0.07</td>
</tr>
<tr>
<td>Milk yield (kg)‡</td>
<td>34.8 ± 3.8</td>
<td>31.4 ± 3.3</td>
<td>29.9 ± 3.3</td>
<td>0.61</td>
</tr>
<tr>
<td>BCS†</td>
<td>2.8 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 5.2. Distribution of progesterone concentrations (LSM ± SEM) across treatment groups at the time of the first GnRH (GnRH1), PGF$_{2\alpha}$ and the second GnRH (GnRH2) of Ovsynch.

*At the time of the second GnRH (GnRH2) of Ovsynch, cows were blocked by parity and randomly assigned to receive 1 of 3 treatment groups: 1) i.m. injection (2 mL) of sterile water (CON, n=7), 2) 100 µg of GnRH given i.m. (IM, n=8), or 3) 100 µg GnRH infused in the uterus (IU, n=8).

†Blood samples for determination of serum progesterone concentrations were collected at the time of each hormonal administration of Ovsynch and 7 d after GnRH2 administration for confirmation of ovulation.

‡Only cows that ovulated within 7 d after GnRH2 were included (CON = 7/7; IM = 8/8; IU = 7/8).

<table>
<thead>
<tr>
<th>Items</th>
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<th>IM (n=8)</th>
<th>IU (n=8)</th>
<th>P</th>
</tr>
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<tr>
<td>Concentrations of P4 (ng/mL)†</td>
<td>2.5 ± 0.6</td>
<td>3.2 ± 0.5</td>
<td>2.1 ± 0.6</td>
<td>0.36</td>
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<td>GnRH1</td>
<td>4.06 ± 0.9</td>
<td>3.8 ± 0.9</td>
<td>4.03 ± 0.9</td>
<td>0.98</td>
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<tr>
<td>PGF$_{2\alpha}$</td>
<td>0.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.96</td>
</tr>
<tr>
<td>7 d after GnRH2‡</td>
<td>1.5 ± 0.7</td>
<td>2.6 ± 0.5</td>
<td>1.3 ± 0.6</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 5.3. Distribution of diameters (mm; LSM ± SEM) of the largest follicle and CL at each injection of Ovsynch, proportion (n/n) of animals ovulating after administration of the second GnRH and mean time (h; LSM ± SEM) to ovulation among treatment groups.

* At the time of the second GnRH (GnRH2) of Ovsynch, cows were blocked by parity and randomly assigned to receive 1 of 3 treatment groups: 1) i.m. injection (2 mL) of sterile water (CON, n=7), 2) 100 µg of GnRH given i.m. (IM, n=8), or 3) 100 µg GnRH infused in the uterus (IU, n=8).

† Follicles and CL were measured by transrectal ultrasonography using a 5 MHz linear transducer. Mean diameters (LSM ± SEM) were calculated by averaging perpendicular measurements of the largest cross sectional diameters of each follicle and CL. For CL with a fluid filled cavity, the final diameter was calculated by subtracting the cavity diameter.

§ Proportion of cows that ovulated (disappearance of previously recorded dominant follicle) was assessed based on transrectal ultrasonography performed twice daily (12-h intervals) for 60 h after GnRH2 administration of Ovsynch.

‡ Mean time (h) to ovulation (LSM ± SEM) was calculated only for animals that ovulated within 60 h after GnRH2 administration.
Table 5.3.

<table>
<thead>
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</thead>
<tbody>
<tr>
<td></td>
<td>CON (n=7)</td>
</tr>
<tr>
<td>Diameter of the largest follicle (mm)†</td>
<td></td>
</tr>
<tr>
<td>GnRH1</td>
<td>13.6 ± 1.4</td>
</tr>
<tr>
<td>PGF$_{2a}$</td>
<td>13.7 ± 1.1</td>
</tr>
<tr>
<td>GnRH2</td>
<td>14.9 ± 1.2</td>
</tr>
<tr>
<td>Diameter of CL (mm)‡</td>
<td></td>
</tr>
<tr>
<td>GnRH1</td>
<td>17.1 ± 1.1</td>
</tr>
<tr>
<td>PGF$_{2a}$</td>
<td>20.5 ± 2.4</td>
</tr>
<tr>
<td>GnRH2</td>
<td>15.8 ± 2.2</td>
</tr>
<tr>
<td>Cows ovulating within 60 h (n/n)§</td>
<td>2/7</td>
</tr>
<tr>
<td>Mean time to ovulation (h)‡</td>
<td>52.7 ± 11.8</td>
</tr>
<tr>
<td>Range</td>
<td>40.8-64.5</td>
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CHAPTER 6

Effect of Intrauterine Administration of Gonadotropin Releasing Hormone on
Serum LH Concentrations in Lactating Dairy Cows
Abstract

The objective of the present study was to evaluate the release of LH following the i.u. administration of 200 μg of GnRH plus the addition of glycerol (7% v/v). In addition the follicular dynamics and ovulatory response were evaluated. Cows (N = 71) were presynchronized with 2 injections of PGF$_{2\alpha}$ given 14 d apart (starting at 26 ± 3 DIM) followed by Ovsynch (OV; GnRH-7 d-PGF$_{2\alpha}$-48 h-GnRH) 12 d later. Ovarian structures were recorded and a blood sample collected from each animal at the time of first GnRH injection of OV. Only cows presenting a CL ≥ 15 mm and at least one follicle ≥ 10 mm in diameter continued in the study (N = 33). Additionally, blood samples were collected and ovarian structures recorded at PGF$_{2\alpha}$ treatment of OV. At the time of the second GnRH of OV (h 0; GnRH2), cows were blocked by parity and randomly allocated to 1 of 4 treatment groups: 1) control group (CON; N = 8) received 2 mL of sterile water i.m.; 2) intramuscular group (IM; N = 8) received 100 μg of GnRH i.m.; 3) intrauterine group where cows were infused with 200 μg GnRH into the uterus (IU; N = 9); and 4) intrauterine plus glycerol group (IUG; N = 8) where cows were infused with 200 μg GnRH plus the addition of glycerol 7% v/v. Blood samples for determination of LH serum concentrations were collected at Hours -0.5, 0, 0.5, 1, 1.5, 2, 3, 4 and 5. Ultrasonography for ovulation determination (OD) of the largest follicle recorded at GnRH2 was performed 48 h after treatment administration and was confirmed 7 d later (OC). Serum circulating progesterone concentrations at h 0 did not differ (P > 0.05) among groups. Concentrations of LH were greater (P < 0.05) in IM cows than IU, IUG and CON cows at Hours 1, 1.5, 2, and 3. More cows ovulated by 48 h in IM (8/8), IU
(7/9) and IUG (5/8) cows compared to CON cows (2/8). Although, administration of GnRH through the i.u. route (IU and IUG) resulted in lower serum concentrations of LH than IM cows, the proportion of cows that ovulated by h 48 after treatment administration was similar among cows receiving i.m. or i.u. GnRH. These findings support previous evidences that the i.u. may be an alternative route of delivery for treatment with GnRH to synchronize ovulation in estrous synchronization programs.

**Introduction**

The neuropeptide gonadotropin releasing hormone (GnRH) is a decapeptide with a molecular weight of 1.183 Daltons that was first isolated from the ovine and porcine hypothalamus (Amoss et al., 1971; Matsuo et al., 1971). This hormone is synthesized by neurons located in the hypothalamus and is released into the hypophyseal portal circulation to stimulate the synthesis and release of LH and FSH by gonadotropes localized in the anterior pituitary gland (Conn et al., 1986; Fink, 1988; Page, 1988). Subsequently, LH and FSH enter the systemic circulation and regulate steroidogenesis and gametogenesis in the gonads. In the United States, the use of GnRH in cattle is labeled for the treatment of cystic ovaries. However, because of its central role in the bovine reproductive cycle, GnRH and its analogues are widely used to control the bovine follicular wave emergence and ovulation, prevention of early embryonic mortality, and induction of cyclicity during the early post-partum period (Peters, 2005; Schneider et al., 2006).
The most common routes to deliver reproductive hormones in dairy and beef cattle are intramuscular (i.m.), subcutaneous (s.c.), intravaginal and the oral (p.o.). The i.m. route is routinely used for hormonal treatments in estrous and ovulation synchronization of protocols in cattle. A recent report from the National Animal Health Monitoring System (NAHMS) on injection practices in dairy operations in the United States showed that dairy cows received an average of 14 injections (ranging from 5 to 24) per year, with reproductive hormones accounting for 27% of the total injections (USDA, 2009). Administration of GnRH and its analogues are usually performed intramuscularly; however, because of their low molecular weight, they can also be absorbed subcutaneously or across mucous membranes (Raehs et al., 1988; Donnez et al., 1989). Following i.m. GnRH administration, LH is released from the anterior pituitary with peak concentrations reached within 2 h and followed by ovulation of a dominant follicle 24 to 32 h later (Lucy and Stevenson, 1986; Chenault et al., 1990; Pursley et al., 1995). Alternative routes for GnRH administration have been evaluated in bovine and other species. When GnRH is administered subcutaneously rather than intravenously, higher doses are required to achieve similar effects; s.c. administration results in delayed release of LH (Conn et al., 1986). In addition, the magnitude of LH released is affected by the dose of decapeptide administered and the potency of the analogue used (Chenault et al., 1990; Souza et al., 2009; Dias et al., 2010; Giordano et al., 2012b). In this regard, great variation in the release of LH following the administration of higher GnRH concentrations has been reported. While most studies reported enhanced release of LH following increased GnRH doses (Colazo et al., 2009; Souza et al., 2009; Dias et al.,
2010; Giordano et al., 2012b), others reported no changes (Yamada et al., 2002; Rantala et al., 2009).

Troxel et al., (1983) studied the effects of i.m. GnRH injections and s.c. implants in suckled beef cows and observed similar LH surges patterns between treatments; however, the release of LH was delayed for cows that received GnRH subcutaneously delivered via a gelatin capsule. In Brown Swiss and Holstein heifers the intrauterine (i.u.) administration of 1 and 10 mg of LHRH were able to induce a release of LH similar to that resulting after the intravenous administration of similar doses of LHRH (Dermody et al., 1976a). Similarly, in mature dairy cows, the i.u. administration of 50 µg of a potent GnRH analogue (D-leu6-des-gly NH210 pro-ethylamide) increased serum concentrations of LH compared to 100 µg of synthetic native GnRH administered through the same route (Manns et al., 1980). Furthermore, increasing the concentrations of LHRH delivered through the i.u. route resulted in greater release of LH (Dermody et al., 1976a). More recently, the LH profile and synchrony of ovulation following the i.u. administration of gonadorelin were evaluated in lactating dairy cows (Bas et al., 2012). Results from this study showed that although the i.m. administration of GnRH resulted in greater concentration of LH, cows receiving GnRH through the i.u. route were able to ovulate (Bas et al., 2012). Collectively, these data suggest that the i.u. can be regarded as an alternative route of delivering GnRH in cattle. In addition to increasing the concentration of GnRH administered, another potential approach to increase the bioavailability of GnRH is through the use of penetration enhancers. Besides facilitating drug solubilization, penetration enhances facilitate permeation of drugs across tissues (Williams and Barry, 2004; Costantino et al., 2007). Solvents used for nasal drug delivery
include glycerol, ethanol, and propylene glycol (Costantino et al., 2007). In addition, lipids containing glycerol, such as lysophosphatidylglycerol, has been shown to promote the absorption of peptides administered intravaginally in rats (O’Hagan et al., 1992; Richardson et al., 1992).

Administration of i.u. GnRH administration could present further reproductive benefits if added to the semen extender. Receptors for GnRH have been identified in the bovine uterus and oviducts and a recent study reported that GnRH was able to induce contraction of the bovine uterine mioepithelial cells in vitro during the follicular phase (Singh et al., 2008; Giammarino et al., 2009). Furthermore, it has been proposed that GnRH may play a role in the process of fertilization. Addition of GnRH to in vitro fertilization media resulted in increased cleavage rate of bovine oocytes (Funston and Seidel, 1995). Moreover, in humans, GnRH increased the ability of sperm to bind to the oocyte zona pellucida (Morales, 1998). Similar findings were recently observed in the bovine, where exposure of male and female gametes to a GnRH analogue showed an increased binding of spermatozoa to zona pellucida (Perera, 2010).

In the present study we hypothesized that the administration of 200 μg of GnRH plus the addition of glycerol (7% v/v) would enhance absorption of GnRH through the uterine lining resulting in a release of LH similar to the i.m. administration. Therefore, the objective was to evaluate the release of LH following the i.u. administration of 200 μg of GnRH plus the addition of glycerol (7% v/v). Additionally, the follicular dynamics and ovulatory response were evaluated.
Materials and Methods

Animals, Facilities, and Feeding

This study was conducted from June 2011 to April 2012. Lactating Holstein dairy cows (N = 71), from two commercial dairy herds located in Ohio were enrolled in the present study. Cows were housed in free-stall barns and were milked three times daily at approximately 8 h intervals. Rolling milk yield for the herds ranged between 9,866 kg to 10,267 kg. Cows had free access to water and were fed a TMR diet formulated to meet or exceed dietary nutritional requirements for high-producing lactating dairy cows (NRC., 2001) twice daily, in the morning and afternoon. Individual cow records regarding DIM, milk yield (Kg) and lactation number were collected using commercial on-farm software program (Dairy Comp 305; Valley Agricultural Software, Tulare, CA). The Ohio State University Institutional Animal Care and Use Committee approved the procedures used in the present study.

Enrollment of Cows, Inclusion Criteria, and Treatment Groups

Each week a cohort of 10 to 24 cows were presynchronized with two injections of PGF$_{2\alpha}$ (25 mg; Lutalyse, 5 mg/mL of dinoprostone tromethamine sterile solution, Pfizer Animal Health, NY, USA) given 14 d apart at 32 ± 3 and 46 ± 3 DIM. Twelve days after the second PGF$_{2\alpha}$ administration (58 ± 3 DIM) the ovaries of all cows were screened by transrectal ultrasonography (Fig. 6.1) using a portable ultrasound (E.I. Medical Imaging, Loveland, CO; USA) equipped with a 7.5-MHz linear transducer. Only cows presenting a follicle ≥10 mm and CL ≥15 mm in diameter were included in the study (Bas et al.,
Selected animals were enrolled in an Ovsynch (OV) synchronization program and received the first GnRH (100 µg; Cystorelin, 50 µg/mL of gonadorelin diacetate tetrahydrate, Merial, Duluth, GA; USA) administration of OV (GnRH1). Seven days later (65 ± 3 DIM), an injection of PGF$_{2\alpha}$ was administered to all animals to induce regression of the CL (Fig. 6.1). Forty eight hours later, at the time of the second GnRH administration of OV (GnRH2; study Hour 0), cows were blocked by parity and randomly assigned to 1 of 4 treatment groups (Fig. 6.1): (1) control group (CON; N = 8) cows received an i.m. injection of 2 mL of sterile water (Vedco Inc., Saint Joseph, MO, USA); (2) cows in the intramuscular group (IM; N = 8) were administered an i.m. injection of 100 µg of GnRH; (3) intrauterine group (IU; N = 9) cows were infused with 200 µg GnRH in the uterus; and (4) cow in the intrauterine plus glycerol group (IUG; N = 8) were infused with 200 µg GnRH plus the addition of glycerol 7% v/v (Sigma-Aldrich, St. Louis, MO, USA). Body condition (BCS) was scored in all study cows using a 5-point scoring system at the time of GnRH1 administration (Ferguson et al., 1994). The i.m. injections were administered using 3.75 cm x 18 gauge needles in the semitendinosus muscle and intrauterine treatments were performed using single-use individually wrapped infusion pipettes (Continental Plastic, Delavan, WI, USA).

**Blood Sample Collection and Hormone Assays**

Blood samples were collected for determination of serum progesterone (P4) concentrations via puncture of the coccygeal blood vessels into 8.5 mL evacuated tubes (Vacutainer; Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Samples were collected at the time of each hormonal treatment of OV (GnRH1, PGF$_{2\alpha}$, and GnRH2)
and 7 d after GnRH2. Additionally, for determination of serum LH circulating concentrations, blood samples were serially collected from coccygeal blood vessels at Hours -0.5, 0, 0.5, 1, 1.5, 2, 3, 4 and 5 (Fig. 6.1). Immediately after collection, blood samples were refrigerated and centrifuged at 2,785 x g for 20 min for serum separation within 2 h of collection. Serum samples were stored at –20 ºC until assayed for P4 and LH.

Concentrations of P4 were assessed using a modified commercially available RIA kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) as described elsewhere (Burke et al., 2003). Samples were run in duplicates in three assays. The intra- and inter-assay CV were 1.9% and 10.8%, respectively and the sensitivity of the assay was 0.04ng/mL. Concentrations of LH in serum samples were determined in duplicates in four assays using a double-antibody RIA as previously described (Anderson et al., 1996). The intra- and inter-assay CV were 1.1% and 11.6%, respectively and the sensitivity of the assay was 0.23ng/mL.

Assessment of Ovarian Structures and Measurements

Ovarian structures (CL and follicles) were examined by transrectal ultrasonography. Measurements of all follicles ≥ 5 mm and CL were recorded for all cows included in the study at each injection of the Ovsynch protocol (GnRH1, PGF2α, and GnRH2). Follicular and CL diameters were estimated using on-screen background gridlines comprising squares with 10-mm sides in the portable scanner (Giordano et al., 2012a). If a CL presented a fluid filled cavity, the final diameter was calculated by subtracting the cavity diameter from the CL diameter (Martins et al., 2011; Bas et al.,
Forty eight h after GnRH2, cows were screened through transrectal ultrasonography for ovulation determination (OD; Fig. 6.1). Ovulation was defined as disappearance of the largest follicle (ovulatory follicle) previously recorded and was confirmed 7 d later (OC) by visualization of a CL on the same ovary that previously contained the dominant follicle (Martins et al., 2011; Bas et al., 2012).

Statistical Analyses

Data were arranged in a randomized complete block design. A test for normality of residuals was performed to continuous variables using the UNIVARIATE procedure of SAS (SAS, 2009) prior to statistical analyses. To ensure normality distribution, data transformation (log base 10) was performed if a value < 0.9 was observed in the Shapiro-Wilk test (W > 0.9) and if the studentized residual plot for each variable did not fulfilled the assumption of normality. The distribution of cows with respect to parity, DIM, milk yield (Kg) and BCS at the time of GnRH1 across treatment groups was computed using the MIXED procedure of SAS. Farm was used as a RANDOM effect. Least squares mean ± SEM values were reported. A P < 0.05 value was considered statistically significant. Concentrations of LH and area under the curve (AUC) for LH in addition to P4 concentrations and ovarian structures (Follicles and CL) diameter at each sampling point during the experimental procedure (Fig. 6.1) were analyzed using the MIXED procedure of SAS. Removal of variables from the model was performed one at a time based on the Wald statistic backward selection criterion (P > 0.15). Analysis of concentrations of LH and P4 over time included the REPEATED statement with cows nested within treatments specified in the SUBJECT option. The variables contained in the final model for LH
concentration during the intensive bleeding period included the effect of treatment, hour of sample collection and their interaction. Cow within treatment group was treated as a RANDOM effect and the spatial power covariance structure model was used to adjust for the non-uniform interval of blood collection (every 30 min during the first 2 h of sampling and then every h until the end of the sampling period). The AUC for LH was calculated by using the trapezoidal rule. The final model for AUC included the effect of treatment. A model that included the effect of treatment was used to compare differences in P4 concentrations at each sampling point during the experimental procedure (Fig. 1). Finally, a model including the effects of treatment was used for analysis of diameter of ovarian structures (Follicles and CL). A P < 0.05 value was considered statistically significant.

Results

A total of 33 animals (16 primiparous and 17 multiparous) that met the inclusion criteria (CL ≥15 mm and Follicle ≥10 mm) at GnRH1 were enrolled in the present study. Distribution of animals with respect to lactation number, DIM, milk yield, and BCS at GnRH1 did not differ (P > 0.05) among treatment groups (Table 6.1).

Circulating Hormone Concentrations

Luteinizing Hormone
Serum concentrations of LH for each treatment group (mean ± SE) are illustrated in figure 6.2. Serum concentrations of LH throughout the sampling period were affected by treatment (P < 0.05), hour (P < 0.05) and their interaction (P < 0.05). In the IM group, mean LH concentrations increased (P < 0.05) by Hour 0.5, peaked 1.5 h later (Hour 2; 20.62 ± 3.25 ng/mL), and decreased (P = 0.037) from maximal concentrations thereafter (Fig. 6.2). Concentrations of LH within the IU, IUG and CON did not change (P > 0.05) over time. However, a slight numerical rise in serum LH concentrations was observed for IU by Hour 1 (5.27 ± 2.07 ng/mL) post treatment administration (Fig. 6.2), while cows in IUG evidenced the greatest circulating concentrations of LH at Hour 1.5 (3.77 ± 1.16 ng/mL). In CON no apparent increase in circulating LH concentrations was observed throughout the sampling period (Fig. 6.2).

Among treatment groups, no difference in LH concentrations was observed at Hours –0.5, 0, and 5 (P > 0.05; Fig. 6.2). At Hours 0.5 and 4 serum LH concentrations were greater (P < 0.05) in IM than CON, but no difference (P > 0.05) between IU and IUG concentrations with respect to IM and CON was observed (Fig. 6.2). From Hour 1 through 3, concentrations of LH were greater (P > 0.05) in the IM group when compared to IU, IUG and CON, but no differences (P < 0.05) among the later groups was observed (Fig. 6.2). The AUC for LH differed (P < 0.05) among treatment groups. For cows in the IM group LH AUC was greater (P < 0.05; 3335.03 ± 807.11) when compared with IU, IUG and CON. In addition, LH AUC was greater (P < 0.05) in IU (1019.23 ± 519.38) and IUG (902.06 ± 427.87) treatment groups when compared to CON (594 ± 209.57).

**Progesterone**

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Average P4 concentrations at GnRH1 and PGF$_{2\alpha}$ did not differ (P > 0.05) among treatment groups (Table 6.2). As anticipated, after the administration of the luteolytic dose of PGF$_{2\alpha}$, P4 concentrations declined to less than 1 ng/mL in all treatment groups with no difference in P4 levels observed at GnRH2 (Table 6.2). Seven days after treatment administration, at the time of OC, P4 concentrations did not differ among treatments (Table 6.2).

**Measurement of Ovarian Structures**

Follicle diameter did not differ (P < 0.05) among treatment groups at GnRH1, PGF$_{2\alpha}$, GnRH2, OD and OC (Table 6.3). Similarly, no differences (P < 0.05) in the diameter of CL were observed at GnRH1, PGF$_{2\alpha}$, OD and OC among treatment groups (Table 6.3). However, at GnRH2 the diameter of the largest recorded CL was smaller in IU when compared to IUG and CON but not IM treatment group. All animals (8/8) in the IM group ovulated within 48 h of treatment administration (Table 6.3). Ovulation in cows in the IU and IUG groups was detected in 6 out of 9 and 4 out of 8 cows respectively (Table 6.3). On the other hand, within 48 h of treatment administration, ovulation was only detected in 2 of 8 cows in the CON group (Table 6.3). Seven days after GnRH2, in addition to the cows that ovulated by 48 h after treatment, ovulation was confirmed in 3 additional cows in the CON group and in 2 and 3 additional cows in the IU and IUG groups respectively (Table 6.3).

**Discussion**
The present experiment was designed to evaluate if the administration of 200 μg of GnRH delivered through the i.u. route plus the addition of glycerol (7% v/v) would result in the release of LH comparable to that observed following i.m. administration of 100 μg of GnRH. In addition, the follicular dynamics and the ovulatory response were evaluated. Results from this study revealed that the i.m. administration of GnRH resulted in greater circulating LH concentrations when compared to the IU, IUG and CON groups; in addition, ovulation within 48 h of treatment administration was observed in all cows (8/8) in the IM group. Interestingly, despite the lower concentration of LH observed in cows infused with GnRH into the uterus, ovulation was detected in 7/9 cows in the IU group and in 5/8 cows in the IUG group. Thus, based on the findings of the present experiment, the i.u. administration of 200 μg of GnRH plus the addition of glycerol (7% v/v) did not result in the LH release similar to that observed following the i.m. administration of GnRH.

Previous studies evaluated the LH profile following the i.u. administration of GnRH or its analogues. In Brown Swiss and Holstein heifers the i.u. delivery of higher than labeled doses (1 and 10 mg) of LHRH induced the release of LH comparable to the one observed after the intravenous administration (Dermody et al., 1976a). Similarly, in mature dairy cows, treatment with 50 μg of a potent GnRH analogue (D-leu⁶-des-gly NH₂¹⁰ pro-ethylamide⁹) through the i.u. route resulted in greater circulating concentrations of LH when compared to 100 μg of synthetic native GnRH (Manns et al., 1980). More recently the release of LH following the i.u. and i.m. administration of the prescribed dose (100 μg) of gonadorelin was evaluated in lactating dairy cows (Bas et al., 2012). Results from this study showed that cows receiving i.m. GnRH had greater LH
concentration when compared to cows receiving a similar dose of GnRH through i.u. route (Bas et al., 2012). Together, these data suggest that to achieve circulating concentrations of LH comparable to the ones observed after i.m. treatment, greater doses or more potent GnRH analogues are required.

The administration of greater doses of GnRH or the use of more potent analogues can be regarded as potential approaches to enhance pituitary release of LH in dairy cattle. Chenault et al., (Chenault et al., 1990) evaluated the release of LH following the i.m. administration of varying doses of gonadorelin, fertilin acetate and buserelin and observed that fertilin acetate and buserelin were 10 and 50 times more potent than GnRH respectively as measured by the amounts of LH and FSH released. Another study compared plasma LH concentrations in ovariectomized Holstein cows after the i.m. administration of 50, 100 and 250 μg of gonadorelin, and observed that cows receiving the high GnRH dose had greater mean plasma LH concentration than cows receiving the lesser doses (Colazo et al., 2009). The evaluation of the release of LH and ovulatory response following the administration of 50 or 100 μg of four commercially available gonadorelin products in lactating dairy cows showed greater LH levels in cows that received the higher dose of gonadorelin irrespectively of the product brand (Souza et al., 2009). In agreement, comparison of LH release following the administration of 100 and 200 μg of GnRH in high and low P4 environments, showed that higher concentrations of GnRH resulted in enhanced pituitary LH release (Giordano et al., 2012b). In addition, the use of diverse vehicles may represent an alternative approach to enhance the absorption and bioavailability of drugs. In this regard, glycerol has demonstrated to enhance absorption of peptides drugs through different body surfaces. Therefore, to achieve a
GnRH-induced release of LH comparable to the one observed following the i.m. administration the dose of GnRH was increased from 100 to 200 µg and glycerol was added to enhance absorption of the decapeptide through the uterine lining.

In the present study, following the i.m. administration of GnRH, LH started to increase within Hour 0.5 to reach peak concentrations (20.6 ng/mL) at Hour 2 and decrease thereafter. Similar responses were observed in previous studies where LH reached maximum concentrations within 2 h of treatment with GnRH, and gradually decreased to basal levels 4 h post treatment (Chenault et al., 1990; McDougall et al., 1995; Martínez et al., 2003; Souza et al., 2009). Increasing the concentration of gonadorelin with the addition of glycerol to enhance GnRH absorption with the subsequent increase GnRH-induced pituitary release of LH did not result in a surge-like pattern as the one observed following the i.m. treatment. While circulating concentrations of LH in IU reached maximum levels (5.27 ng/mL) 1 h post-treatment, peak concentrations of LH (3.77 ng/mL) in the IUG treatment group were observed 1.5 h after GnRH administration. As anticipated, throughout the sampling period no apparent increase in systemic LH concentrations was observed in cows in the CON group which had the lowest LH levels. These results are in partial agreement with a recent study where the i.m. administration of 100 µg of gonadorelin resulted in the typical pattern of LH release, while the same dose administered through the i.u. route resulted in a slight increase in LH concentrations with maximum concentrations observed 3 h after treatment (Bas et al., 2012). Similar results were observed by Manns et al., (Manns et al., 1980) in mature cows following the i.u. administration of 100 µg of native GnRH dissolved in semen extender or saline. Throughout the blood collection period the LH AUC was
greater in the IU, IUG treatment groups when compared to CON. Furthermore, LH AUC in IU and IUG almost doubled the AUC observed in CON.

It was speculated that the reduction in circulating concentrations of LH may be the result of the reduced absorption of the GnRH through the uterine wall (Bas et al., 2012). Thus, to overcome this constraint, the dose of GnRH administered was increased and glycerol was added. However, circulating levels of LH were lower in comparison to the ones observed after i.m. administration suggesting that absorption of GnRH was not improved. The incomplete trans-endometrial absorption of GnRH may be due to a combination of poor membrane permeability and metabolism by local uterine enzymes at the absorption site (Golomb et al., 1995). Additional, potential explanations for the decreased absorption of GnRH may include leakage of the drug through the cervix, instability of the peptide at the uterine lumen and enzymatic degradation during passage through the uterine wall (Hoffman et al., 1995). The potential capability of the reproductive tract mucosa for absorption of GnRH warrants further investigation.

Serum P4 concentrations did not differ among treatment groups at GnRH1, PGF$_{2\alpha}$ or GnRH2. Presence of a functional CL at GnRH1 (inclusion criteria) was confirmed by elevated circulating P4 concentrations (> 1 ng/mL). Serum concentrations of P4 remained elevated in all groups at the time of PGF$_{2\alpha}$, and decreased (< 0.5 ng/mL) in all treatment groups in response to the luteolytic dose of PGF$_{2\alpha}$. During the periovulatory period, the increasing estradiol concentrations stimulate the hypothalamic release of GnRH and the synthesis and insertion of GnRH-R into the pituitary, thus increasing the sensitivity of the anterior pituitary to GnRH (Schoenemann et al., 1985; Moenter et al., 1991). However, elevated P4 concentrations have been negatively associated with mRNA concentrations.
of GnRH-R and number of receptors for GnRH at a pituitary level; thus decreasing the amount of LH released (Laws et al., 1990; Turzillo et al., 1998). Previous studies suggested that high P4 concentrations negatively affected the magnitude of the GnRH induced release of LH in beef cows and heifers (Colazo et al., 2008; Dias et al., 2010). More recently, it was observed that P4 concentrations at GnRH treatment affected the GnRH-induced release of LH in lactating dairy cows (Giordano et al., 2012b). In the present study, regression of the CL was observed in all cows in response to the luteolytic dose of PGF2α by the time of treatment administration (GnRH2) as observed by the reduced levels of P4 in all cows (< 0.42 ng/mL).

Follicular diameter did not differ among treatment groups throughout the experiment. Similarly, no differences in CL diameter were observed at GnRH1 and PGF2α. However, at GnRH2, the diameter of the largest recorded CL was smaller in IU when compared to IUG and CON but not IM treatment group. Nevertheless, evaluation of functional regression of the CL as determined by P4 concentrations did not differ among treatment groups. At the time of the second GnRH injection of OV cows are expected to have at least one dominant follicle that expresses LH receptors in the granulosa cells and that is capable to ovulate in response to the GnRH-induced release of LH (Perera, 2010). Following the exogenous i.m. administration of GnRH, the preovulatory LH surge occurs within 2 h and ovulation of the dominant follicle is expected to occur within 24-32 h later, with reported ovulatory responses ranging from 75 to 92% (Pursley et al., 1995; Galvão and Santos, 2010). In the present study, at the time of treatment administration (GnRH2), all cows had at least one dominant follicle (≥ 8.5 mm). Ovulation was detected in all animals (8/8) in the IM group within 48 h of
treatment administration (OC). Interestingly, despite the low concentrations of LH following the i.u. administration of GnRH, ovulation was observed in 6/9 and 4/8 cows in the IU and IUG groups respectively. These results are in partial agreement with an earlier study that evaluated the ovulatory response following i.u. treatment with 100 μg of gonadorelin in lactating dairy cows (Bas et al., 2012). However, in that study ultrasonography of the ovaries for detection of ovulation was continued for 60 h post GnRH administration. Furthermore, similar ovulatory responses were observed in a recent study following the i.m. administration of GnRH in a high P4 environment that resulted in a low release of LH (Giordano et al., 2012b). As anticipated, the ovulatory response was lower for cows in the CON group were ovulation was detected in only 2 of 8 animals. In lactating dairy Holstein cows, the mean interval from treatment with PGF$_{2α}$ to ovulation ranged from 87 to 123 h (Martins et al., 2011); thus, ovulation may have occurred in response to the PGF$_{2α}$ dose administered 48 hours prior to treatment administration. At the time of OC, 7 days after treatment administration, in addition to the cows where ovulation was initially diagnosed, 4, 2 and 3 extra cows in the CON, IU and IUG treatments groups respectively ovulated. Whereas ovulation in the IM group was confirmed in all cows.

In conclusion, results from the present study revealed that increasing the dose of GnRH plus the addition of glycerol to enhance endometrial absorption of GnRH and optimize the LH release from the anterior pituitary was not successful in lactating dairy cows. Surprisingly, despite the lower concentrations of LH observed following the i.u. treatment with GnRH as opposed to the i.m. administration of the prescribed dose of the hormone, ovulation within 48 h of treatment administration was observed in 6/9 and 4/8
cows in the IU and IUG treatment groups respectively. Thus, further investigation administering various GnRH doses or more potent GnRH analogues in the uterus of cows are required to optimize the release of LH and ovulatory response in cattle following i.u. treatment with GnRH. The administration of GnRH at AI via semen extender could provide substantial practical benefits for cattle. The potential of addition of GnRH to semen and its impact on fertility in lactating Holstein dairy cows or heifers warrants future investigation if an efficacious method of IU GnRH delivery can be developed.
Figure 6.1. Schematic diagram of the experimental design. Cows were presynchronized with two injections of PGF$_{2\alpha}$ given 14 d apart. Twelve days after the second PGF$_{2\alpha}$ the ovaries of all cows were screened by ultrasonography (US). Only cows presenting at least one follicle $\geq$10 mm and CL $\geq$15 mm in diameter continued in the study. Animals were enrolled in an ovsynch (OV) protocol and received the first GnRH (GnRH1) treatment followed, 7 d later by an injection of PGF$_{2\alpha}$. Forty eight hours later, at the time of the second GnRH of OV (GnRH2; study Hour 0), cows were blocked by parity and randomly allocated to receive an i.m. injection (2 mL) of sterile water (CON), 100 $\mu$g of GnRH i.m. (IM), 200 $\mu$g GnRH infused in the uterus (IU) or an i.u. infusion of 200 $\mu$g GnRH plus the addition of glycerol 7% v/v (IUG). Blood samples (BS) were collected at the time of each hormonal treatment of OV and 7 d after treatment administration. Additionally, BS were collected from coccygeal blood vessels at Hours -0.5, 0, 0.5, 1, 1.5, 2, 3, 4 and 5. Ovarian structures were examined US at all injections of the OV (GnRH1, PGF$_{2\alpha}$, and GnRH2). Ovulation was determined (OD) 48 h after GnRH2 and was confirmed (OC) 5 d later by transrectal US.
Figure 6.1.

GnRH2

CON
(n = 8)

IM
(n = 8)

IU2
(n = 9)

IU2G
(n = 8)

OC
US
BS

PGF$_{2\alpha}$

US
BS

PGF$_{2\alpha}$

US
BS

PGF$_{2\alpha}$

US
BS

PGF$_{2\alpha}$

US
BS

7d

48 h

BS at -0.5, 0, 0.5, 1, 0.5, 1, 1.5, 2, 3, 4 & 5

US at 0 h & at 48 h (OD)

32 ± 3
46 ± 3
58 ± 3
65 ± 3
74 ± 3

67 ± 3
Table 6.1. Distribution (LSM ± SEM) of lactation number, DIM, milk yield (kg) and BCS across treatments groups at the time of the first GnRH (GnRH1) administration of Ovsynch.

<table>
<thead>
<tr>
<th>Treatment Groups*</th>
<th>CON (N = 8)</th>
<th>IM (N = 8)</th>
<th>IU (N = 9)</th>
<th>IUG (N = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactation number</td>
<td>1.7 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>0.56</td>
</tr>
<tr>
<td>DIM</td>
<td>69.2 ± 6.8</td>
<td>66.0 ± 6.8</td>
<td>69.4 ± 6.9</td>
<td>67.9 ± 6.8</td>
<td>0.10</td>
</tr>
<tr>
<td>Milk yield(kg)a</td>
<td>31.5 ± 5.8</td>
<td>37.9 ± 5.8</td>
<td>39.9 ± 6.4</td>
<td>38.4 ± 5.9</td>
<td>0.48</td>
</tr>
<tr>
<td>BCSb</td>
<td>2.8 ± 1.0</td>
<td>2.9 ± 1.0</td>
<td>2.9 ± 1.0</td>
<td>3 ± 1.0</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*At the time of the second GnRH (GnRH2) of Ovsynch (OV), lactating Holstein cows were blocked by parity and were randomly assigned to receive an i.m. injection (2 mL) of sterile water (CON), 100 µg of GnRH i.m. (IM), a 200 µg 6.1GnRH infused in the uterus (IU) or 200 µg GnRH plus the addition of glycerol 7% v/v (IUG).

Milk yield (Kg) recorded at the closest dairy herd improvement test relative to GnRH1

Body condition score at the time of GnRH1 administration was recorded using a 5-point scoring system as previously described (Ferguson et al., 1994)
Figure 6.2. Effect intramuscular (IM), sterile water (CON) and intrauterine (IU) treatments on mean (± SEM) serum LH concentrations relative to treatment administration (h 0) in lactating dairy cows.
<table>
<thead>
<tr>
<th>Treatment Groups*</th>
<th>CON (N = 8)</th>
<th>IM (N = 8)</th>
<th>IU (N = 9)</th>
<th>IUG (N = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P4 Concentrations</strong> (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GnRH1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.2 ± 0.7</td>
<td>3.6 ± 0.6</td>
<td>3.4 ± 0.5</td>
<td>3.8 ± 0.7</td>
<td>0.91</td>
</tr>
<tr>
<td>PGF&lt;sub&gt;2α&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.7 ± 0.8</td>
<td>5 ± 0.8</td>
<td>3.3 ± 0.8</td>
<td>3.8 ± 0.8</td>
<td>0.15</td>
</tr>
<tr>
<td>GnRH2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.3 ± 0.05</td>
<td>0.2 ± 0.06</td>
<td>0.2 ± 0.06</td>
<td>0.3 ± 0.06</td>
<td>0.15</td>
</tr>
<tr>
<td>OC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.8 ± 0.3</td>
<td>2.3 ± 0.4</td>
<td>2.3 ± 0.5</td>
<td>1.9 ± 0.4</td>
<td>0.77</td>
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Table 6.2. Distribution of progesterone concentrations (mean ± SEM) across treatments at the time of the first GnRH (GnRH1), the PGF<sub>2α</sub> (PGF) and the second GnRH (GnRH2) of Ovsynch (OV).

*At the time of the second GnRH (GnRH2) of Ovsynch (OV), cows were blocked by parity and randomly assigned to receive an i.m. injection (2 mL) of sterile water (CON), 100 µg of GnRH i.m. (IM) or a 100 µg GnRH infused in the uterus (IU).

<sup>a</sup>GnRH1 = first GnRH of OV.

<sup>b</sup>PGF<sub>2α</sub> = prostaglandin of OV.

<sup>c</sup>GnRH2 = treatment administration (Hour 0 of study).

<sup>d</sup>OC = ovulation confirmation.
Table 6.3. Leastsquare mean (± SEM) diameter (mm) of the largest follicle and CL at the time of the first GnRH (GnRH1) and PGF$_{2\alpha}$ of Ovsynch and at treatment administration (GnRH2) across treatments, and proportion (N/N) of animals ovulating within 48 h and 7 days after treatment administration among treatment groups.

*At the time of treatment administration (GnRH2), cows were blocked by parity and randomly assigned to receive an i.m. injection (2 mL) of sterile water (CON), 100 µg of GnRH i.m. (IM), 200 µg GnRH infused in the uterus (IU) or an i.u. infusion of 200 µg GnRH plus the addition of glycerol 7% v/v (IUG).

† Follicles and CL were measured by transrectal ultrasonography. Diameters were calculated by averaging perpendicular measurements of the largest cross sectional diameters of each follicle and CL. For CL presenting a fluid filled cavity, the final diameter was calculated by subtracting the cavity diameter. Largest follicles and CL diameters are reported.

*GnRH1 = first GnRH of OV.

bPGF$_{2\alpha}$ = prostaglandin of OV.

cGnRH2 = treatment administration (Hour 0 of study).

dThe Proportion of cows that ovulated within 48 h of treatment administration was assessed by US. Ovulation was defined as disappearance of the largest follicle (ovulatory follicle) previously recorded (OD) and was confirmed 7 d (OC) later by visualization of a CL on the same ovary that previously contained the dominant follicle

a,b Different superscript letter within a row differ significantly at $P < 0.05$. 

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Table 6.3.

<table>
<thead>
<tr>
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<th>Treatment Groups*</th>
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<tr>
<td></td>
<td>CON</td>
<td>IM</td>
<td>IU</td>
<td>IUG</td>
<td>P</td>
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<tr>
<td></td>
<td>(N = 8)</td>
<td>(N = 8)</td>
<td>(N = 9)</td>
<td>(N = 8)</td>
<td></td>
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<tr>
<td>Follicle Diameter†</td>
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<tr>
<td>GnRH1a</td>
<td>17.1 ± 1.0</td>
<td>16.3 ± 1.1</td>
<td>14.1 ± 1.1</td>
<td>17 ± 1.0</td>
<td>0.19</td>
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<tr>
<td>PGFb</td>
<td>12.7 ± 0.9</td>
<td>12.2 ± 0.9</td>
<td>14.1 ± 0.9</td>
<td>12.9 ± 1.0</td>
<td>0.41</td>
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<tr>
<td>GnRH2c</td>
<td>14.9 ± 0.9</td>
<td>15.5 ± 0.9</td>
<td>15.1 ± 0.9</td>
<td>17.1 ± 1.0</td>
<td>0.41</td>
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<tr>
<td>CL Diameter†</td>
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<tr>
<td>GnRH13</td>
<td>24.9 ± 1.5</td>
<td>25.6 ± 1.4</td>
<td>23.2 ± 1.3</td>
<td>23.3 ± 1.5</td>
<td>0.56</td>
</tr>
<tr>
<td>PGF2α4</td>
<td>25 ± 1.4</td>
<td>25.3 ± 1.5</td>
<td>24.4 ± 1.3</td>
<td>25 ± 1.5</td>
<td>0.98</td>
</tr>
<tr>
<td>GnRH25</td>
<td>23.1 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.9 ± 1.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>16 ± 1.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.4 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.01</td>
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<td>Cows (N/N) ovulating</td>
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<td>at OD&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2/8</td>
<td>8/8</td>
<td>6/9</td>
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<tr>
<td>Cows (N/N) ovulating</td>
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<tr>
<td>at OC&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6/8</td>
<td>8/8</td>
<td>9/9</td>
<td>8/8</td>
<td>-</td>
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</table>

<sup>a</sup> denotes significance at P < 0.05.
CHAPTER 7

General Discussion

The overall goal of this dissertation was to develop strategies to improve reproductive performance in dairy cattle. The specific aims were: 1) To evaluate the effectiveness of using AI cover sheaths at the time of AI on fertility of lactating dairy cows, and 2) To evaluate the release of LH and ovulatory response following the intrauterine administration of GnRH in lactating dairy cows.

According to the NAHMS, AI is the most common practice used to breed dairy cows in the United States with approximately 78% of the services performed through AI (USDA, 2009). Placement of semen into the uterus of a cow in a timely manner and using an adequate technique should be the final step for successful reproductive outcomes in Timed-AI or estrous detection programs. However, this culminating step is often time overlooked. The first aim of this dissertation was to improve the method of AI through the use of PS and for this purpose two experiments were designed (Chapters 3 and 4). We hypothesized that using PS at the time of AI would reduce contamination of the AIC and improve PAI with the consequent improved economic return. A significant reduction in the number of bacteria isolated from the AIC was observed when inseminations were performed using PS. In addition, protection of the AIC with disposable PS not only
minimized contamination of the AIC, but also reduced the density of bacterial growth. *Escherichia coli*, a recognized uterine pathogen, was the most prevalent bacteria isolated from the AIC. Fertility data revealed that PAI were greater for those animals where the insemination catheters were protected with PS at the time of AI compared to control animals. Interestingly, PAI were only improved for second and greater services with no apparent benefit observed for first post partum inseminations. The economic analysis of using PS revealed that protecting the AIC at the time of AI resulted in an additional $4 cow/yr (or $4,000/yr for a 1,000-cow herd) for each point increase in CR. These findings showed that the use of PS increased profitability of dairy herds at a minimal cost by improving PAI for lactating dairy cow without the need of additional handling of animals or timing of hormonal injections.

During AI, in addition to semen, bacteria from the perineal region and from the vagina can be introduced into the uterine lumen leading to a post-breeding inflammatory response with the consequent reduction in fertility (Maischberger et al., 2008). Presence of pathogens within the uterus will trigger the production of proinflammatory cytokines and chemokines that in the bovine will lead to the influx of PMN into the uterus (Zerbe et al., 2003). A higher proportion of PMN immediately before and 4 h post-AI was associated with a reduction in fertility (Kaufmann et al., 2009). Furthermore, E2 is associated with the uterine immune function and it has shown to regulate leukocyte migration into the cervix, the uterus, and the extracellular matrix of the reproductive tract (Ramos et al., 2000; Stygar et al., 2006); thus, reduced level of estradiol at the time of AI may lead to poor immunological response with a reduce influx of PMN cells into the uterus (Lacetera et al., 2004; van Knegsel et al., 2007). It is tempting to speculate that the
use of PS reduced contamination of the AIC with the subsequent reduction of pathogens introduced into the uterine lumen. Recognized uterine pathogens like *E. coli* and *A. pyogones* not only affect ovarian function, but also have the potential to affect the uterine environment creating an inhospitable environment for an embryo to survive. Furthermore, animals that conceived to the first post-partum insemination would have become pregnant regardless of the use of PS. Most likely these cows represent a population of healthy animals that underwent a transition period without any health problems. On the other hand, lactating dairy cows that failed to conceive to the first post-partum inseminations, may have suffered conditions such as difficult calving, metabolic disorders (e.g., hypocalcemia, ketosis), uterine diseases (e.g., metritis, endometritis) and mastitis which are known risk factors for poor reproductive performance in dairy cows. Animals affected by any of these health events may develop an immunological disadvantage with respect to healthy animals; thus benefiting from the use of PS at the time of AI.

Further research is needed to understand the underlying mechanisms of improved reproductive performance when using disposable PS at the time of AI. Evaluation of steroid hormone profiles (i.e., estradiol and progesterone) around the time of insemination may provide useful information regarding the potential role of these hormones in the development (or prevention) of post breeding inflammatory reactions and the potential benefit of using disposable PS. It would also be interesting to evaluate the presence of PMN cells immediately before and after AI. This approach may provide useful information to respond if by using PS the development or severity of a post-AI inflammatory process is avoided with the subsequent improvement in fertility of dairy
cows. In addition, the use of PS should be evaluated under different managerial conditions with different AI technicians.

The second specific aim was to evaluate the release of LH and ovulatory response following the i.u. administration of GnRH in lactating dairy cows. The hypothesis was that the release of LH and ovulatory response would be similar in cows receiving the i.u. dose of the GnRH analogue when compared to cows receiving GnRH by the i.m. route. The final goal of this specific aim was to incorporate GnRH into the semen extender; thus GnRH would be delivered at the time of AI. Two experiments (Chapter 5 and 6) were designed to test the proposed hypothesis. The first experiment (Chapter 5) aimed to evaluate the preovulatory serum LH concentrations and synchronization of ovulation following i.m. or i.u. administration of the second GnRH of ovsynch in lactating dairy cows. Results from this study showed that despite the low concentrations of LH in the i.u. group (when compared to the i.m. group), cows were able to ovulate. Ovulation was observed in all (8/8) cows receiving GnRH through the i.m. route and in 7 out of 8 cows infused with GnRH into the uterus within 42.6 h and 51.8 h after GnRH administration respectively. From this study, it was concluded that the lack of an increase in circulating LH concentrations may have been due to a reduced absorption of the decapeptide through the uterine wall. This reduction in GnRH absorption may have been due to the presence of proteolytic enzymes within the uterine lumen and during passage through the uterine wall that directly affects the bioavailability of the drug following i.u. administration (Hoffman et al., 1995). In addition, it was speculated that by increasing the dose of GnRH delivered through the i.u. route, release of LH would be improved.
Based on these results, a second experiment (Chapter 6) was designed in an attempt to enhance GnRH absorption after i.u. delivery. For this purpose, the dose of GnRH was doubled (from 100 to 200 µg) and glycerol (7% v/v) was added to GnRH. Release of LH was not improved by increasing the GnRH dose infused or by adding glycerol. Interestingly, despite the lower concentration of LH, cows receiving GnRH through the i.u. route were able to ovulate as observed in our previous experiment.

Collectively, these data suggest that the i.u. can be regarded as an alternative route of delivering GnRH in cattle. However, strategies aimed to increase the amounts of LH release following administration of GnRH through the i.u. route need to be evaluated. The administration of more potent analogues can represent a potential approach. Buserelin is 50 times more potent than GnRH and its administration would likely result in LH profiles comparables to those observed following the i.m. administration of GnRH. Increasing the amount of LH released after i.u. GnRH delivery may lead to improved ovulatory response and timing to ovulation. Since our final goal is to add GnRH into semen extender, it would be interesting to determine if the addition of GnRH has any detrimental effects on semen characteristics (e.g., motility, viability, acrosome integrity). Delivery of GnRH with the semen extender would provide an alternative way to reduce the number of hormonal injections during synchronization programs. Most timed AI protocols resulted in satisfactory CR; however, these protocols require additional handling of animals and timely use of hormones (e.g., GnRH, progesterone, and prostaglandin) with the subsequent increase in the number of injections per animal.

In conclusion, this dissertation provides alternative strategies to improve reproductive performance in dairy herds without the need of increasing handling of
animals. A unique aspect of this dissertation is the integration of studies aimed at improving reproductive efficiency of dairy operations through the use of PS at the time of AI, and intrauterine delivery of GnRH.


Pinedo, P.J., and A. De Vries. 2010. Effect of days to conception in the previous lactation on the risk of death and live culling around calving. J. Dairy Sci. 93:968-977.


