EFFECTS OF INDUCTION OF OVULATION DURING EARLY LACTATION ON UTERINE HEALTH AND FERTILITY IN DAIRY COWS

By

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFC	Antral follicle count
AI	Artificial insemination
AKT	Protein Kinase B
AMH	Anti-Müllerian hormone
BCS	Body condition score
BHBA	β-hydroxybutyric acid
BMP5	Bone morphogenetic protein 5
CE	Clinical endometritis
CI	Confidence interval
CL	Corpus luteum
Cox-2	Cyclooxygenase 2
CR	Conception rate
CTE	Cytological endometritis
DF	Dominant follicle
DIM	Days in milk
FSH	Follicle-stimulating hormone
FOXO3	Forkhead box O3
GC	Granulosa cells
GDF 9	Growth differentiation factor 9
GH	Growth hormone
GnRH	Gonodotropin releasing hormone
HR	Hazard ratio
IGF-1	Insulin-like growth factor 1
LH	Luteinizing hormone

LHR	Luteinizing hormone receptor
mTOR	Mammalian target of rapamycin
NEB	Negative energy balance
NEFA	Non-esterified fatty acids
OR	Odds ratio
P/AI	Pregnancy per artificial insemination
PGF2α	Prostaglandin F2α
PL	Pregnancy loss
PP	Postpartum
SOP	Standard operation procedure
TC	Theca cells
TAI	Timed artificial insemination
Tbp2	TATA-binding protein 2
TaF4b	TATA box binding protein-associated factor
UK	United Kingdom
US	Ultrasonography examination
VWP	Voluntary waiting period

Abstract of Thesis Presented to the Graduate School
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Early ovulation postpartum is fundamental for optimal fertility. Cows having more estrous cycles before matting have higher hazard of pregnancy after the voluntary waiting period. The objective was to evaluate the effect of GnRH given early postpartum on induction of ovulation, uterine health and fertility in dairy cows. Holstein cows without corpus luteum (CL) at 17 DIM were randomly assigned to receive i.m. GnRH (n = 245) at 17 and at 20 DIM or remain as control (n = 245). Cows' ovaries were scanned by ultrasonography twice a week totaling four ultrasonographies (US). Ovulation was characterized by the appearance of a CL ≥ 20 mm at any US or CL < 20mm in two consecutives US. Clinical and cytological endometritis were diagnosed at 35 DIM. Data were analyzed using the LOGISTIC and PHREG procedures of SAS adjusting for the effects of dairy, parity, calving related problems, metabolic problems, and metritis. GnRH increased ovulation up to 3.5 d after the last treatment (78.7% vs. 45.0%; $P < 0.001$), and did not affect the prevalence of clinical endometritis (23.9% vs. 18.6%, GnRH vs. control respectively; $P = 0.2$) or cytological endometritis (30.9% vs. 32.8%, GnRH vs. control respectively; $P = 0.6$). Prevalence of clinical endometritis increased in cows that had calving problems (32.6% vs. 15.9%; $P = 0.001$) and metritis (40.6% vs. 15.8%; $P < 0.001$). Metritis increased prevalence of cytological endometritis (50.7% vs. 23.5%; $P < 0.001$). Treatment with GnRH did not affect

conception rate at 32 (37.6% vs. 38.6%; $P = 0.2$) or 74 d after AI (35.0% vs. 31.5%; $P = 0.5$), but reduced pregnancy loss (6.8% vs. 18.1%; $P = 0.002$). Interaction between GnRH treatment and ovulation showed that GnRH-treated cows that ovulated had increased hazard of pregnancy by 300 DIM compared to GnRH and control cows that did not ovulate (HR = 2.0 and HR = 1.3, $P < 0.05$, respectively), but hazard of pregnancy was similar to control cows that ovulated ($P = 0.7$). GnRH early postpartum induced ovulation without affecting uterine health, but failed to improve reproductive performance although it reduced pregnancy loss.

CHAPTER 1
LITERATURE REVIEW

Estrous Cycle

The estrous cycle in cattle is a dynamic process representing the cyclical pattern of ovarian activity between ovulations that allows females to move from a reproductive period of non-receptivity to receptivity given repeated opportunities to the cow or heifer to become pregnant (Forde et al., 2011). The bovine female is non-seasonal polyestrous cyclic animals with a uniform distribution of estrous cycles throughout the year that allows them to conceive year round. The estrous cycle lasts for approximately 18 to 24 days and compromise the period in between two consecutive estrus events, which normally is composed by two or three follicular waves (Savio et al., 1988; Ginther et al., 1989; Endo et al., 2012). Its onset occurs at the time of puberty and the cycle is normally divided into two distinct phases, the luteal and the follicular phase. The luteal phase starts right after ovulation with CL formation during the metestrus, characterized by low concentrations of estrogen and progesterone, until the fully formed CL develops and regresses also known as the diestrus, characterized by the fully functional activity of the CL relative to progesterone production and lasts for approximately 13-15 days (Forde et al., 2011). The follicular phase initiates with the onset of CL lysis and decline in concentration of progesterone, also known as proestrus, and is complete during the acceptance of mating, known as estrus (Lee et al., 1988). The anterior pituitary under influence of GnRH will induce release of FSH and LH, and in concert with adequate concentrations of IGF-1 and insulin, follicles are recruited and grow during the luteal phase. However, follicles are not able to ovulate because progesterone exerts a negative feedback in the hypothalamus blocking the GnRH/LH surge naturally induced by high concentrations of estradiol (Figure 1-1; Forde et al., 2011).

Once the main source of progesterone is removed by lysis of the CL, the concentration of estradiol, produced by the dominant follicle, increases and induces estrus behavior and exerts a positive feedback on the hypothalamus to cause the GnRH/LH surge, which will induce ovulation of the dominant follicle 24-30 hours later (Kesner et al., 1981; Vailes et al., 1992).

After ovulation, the newly ovulated follicle undergoes cellular and structural remodeling, giving rise to the transient corpus hemorrhagicum, present from right after ovulation up to 3 days later, and finally later the formation of the corpus luteum, in which significant levels of progesterone will be produced about one week after ovulation. There are several processes and factors involved with CL growth, maintenance and regression as described by Skarzynski et al. (2008a) and shown in Figure 1-2. After ovulation, the CL forms from the wall of the ruptured follicle, and its growth and vascularization occurs rapidly. In the cow, the weight of the CL 3 days after ovulation averages 640 mg, whereas on day 14, the average is 5.1 g (Fields and Fields, 1996) and most of this rapid mass increase is due to hypertrophy of granulosa (GC) and theca cells (TC), and also TC mitotic division. Endothelial cells and fibroblasts mitosis and growth also contribute to this rapid growth. The CL is a complex tissue composed of parenchymal (small and large steroidogenic) and non-parenchymal (fibroblast, vascular smooth muscle, pericytes and endothelial) cells (Reynolds and Redmer, 1999). The preovulatory LH surge results in luteinization of GC and TC. Within a few hours post ovulation, the GC give rise to the large luteal cells and the TC give rise to the small luteal cells. With the disruption of the basement membrane, these two cell types will be intermixed and remain in close contact during the reorganization of the follicle into the CL (Stocco et al., 2007). Luteinization of GC and TC alters their steroidogenic pathway, in which progesterone will be the primary steroid produced. Before ovulation, the GC aromatize androgens produced by the theca cells, and estradiol was the main

steroid being produced (Fortune and Quirk, 1988). Progesterone mainly produced by the CL will influence several organs and tissues preparing the uterus for gestation and maintenance of pregnancy by induction of a quiescent state in the myometrium (Csapo and Pulkkinen, 1978), stimulation of the uterine glands, and suppression of the maternal immune response to fetal antigens (Siiteri et al., 1977; McCracken et al., 1999). After exposure to estradiol, exposure to progesterone upregulates progesterone receptors in the reproductive tract. In contrast, progesterone downregulates receptors for estradiol and thereby blocks many of the actions of estrogens that generally act as mitogenic factors (reviewed by Niswender et al., 2000). Luteinizing hormone and growth hormone play an important role in the maintenance of the CL and progesterone production. Hypophysectomized ewes treated with growth hormone had circulating progesterone concentrations higher than untreated ewes. When LH was also replaced the hypophysectomized ewes had similar parameters of luteal function as pituitary-intact control ewes (Juengel et al., 1995).

The process of the CL lyses initiates with progesterone losing the ability of block the formation of oxytocin receptors in the uterus, and the rise in estradiol combined with the increase in oxytocin and oxytocin receptors will act through a COX-2 pathway to induce the pulsatile release of the luteolysin prostaglandin F2α (PGF2α) (McCracken et al., 1999). The PGF2α pulses start to increase and through a countercurrent mechanism PGF2α is able to reach the ovary and trigger the lysis of the CL (Thorburn and Nicol, 1971; McCracken et al., 1972).

The lyses of the CL follows a fashion in which first there is an inhibition of progesterone synthesis and a reduction of the blood flow into the CL characterizing the functional luteolysis. In addition, later the structural luteolysis takes place in which the immune response leads to the programmed cell death of the luteal tissue (McCracken et al., 1999). The lifespan of the CL

normally last for 15 to 17 days in a normal cycle, and lasts the duration of gestation in pregnant cows. If, by day 15-17 of the oestrous cycle, the maternal recognition of pregnancy signal (interferon tau) has not been detected, luteolysis occurs. With the absence or low concentrations of progesterone in blood, the block on LH surge is removed, leading to LH surge and ovulation.

With the advances of ultrasonography, a better understanding of uterine and ovarian dynamics throughout the estrous cycle and pregnancy has been achieved. Three functionally critical follicular sizes have been characterized during the stages of antral follicular growth (Figure 1-3): emergence (approximately 4 mm), deviation (approximately 9 mm), and ovulation (from 10 to 20 mm). These classifications helped to unravel the mechanisms associated with anovulatory conditions and led to corrective interventions (Lucy et al., 1992; Thatcher et al., 1996; Wiltbank et al., 2002; Sartori et al., 2004).

Folliculogenesis

The total reservoirs of available follicles during the entire reproductive life period of the female are the primordial germ cells, which give rise to primordial follicles. Anti-Mullerian hormone (AMH) maintains the balance between the number of primordial follicles remaining in the arrested pool and the follicles being activated by gonadotropins (Sánchez and Smitz, 2012). The mechanism involved in the follicular growth from primordial to primary stages is not completely known, although advances in molecular biology identified several pathways. Growth factors and Kit-ligans will activate the PI3K (phosphatidylinositol 3 kinase) pathway. Through a cascade of events, AKT (a serine/threonine protein kinase that enhances cellular proliferation and survival) is able to phosphorylate FOXO3 (Forkhead box 3, a transcription factor that leads to apoptosis and cell cycle arrest) and PI3K also phosphorylates and inactivates an inhibitor of mTOR (Mammalian target of rapamycin, a serine/threonine protein kinase that regulates cell growth). Phosphorylated FOXO3 will not be able to enter into the nucleus and therefore will not

be able to promote follicle arrest. Concomitantly, activated mTOR will signal protein translation and follicle development will progress (Sánchez and Smitz, 2012). Progression from primary follicles to secondary follicles is driven by several intraovarian factors produced by the oocyte, GC and TC (Kol and Adashi, 1995). Growth differentiation factor 9 (GDF9) and Bone morphogenetic protein 15 (BMP15) promote GC proliferation (i.e., GDF9) in the early stages beyond the primary stage and stimulating the proliferation of GC in a FSH-independent manner (i.e. BMP15; Yan et al., 2001). Two other transcription factors are required for the progression of early preantral follicle development, TATA-binding protein 2 (Tbp2) and TATA box binding protein-associated factor 4 (Taf4b). Once the secondary follicles are developing, FSH and LH receptors start to be expressed and a rapid follicular growth takes place. Insulin like growth factor (IGF) also is important for follicle development (Sánchez and Smitz, 2012). With the abrupt proliferation of GC, activin production decreases and inhibin production increases to avoid the uncontrolled proliferation of GC (Findlay et al., 2000). The follicle, at this point of development, becomes an independent unit that is less affected by local stimulus but is now dependent on gonadotrophin hormones. The antral follicle is characterized by differentiation of GC into cumulus cells and mural cell compartments, and the antrum formation and accumulation of estradiol. Granulosa cells utilize androgens produced by theca cells in response to LH stimulation as a substrate for estradiol production. This process is known as the "Two cells two gonadotrophin model" (Fortune and Armstrong, 1976; Hillier et al., 1994).

Elevated FSH levels released by the anterior pituitary induce recruitment of a cohort of follicles from the gonadotrophin sensitive pool within the ovary. Estradiol produced by these antral follicles, in conjunction to low levels of inhibin, will exert a positive feedback on the hypothalamus-pituitary axis to stimulate basal LH secretion. As the follicles grow, inhibin

secretion increases to the point in which combined with estradiol causes a negative feedback on the anterior pituitary to reduce FSH secretion (Haughian et al., 2013). At this point the deviation of the dominant follicle occurs at approximately 8 mm of diameter (Ramasharma et al., 1981). All the events involved in selection and dominance of the dominant follicle are not completely understood. The dominant follicle is the faster growing follicle that will express LH receptors in the granulosa cells at approximately 8 mm in diameter. By approximately 8 mm in diameter, the follicles express LH receptors (Figure 1-4) that will be functionally competent when the follicle reaches the size of approximately 10 mm and is capable of ovulating in response to an LH surge or injection of GnRH that stimulates the release of LH (Sartori et al., 2004; Beg and Ginther, 2006). The time from primordial to preovulatory stage is approximately 180 days and the time from the antral to the preovulatory stage is approximately 40 days (Lussier et al., 1987). In an environment of low progesterone concentrations, high estradiol lead to a GnRH surge, leading to the preovulatory LH surge, this will lead to disruption of the gap junctions of the oocyte-granulosa cells, termination of the meiotic arrest and ovulation (Conti et al., 2012). Alternatively, in a high progesterone environment, the GnRH/LH is prevented and the dominant follicle becomes atretic allowing for the release of FSH and recruitment of a new cohort of follicles (Forde et al., 2011). Luteinizing hormone (LH) is important for the final growth stages of the follicle prior to ovulation and for the ovulation process (Ireland and Roche, 1983; Ginther et al., 2013).

A recent study evaluated the antral follicle count (AFC), which is defined as the number of follicles present in the ovaries with a diameter equal or greater than 3 mm, and is classified as low ($\leq$ 15 follicles) or high ($\geq$ 25 follicles). Follicles from ovaries with a low AFC have GC with a poor response to FSH stimulation compared to follicles in the high AFC group. Consequently,

cows of the low AFC group have lower estradiol and Anti-Müllerian hormone (AMH) production. This new findings provide insight into the potential mechanisms involved with ovarian dysfunction in females with low AFC (Scheetz et al., 2012).

AMH is expressed only in the gonads, acts to inhibit the recruitment of primordial follicles into the pool of growing follicles, and avoids premature exhaustion of the ovarian follicular reserve, and AMH expression is high in small healthy follicles. AMH becomes a reliable endocrine marker for the prediction of a high and healthy population of AFC responsive to gonadotropins (Monniaux et al., 2013). Discovery of more regulatory factors of the follicle growth were made recently and strongly suggest a role of angiotensin II in follicular selection and development, and ovulation (Gonçalves et al., 2012).

Resumption of Ovarian Cyclicity Postpartum

The first follicular waves postpartum in dairy cows usually start about 5-10 days after parturition and it is followed by clearance of gestational estradiol from blood (Beam and Butler, 1997), which removes the inhibition on FSH release from the anterior pituitary gland. Consequently, FSH leads to follicular recruitment (Adams et al., 1992). Follicles can then grow in 2 different patterns (Figure 1-5): 1- with adequate levels of IGF-1 and insulin leading to ovulation; the follicle will continue to grow beyond 5 mm with the indirect stimulation of GnRH that will induce FSH and LH pulses from the anterior pituitary, and after deviation and selection the rise in E2 induces the surge of LH from the GnRH surge center in the brain that leads to ovulation (Wiltbank et al., 2002); 2- with inadequate levels of IGF-1 and insulin leading to anovulation. Anovulation can be subdivided in three categories. In the first category, follicles are recruited and grow to the point of acquisition of dominance and ovulatory capacity, but lack GnRH/LH surge due to inadequate levels of estradiol produced by the dominant follicle. This condition is associated with lower concentrations of IGF-1, glucose, and high concentrations of

NEFA characteristic of negative energy balance (Chagas et al., 2007). In the second category, follicles do not develop beyond the selection phase. This condition is a result of severe undernourishment. The GnRH neurons become very sensitive to the negative feedback of estradiol. GnRH and consequently FSH/LH release is compromised which prevent follicle growth beyond the selection phase (Murphy et al., 1990). In the third category follicles reach ovulatory size but fail to ovulate. This condition is called ovarian cystic degeneration, cystic ovarian disease or cystic follicles. This pathologic condition affects approximately 9.5 % to 25 % of the dairy cows (Whitmore et al., 1974). Cystic follicles are believed to be formed after an LH surge that fails to induce ovulation (De Silva and Reeves, 1988; Hamilton et al., 1995; Garverick, 1997; Gümen and Wiltbank, 2002; Kaneko et al., 2002; Todoroki et al., 2004).

Negative Energy Balance Postpartum

Close to parturition, dairy cows undergo a period of complex endocrine and metabolic changes to adjust to milk production right after calving. The nutrient demands are five fold higher 4 days postpartum compared to 250 days of gestation (Bell, 1995). While the energy demands for production increase rapidly, dry matter intake increases slowly which puts cows in a state of negative energy balance (NEB). To compensate for this gap of required nutrients, there is a massive mobilization of body fat as nonesterified fatty acids (NEFA) into the bloodstream. The liver is challenged to produce high amounts of glucose and to oxidize fatty acids (Butler et al., 2006). The NEFA are utilized to make upwards of 40% of milk fat during the first days of lactation (Bell, 1995). Given that plasma NEFA concentrations increase in response to increased energy needs accompanied by inadequate feed intake, DMI and plasma NEFA concentrations usually are inversely related. The liver takes up NEFA in proportion to their supply (Pullen et al., 1989; Reynolds et al., 2003); therefore, cows are predisposed to accumulate NEFA as triglycerides within liver when large amounts of NEFA are released from adipose tissue into the

circulation (Emery et al., 1992). Nonesterified fatty acids are metabolized in the liver to more available substrates such as ketone bodies. The three endogenous ketone bodies are acetone, acetoacetic acid, and beta-hydroxybutyric acid (BHBA), although BHBA is not technically a ketone but a carboxylic acid. Ketone bodies can be used as an energy source for muscle and nervous tissues; however, its excess is associated with depression in feed intake and increases the risk of clinical diseases such as fatty liver, ketosis, displaced abomasum, metritis, etc, and subsequent decrease of milk production (Duffield et al., 2009). BHBA administration has been shown to reduce feed intake in pigs (Müller et al., 1984) and ewes (Schlumbohm and Harmeyer, 2004). Nonetheless, a recent study fail to show a decrease in feed intake with infusion of BHBA in mid lactation dairy cows (Zarrin et al., 2103). Although feed intake was not reduced, glucose concentrations in blood were reduced because of a decrease in glucagon concentrations.

The severity of the NEB is directly related to the decrease in dry matter intake (DMI), and it is associated with lower blood concentrations of metabolic hormones, insulin and IGF-1 (Butler et al., 2006). Growth hormone stimulates IGF-1 which in turn stimulates the development of follicles, whereas high concentration of NEFAs inhibit the proliferation rate of granulosa cells and increase theirs apoptotic rate in vitro (Jorritsma et al., 2004; Vanholder et al., 2005**)**. Severe NEB reduces the pulse frequency of LH, hence compromising estradiol production, follicular selection, maturation and ovulation (Butler, 2006). Ovulation occurs on average two weeks after the nadir in NEB (Butler, 2003). Longer periods of NEB are detrimental to reproduction because of delay in first ovulation (Figure 1-6) and negative effects on oocyte quality (Figure 1-7).

The extent of NEB is correlated to body condition score (BCS) loss early postpartum, and cows experiencing higher losses of BCS in the first month of lactation have the most severe NEB and consequently have longer intervals to first ovulation, reduced risk of conception and

increased risk of culling (Butler, 2012). Another carryover negative effect of NEB is the lower circulating levels of IGF-1 that may be associated with reduced fertility, or also lower quality and lower viable embryos recovered from high milk yield cows in early postpartum stages (Sartori et al., 2002).

Characterization of metabolic profiles of cows that did or did not ovulate the follicle of the first follicle wave postpartum demonstrated that ovulatory cows had lower concentration of growth hormone and NEFAs, higher concentration of IGF-1 and insulin, and delayed decline in IGF-1 (related to the acquisition of follicular dominance), compared to cows that fail to ovulate. The ovulatory cows had also higher concentrations of estradiol and LH during the follicular phase (Kawashima et al., 2012). Collectively, these hormonal and metabolic changes reinforce the extreme importance of metabolic status on reproduction.

Early Ovulation Postpartum

Early postpartum (PP) resumption of ovarian cyclicity leads to higher fertility (Thatcher and Wilcox, 1973; Stevenson and Call, 1988; Staples et al., 1990; Darwash et al., 1997; Tanaka et al., 2008; Santos et al., 2009; Galvão et al. 2010). Fewer services per conception were necessary for cows to become pregnant when estrus activities occurred in the first month postpartum (Thatcher and Wilcox, 1973) and longer intervals from first ovulation PP to the first service increased pregnancy to the first AI (Darwash et al., 1997).

Several studies showed positive effects of resumption of ovarian cyclicity before the first AI on first service conception rate (Darwash et al., 1997; Chebel et al., 2006; Santos, et al., 2009; Galvão et al. 2010). Furthermore, cows ovulating within 21 DIM had higher hazard of pregnancy by 300 DIM than cows that started cycling from 21 to 49 DIM or anovular cows by 49 DIM (Galvão et al., 2010). Kim et al. (2012) showed similar patterns of enhanced reproductive performance in Korean dairy herds, for cows ovulating early postpartum. Increased fertility in

cows that resumed ovulation early postpartum is believed to be influenced by the increased frequency of estrous cycles before first artificial insemination (Thatcher and Wilcox, 1973). More cycles would provide progesterone priming and uterine cleansing during estrus. Failure of ovulation of the dominant follicle early postpartum is associated with profound negative energy balance and long interval to its nadir (Beam and Butler 1997, 1998). Uterine diseases such as clinical and cytology endometritis are positively associated with NEFA and BHBA (Hammon et al., 2006); therefore, early ovulation may be a marker of good overall health status.

Resumption of ovulation postpartum is highly variable (Butler and Smith, 1989) and there is still 20% to 40% of anovular cows at the end of the voluntary waiting period (Walsh et al., 2007; Santos et al., 2009; McDougall, 2010; Bisinotto et al., 2010b). Lack of adequate transition period management, inadequate energy balance, and poor health status are directly related to high proportion of anovular cows at the end of the voluntary waiting period.

Controversially, early ovulation postpartum has been hypothesized to have detrimental effects on uterine involution (Padula and Macmillan, 2002) and also on reproductive performance. Prolonged luteal phase (defined as elevated progesterone for more than 25 days) is correlated with a greater risk of developing pyometra (Farin et al., 1989) and was associated with lower fertility (Smith and Wallace., 1998). In fact, a side effect of early induced ovulation in response to GnRH injections (15 days postpartum) was an increase in the subsequent incidence of pyometra and prebreeding anestrous. Thus, treatment with GnRH alone increased calving to first estrous and calving to first breeding intervals, and resulted in a tendency for an increased calving to conception interval (Etherington et al., 1984).

Studies performed using continual exposure to a synthetic GnRH implant delayed ovulation postpartum during heat stress periods and successfully increased rates of uterine

involution (Silvestre 2003, 2009). The chronic treatment with a potent and high affinity GnRH agonist, deslorelin (Karten and Rivier, 1986) first induces an LH surge from the pituitary; however, downregulates the GnRH receptors on the gonadotroph cells altering the responsiveness of the pituitary to endogenous GnRH. The pituitary gland becomes refractory to gonadotrophin-releasing hormones and LH pulsatility secretion will be diminished resulting in block of ovulation (D'Occhio et al., 2000). However, there was a high variation in resumption of ovarian cyclicity once the block in ovulation was removed with almost half of the cows still not having ovulated by the beginning of the Ovsynch program. This delay in cyclicity compromised P/AI at first AI and negatively affected milk production (Silvestre, 2009). Early resumption of postpartum activity and enhanced fertility to first service is a challenge when coupled with non-optimal management of nutrition during the transition period, inadequate energy balance postpartum, and poor health status.

GnRH and Ovulation

Gonadotropin releasing hormone, a decapeptide, produced by the hypothalamus is mainly known for its role in reproduction with an important role in the ovulation process. However, the presence of GnRH receptors outside of the hypothalamic-pituitary-reproductive axis has been demonstrated (Skinner et al., 2009). At the molecular level, once GnRH reaches the pituitary gland, it binds to its G-coupled protein receptor on the surface of the gonadotrophs cells. This receptor has a unique characteristic because it lacks an intracellular cytoplasmatic tail allowing slower internalization (Hislop et al., 2001). Once the ligand is bound to the G-receptor, there is activation of the G-proteins (Stanislaus et al., 1997) that subsequently activate its subunits, G-protein αq11 and phospholipase Cβ (PLCβ). These subunits then activate protein kinase C (PKC) and inositol 1, 4, 5 triphosphate (IP3) and the later induces calcium influx to the cytosol from the extracellular fluid and also from the endoplasmic reticulum. PKC acts through a cascade of

phosphorylation and activates mitogen-activated kinases (MAPK), in which MAPK goes to the nucleus to activate the transcription steroidogenic factor-1 (SF-1). Immediate-early gene (Egr-1), another transcription factor of LHβ gene, also becomes active and the synergistic interaction among SF-1, Egr-1 and a third LHβ transcription factor, the pituitary-specific HD protein (Pitx-1), results in activation of LHβ gene expression in the nucleus. Calcium also plays a role on the LHβ gene activation however in lower significance compared to MAPK pathway (Dorn et al., 1999; Haisenleder et al., 1997). The calcium in the cytosol then interact to calmodulin and activates calcium – calmodulin kinases, in which phosphorilate and activate some of the transcription factors for FSHβ gene (Haisenleder et al., 2003; Liu et al., 2002) resulting in the synthesis of FSH hormone. FSHβ gene is mainly dependent on the calcium-calmodulin mediated pathway and FSH is only stored in secretory granules in the cytoplasm for short periods, whereas LH is stored for longer periods during the estrous cycle (Farnworth, 1995).

GnRH neurons in the cow are located in two different areas of the brain with species variations. The first group of GnRH neurons is located at the ventromedial and arcuate nuclei of the hypothalamus, which is known as the tonic GnRH center. The tonic center is responsible for the basal secretion, in which various small pulses of different frequencies and amplitudes of GnRH are released in the female and also in the male. The second group of GnRH neurons – the preoptic and suprachiasmatic nuclei center - is localized on the superior-anterior area of the hypothalamus. This area is called the surge center or preovulatory center because it is where the preovulatory GnRH surge occurs (Sakakibara et al., 2013). The surge center is developed and active only in the female but not in males. In the female fetuses, alpha-fetal protein binds estradiol and prevents it from reaching and crossing the blood brain barrier; therefore, the surge center of the hypothalamus develops. In male fetuses, testosterone is converted to estradiol in

brain and defeminizes or prevents the differentiation of the GnRH surge center in the hypothalamus. Regulation of GnRH release by steroids is not entirely due to the direct effect of the steroids on GnRH neurons. In fact steroids affect others neurons that are in communication with GnRH neurons. GnRH acts on the pituitary to stimulate the synthesis and release of FSH and LH, and it is negatively affected by estradiol in the tonic center (Ginther, 2000; Takumi et al., 2012). However, in the surge center, estradiol stimulates the surge in GnRH and ultimately LH. Progesterone suppresses the GnRH surge center, although the full mechanism is not completely understood. Both estradiol and progesterone act on GnRH neurons through the Kisspeptin pathway. Estradiol up-regulates the expression of Kiss 1 gene and stimulates GnRH release at the surge center but downregulates the expression of Kiss 1 gene in the tonic center (Alçin et al., 2103). Progesterone down-regulates the expression of Kiss-1 gene and decrease GnRH release in the tonic center (Radovick et al., 2012). Nutritional status also plays an important role in the control of GnRH production and release (Santos et al., 2010). The neurotransmitter neuropeptide Y (NPY) is an orexigenic peptide that stimulates appetite and is stimulated by low energy levels in the blood associated with low leptin concentrations. NPY also is an inhibitor of GnRH neurons, and can be blocked by leptin that is produced by the adipose tissue (Schwartz et al., 1996). Leptin concentration functions as a sensor of body fat status in the body that ultimately partially integrates metabolic status with GnRH release. Under favorable metabolic condition, leptin upregulates Kiss 1 leading to kispeptin production, which stimulates GnRH release from the GnRH tonic center leading to FSH and LH release by the pituitary, which affects follicle growth leading to increased estradiol concentration which will up-regulate the Kiss-1 neurons on the surge center resulting in the GnRH-LH surge and ovulation (Garcia-Galiano et al., 2012).

Treatment with GnRH is a means to induce ovulation (Britt et al., 1974; McDougall et al., 1995; Gümen and Seguin, 2003; Amaya-Montoya et al., 2007) and is used routinely within a variety of synchronization of ovulation protocols. However, the use of GnRH to induce ovulation early postpartum does not consistently result in improvement of fertility. In fact, early ovulation postpartum has been hypothesized to have detrimental effects on reproduction (Padula and Macmillan, 2002). Etherington et al. (1984) reported increased frequency of pyometra and increased calving to first estrus and calving to first breeding intervals for cows treated with GnRH at 15 DIM. Stevenson and Call (1988) reported increased calving to conception interval with early postpartum GnRH administration in cows with reproductive disorders. Others failed to improve reproductive performance with GnRH administration in healthy postpartum cows (Foote and Riek, 1999). On the other hand, Benmrad and Stevenson (1986), and Nash et al. (1980) showed a reduction on days to conception and fewer services per conception for GnRH treated cows between 10-15 days PP. Cows treated with GnRH between 13 to 15 days PP had increased conception to the first AI (74% versus 56% respectively), better overall conception rate (70.6 vs. 51.1% respectively) and a lower number of services per conception (1.23 vs. 1.74 AIs/pregnancy) compared to control cows (Nash et al., 1980). Reduction of culling rate because of low reproductive performance was also achieved with the use of GnRH in early lactation (Britt et all., 1977).

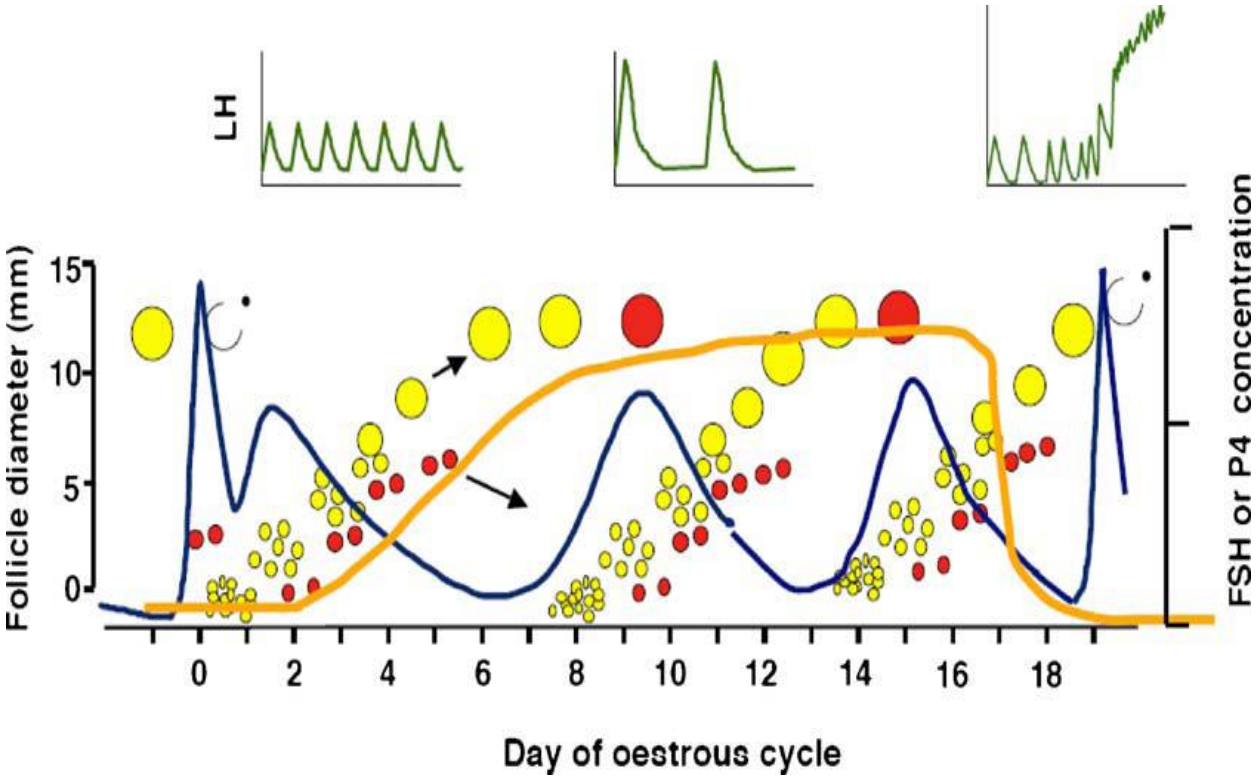

Figure 1-1. Schematic depiction of the pattern of secretion of FSH, LH and progesterone; and pattern of growth of ovarian follicles during the estrous cycle in cattle. Each wave of follicular growth is preceded by a transient rise in FSH concentrations. Healthy growing follicles are shaded in yellow, atretic follicles are shaded red. A surge in LH and FSH concentrations occurs at the onset of estrus and induces ovulation. The pattern of secretion of LH pulses during an 8-h window early in the luteal phase (greater frequency, lesser amplitude), the mid-luteal phase (lesser frequency, lesser amplitude) and the follicular phase (high frequency, building to the surge) is indicated in the inserts in the top panel (Forde et al., 2011).

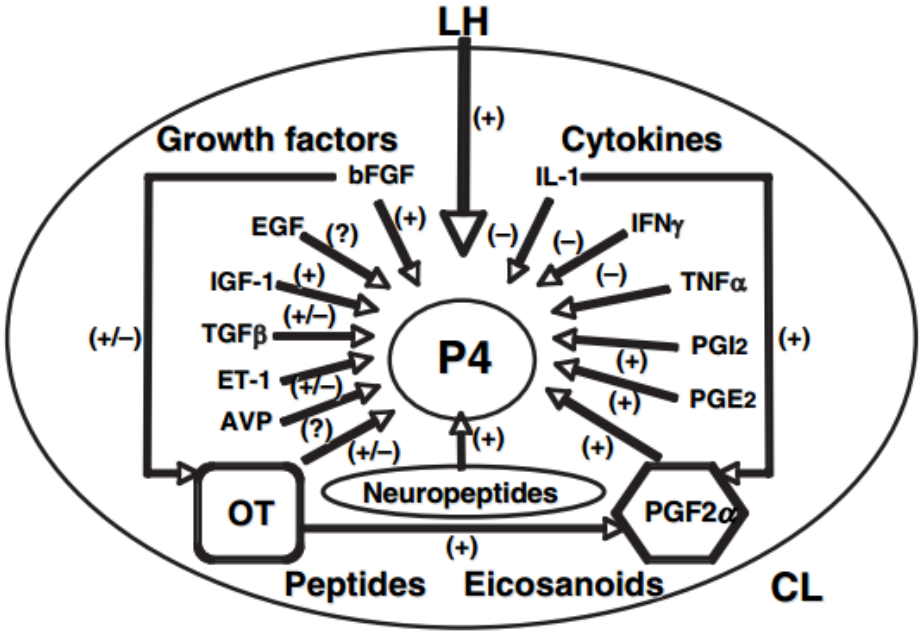

Figure 1-2. Schematic presentation of factors involved in the growth, maintenance and regression of the CL (Skarzynski et al., 2008).

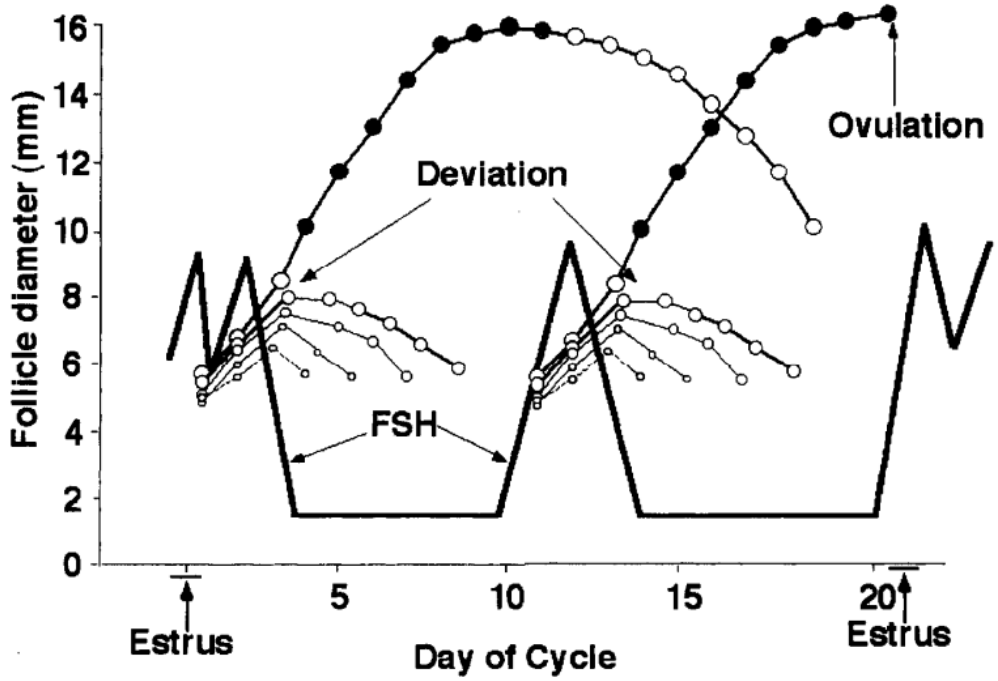

Figure 1-3. Schematic of follicle growth and FSH for a cow that has two follicular waves during a 21 -d estrous cycle (Wiltbank et al., 2002).

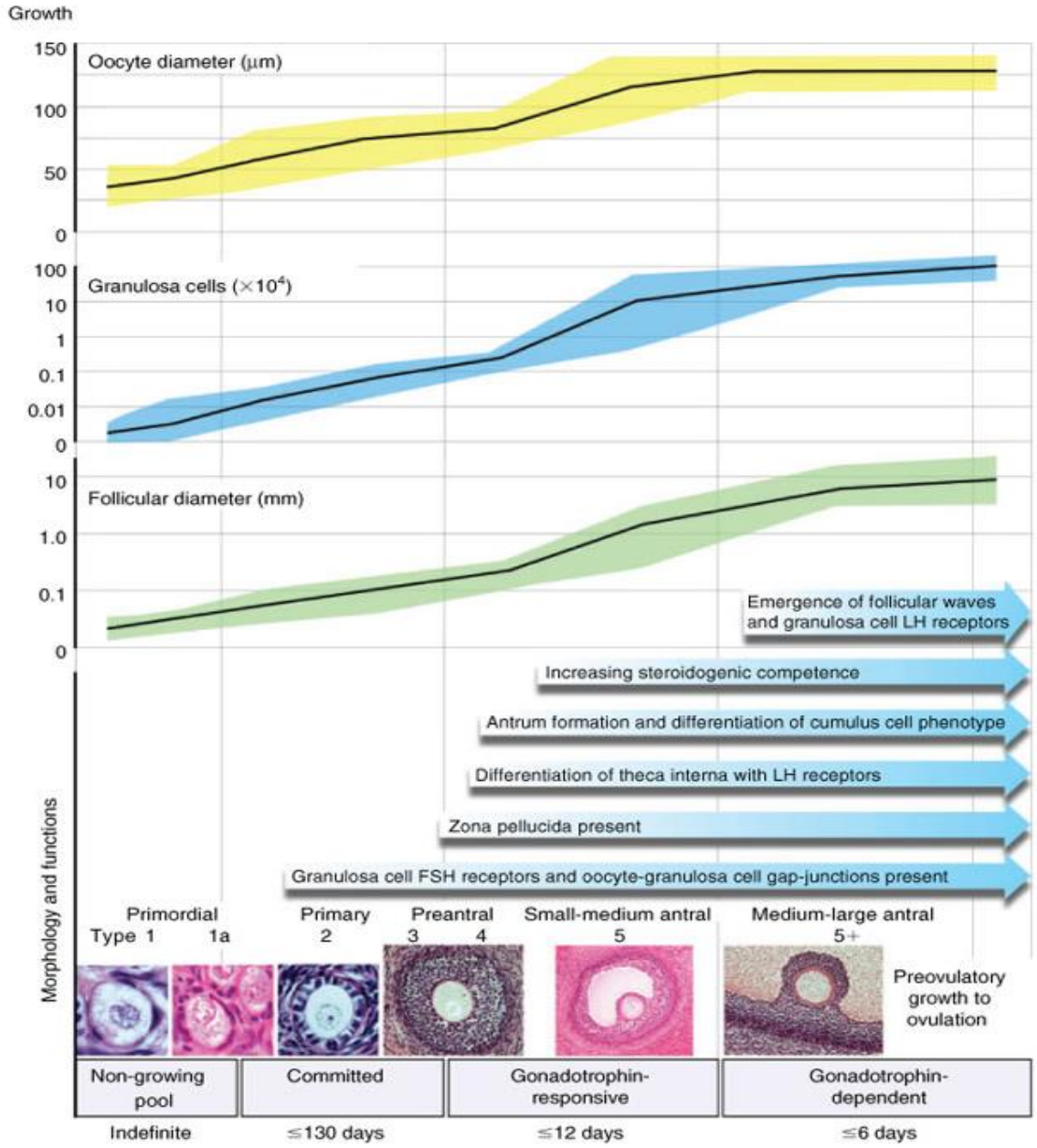

Figure 1-4. A summary of folliculogenesis in the ewe, developed with data from several sources (Lundy et al. 1999; K. P. McNatty, unpubl. data). The upper panel shows the mean (central line) and range (shaded band) time lines of growth of the follicle and oocyte and the number of granulosa cells, from primordial to ovulatory stages. The lower panel shows the progressive emergence of several critical functional and morphological characteristics of follicles as they develop. The stages of development have been defined by two different systems – one based on morphology and the other on functional characteristics of follicles (Scaramuzzi et al., 2011).

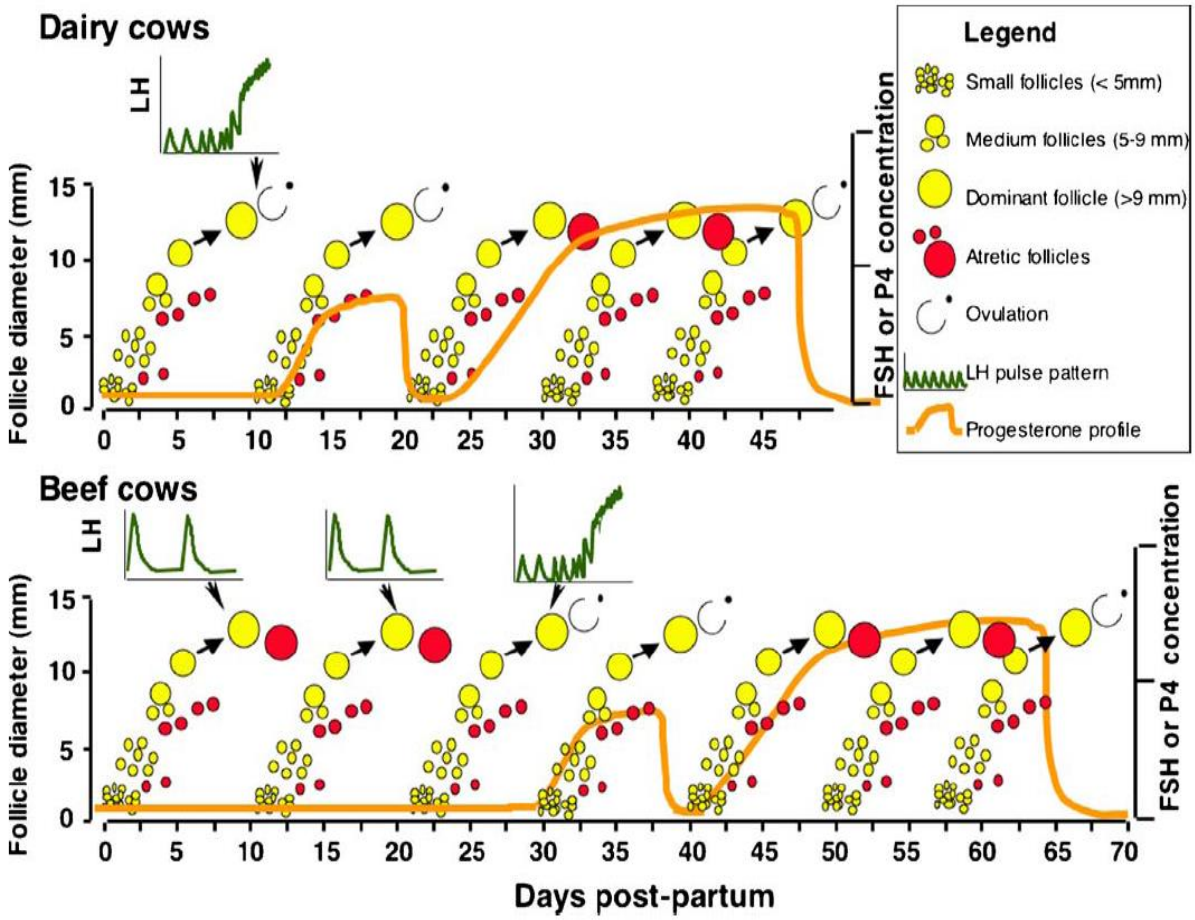

Figure 1-5. Diagrammatic scheme of resumption of dominant follicles and ovarian cycles during the postpartum period in dairy and beef suckled cows not nutritionally stressed. LH pulse frequency is that occurring during an 8-h window where cows are blood sampled every 15 min. Short oestrous cycles do not always occur after first ovulation (Forde et al., 2011; reprinted from Crowe, 2008).

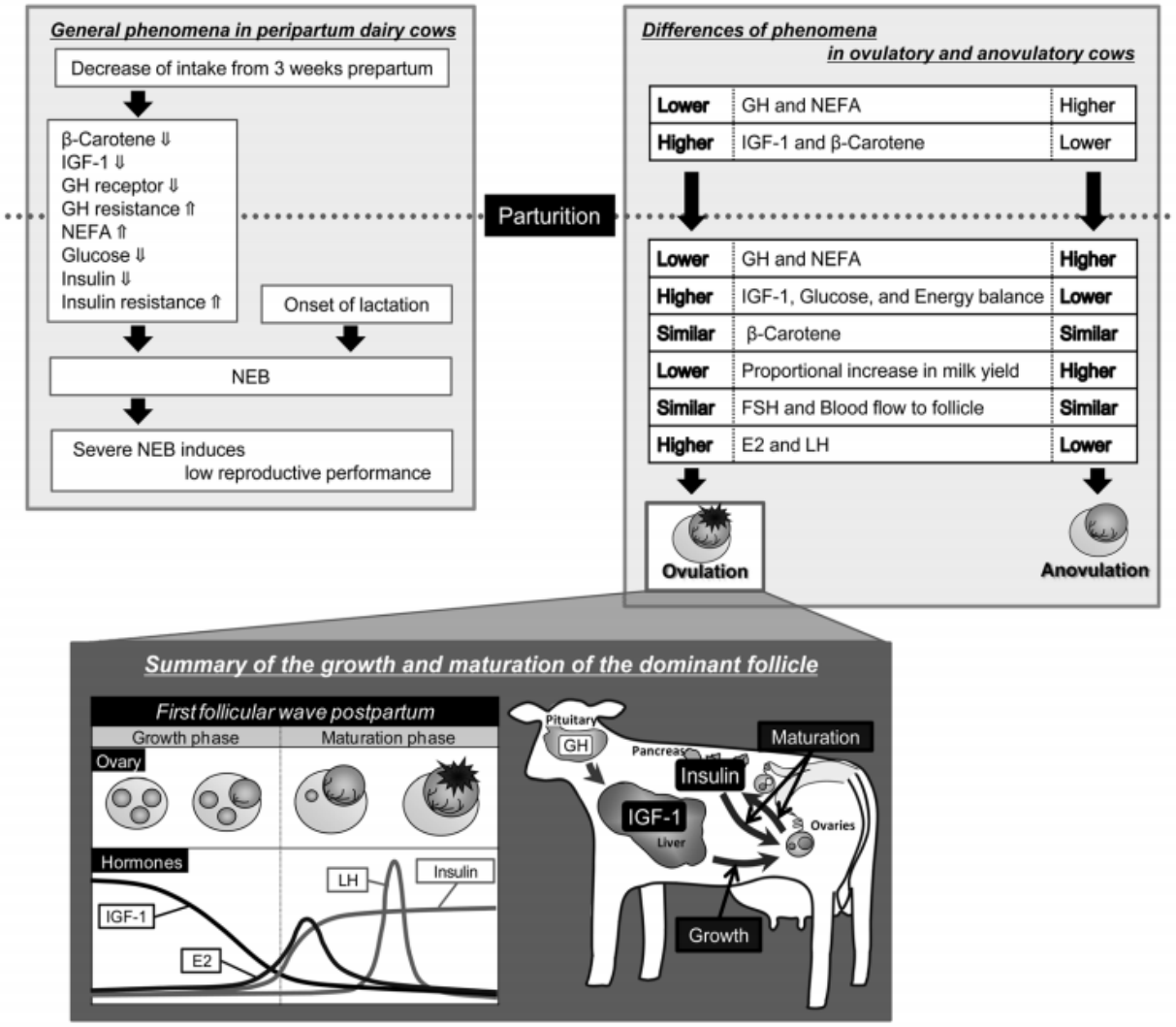

Figure 1-6. Schematic representation of nutritional status during peripartum, lactation and reproductive function during early postpartum in anovulatory and anovulatory cows at first follicular wave. GH, NEFA, β-caroteno, IGF-1, glucose and energy balance influencing ovarian and follicle dynamics that lead or not to ovulation (adapted from Kawashima et al., 2012).

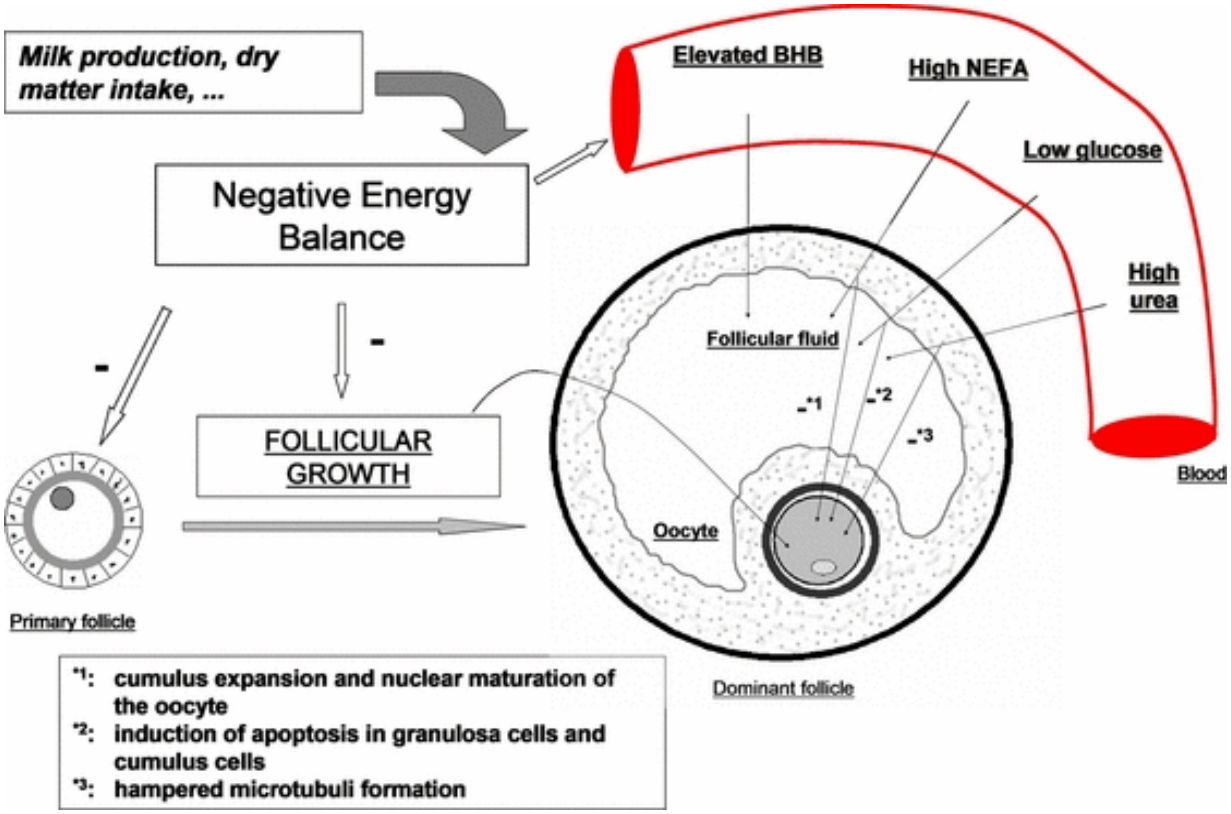

Figure 1-7. Metabolic mechanisms linking negative energy balance and oocyte quality in high producing dairy cows. A status of negative energy balance is hypothesized to affect the health of the primary follicles which may have a carry-over effect on oocyte quality. An altered follicular growth pattern might impair oocyte developmental competence. Biochemical parameters, associated with a negative energy status, are well reflected in the follicular fluid and can directly affect oocyte competence. NEFA: non-esterified fatty acids, β-OHB: β-hydroxybutyrate (Leroy et al., 2008).

CHAPTER 2
INTRODUCTION

It is well documented that reproductive performance affects profitability of the dairy enterprise (De Vries et al., 2006; Galvão et al., 2013). Having high reproductive performance (i.e. high pregnancy per AI and low pregnancy loss) will increase the proportion of cows conceiving immediately after the voluntary waiting period which can increase the herd average daily milk yield by increasing the proportion of cows in the early stages of lactation in the subsequent lactation (Ribeiro et al., 2012). Furthermore, high reproductive performance lead to reduced culling rates due to reproductive failure (Pinedo and de Vries, 2010), and maximize farm revenue (Risco et al., 1998; De Vries et al., 2006; Galvão et al., 2013). Reports using the average herd producing of 9,000 Kg of milk in the United States of America showed that an extra day open beyond 90 days costs on average of $ 3.00 and this value increases with increased days to conception (De Vries, 2008).

Treatment with GnRH is not a new tool to induce ovulation (Britt et al., 1974; McDougall et al., 1995; Gümen and Seguin, 2003; Amaya-Montoya et al., 2007) and it is used routinely within a variety of synchronization of ovulation protocols. However, effects of GnRH administration early postpartum on fertility are not consistent. Fernandes et al. (1978) and Kesler et al. (1977) recommended the use of GnRH after 7 to 10 days postpartum when the pituitary had restore responsiveness to GnRH. Padula and Macmillan (2002) and Etherington et al. (1984) reported detrimental effects on reproductive performance (ie. increased frequency of pyometra, increased calving to first estrus and to the first breeding interval) with the use of GnRH at 15 DIM. Administration of GnRH between 18 and 25 DIM to cows with reproductive disorders resulted in increased calving to conception interval (Stevenson and Call, 1988). Others failed to improve reproductive performance with GnRH administration at 13 or 14 days postpartum in

healthy postpartum cows (Foote and Riek, 1999). Nonetheless there are beneficial effects demonstrated by GnRH use in early postpartum as demonstrated with an increase in conception rate, a reduction in days to conception and in services per conception when GnRH treatment occurred between 10 to 15 days postpartum (Benmrad and Stevenson, 1986; Nash et al., 1980). Reduction of culling rate because of low reproductive performance was also achieved with use of GnRH between 8 to 23 days of lactation (Britt et al., 1977). Studies had shown increased ovulation rates for cows treated with GnRH early in lactation. In one study (Benmrad and Stevenson, 1986) 75% of the cows that received GnRH between 10 to 14 DIM ovulated and only 28% of the non-treated cows ovulated within 7 days post treatment. Britt et al. (1974) obtained 90% of ovulation rate in GnRH treated dairy cows at 14 DIM, however, less than 20% of control cows that received saline, ovulated within 7 days post treatment. More recently a study performed in Korea (Jeong et al., 2013) showed that GnRH administration around 30 days postpartum limited to cows that did not experience any peripartum or metabolic problem such as dystocia, retained placenta, abortion, metritis or endometritis, increased ovulation rate compared to non-treated cows (ovulation was measured in a subset of cows 14 days after GnRH injection; 70.9% vs. 53.0%; $P < 0.001$). The GnRH group also had increased hazard of pregnancy by 210 DIM (HR = 1.3; 95 % CI = 1.06 to 1.61; $P = 0.01$) compared to control cows.

None of the previous studies used modern synchronization methods such as the Presynch-Ovsynch as part of the reproductive management. The inconsistent results, the small sample sizes combined to the inconsistent characterization of uterine health in the previous studies evaluating the use of GnRH during the first month postpartum justify further investigation in this field and the novel of the proposed research is the treatment with GnRH performed in cows that had protocol of ovulation synchronization to be timed artificially inseminated. The hypothesis of

the study was that administration of exogenous GnRH early postpartum in cows without a CL would induce ovulation without detrimentally affecting uterine health and would induce resumption of cyclicity in anovular cows, which is expected to increase fertility. The main objective of the study was to evaluate the effects of administration of GnRH at 17 and 20 ± 3 DIM in Holstein dairy cows without a CL on induction of ovulation, uterine health, and reproductive performance.

CHAPTER 3
MATERIALS AND METHODS

Cows, Housing and Feeding

A randomized clinical experiment was conducted on two freestall dairy farms located in North Central Florida from December 2010 through August 2012. All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee (IACUC-UFL).

Dairy 1, the Dairy Unit from the University of Florida, milked approximately 450 Holstein cows twice daily with a rolling herd average of ~10,500 Kg/milk/cow. A single lactating total mixed ration (TMR) was fed twice daily to match or exceed the requirements of second lactation Holstein cows with the following characteristics: 650 Kg of body weight, producing 45 Kg/day with 3.7% fat-corrected milk and 3.15% of protein at 60 DIM; fresh water was available ad libitum. The freestall barns were cleaned twice daily and freestalls bedded twice a week with sand. Fans with misters and sprinklers over the feed line were present in the barns. The AfiMilk system was comprised of AfiMilk Pedometer, AfiLab and AfiFarm software (AfiMilk System, SAE Afikim; Kibbutz, Israel) to monitor cow health, occurrence of estruses and milk measurements (i.e., milk weight; % fat; % protein; % lactose, somatic counting cell; etc.) daily of individual cows. Cows were restrained in head lock gates along the feed line when all experimental procedures were conducted. The second dairy (Dairy 2) was a private owned dairy milking approximately 4,800 Holstein cows thrice daily with a rolling herd average of ~11,500 Kg/milk/cow. Cows had ad libitum fresh water available, and TMR offered twice a day formulated to meet or exceed the requirements of a second lactation Holstein dairy cow with the following characteristics: 680 Kg of body weight producing 52 Kg of milk/day with 3.5% fat corrected milk and 3.0% of protein at 60 DIM. In Dairy 2, cows were housed in tunnel-ventilated

freestall barns, freestall were cleaned thrice daily and bedded twice a week with sand. PCDart software (Dairy Records Management Systems, Raleigh, NC) and Allflex RFID ear tags (Allflex USA, Dallas, TX) were used as software program and electronic devices for identification, collection and recording of milk yield and health/reproductive events. All procedures were performed while cows were restrained at a palpation rail after the milking on the afternoon shift.

In both dairies primiparous and multiparous were housed separately. Cows were vaccinated and treated for common diseases according to standard operating procedures (SOP) developed with participation of the veterinarians from University of Florida/College of Veterinary Medicine (Food Animal Reproduction and Medicine Service).

Study Groups, BCS and Ultrasonography of the Ovaries

Once weekly, on Tuesdays, a cohort of lactating cows within 17 ± 3 DIM was enrolled in the study for 65 consecutive weeks in Dairy 1, and for 16 consecutive weeks in Dairy 2. A total of 637 Holstein lactating dairy cows were initially examined by ultrasonography (US) at day 17 ± 3 of lactation and 23.4% of them had a CL (n = 147). Cows with a corpus luteum (CL) detected on the first US at 17 ± 3 DIM were excluded from enrollment in the experiment, although used in some univariable analyses. The remaining cows with no detected CL (n = 490) were stratified by parity and randomly allocated (by the tossing of a coin) to one of the two treatments: GnRH (n = 245) i.m. injection of 100 µg gonadotropin releasing hormone (gonadorelin hydrochloride, Factrel®, Zoetis, Madison, NJ) at 17 ± 3 DIM (GnRH-1) and again 3.5 days later (Friday afternoon), at 20 ± 3 DIM (GnRH-2); or control (n = 245), no GnRH treatment. Figure 3-1 shows the schematic diagram of study activities.

Body condition (BCS), based on a 5 points scoring system (Ferguson et al., 1994) was scored three times during the study, at calving, at enrolment on day 17 ± 3 DIM and at 35 DIM. Descriptive statistic of variables of interest shown in Table 4-1.

Ovarian ultrasonography were performed twice a week using a portable ultrasonography scanner (Ease Scan, BCF Technology, Livingston, UK) with a 7.5-MHz linear transducer, starting at 17 ± 3 DIM and continued until ovulation was detected or the cow began an ovulation synchronization protocol in Dairy 1. In Dairy 2, ultrasonography was also done twice a week for up to 28 ± 3 DIM. Size of follicles and corpus luteum/corpora lutea (CL) were measured and recorded. Ovulation was characterized by the appearance of a CL ≥ 20mm in any US or when CL < 20 mm appeared in two consecutive US. Ovulation to GnRH-1 was characterized by appearance of a CL in the US at 20 ± 3 DIM or the disappearance of a follicle > 10 mm present in the US at 17 ± 3 DIM but absent in the US at 20 ± 3 DIM and the appearance of a CL in the US at 24 ± 3 DIM. After excluding cows that had already ovulated to GnRH-1, ovulation to GnRH-2 was evaluated following the same criteria as ovulation to GnRH-1; however, evaluation started on the day of the GnRH-2 and cows either had to have the appearance of a CL in the US at 24 ± 3 DIM or the disappearance of a follicle > 10 mm that was present in the US at 20 ± 3 DIM but absent in the US at 24 ± 3 DIM, and the appearance of a CL in the US at 28 ± 3 DIM. Therefore, ovulation to GnRH-1 or GnRH-2 was the overall ovulation from 17 ± 3 DIM up to 24 ± 3 DIM.

Uterine Health

Clinical endometritis (CE) was diagnosed in both dairies at 5 weeks post-partum. Cytological endometritis (CTE) was only diagnosed in Dairy 1. Clinical endometritis was determined using a Metricheck device (Simcro Tech Ltd. Hamilton, New Zealand). After cleaning of the vulva, the Metricheck device, a 50 cm long stainless steel rod with a silicon cup in one end (4 cm in diameter) was inserted into the vagina until the tip reached the fornix of the vagina. The device was then retracted. Material adhered on the internal surface of the silicon cup

was assessed visually and classified according to the method of Williams et al. (2005) on a scale from 0 to 3. Clear or translucent mucus was scored as 0; score 1was described as mucus containing flecks of white or off-white pus; score 2 is a discharge containing less than 50% of white or off-white mucopurulent material and sanguineous discharge or discharge composed by more than 50% of white or yellow pus was scored 3 (Williams et al., 2005). Scores equal or greater than 2 were considered positive for CE (Sheldon et al., 2006).

Cytological endometritis was characterized by neutrophil percentage ≥ 10% on cytology (Sheldon et al., 2006). Basically, after cleaning and disinfecting the external vulva with alcohol 70%, a Cytobrush® Plus Cell collector (CooperSurgical, Inc., Trumbull, CT) attached to a stainless steel modified AI gun and protected by a plastic sheath protector (Continental Plastic Corp.; Delavan, WI) was carefully introduced in the vagina, through the cervix and into the body of the uterus, then the brush was inserted past the sheath protector and pressed against the endometrium while being rotated for three times. The brush was retrieved into the stainless steel AI gun and removed from the animal. The brush was then disconnected and smeared onto a glass slide and left to air dry. Smeared slides were then Wright-Geimsa stained with Camco Stain Pak 702 (Cambridge Diagnostic Products Inc.; Fort Lauderdale, FL) and left in room temperature to dry. Reading of the slides was done in a manner so that investigator was unaware of the treatments. A total of 200 cells per slide were counted, including all leukocyte types and epithelial cells, but excluding erythrocytes. The proportion of PMN was determined and cytological endometritis considered with ≥ 10 % of PMN, as described previously (Kasimanickam et al., 2004; Sheldon et al., 2006).

Reproductive Management

Cows were managed under the respective dairies reproductive programs (Figure 3-1): in Dairy 1 cows were presyncronized with 2 injections of prostaglandin F2α (PGF2α) 14 days apart

(PreSynch) at 41 and 55 ± 3 DIM, followed 12 days later (67 ± 3 DIM) by Ovsynch-56 TAI protocol (GnRH, 7 days later PGF2α, 56 hours later GnRH followed by timed artificial insemination 16-20 hours). Pregnancy diagnosis was made by transrectal ultrasonography at day 32 after AI (i.e., pregnancy was characterized by the presence of an amniotic vesicle containing a live embryo - heart beat present during ultrasonography) and reconfirmation of the pregnancy by transrectal palpation was performed on day 74 after conception. Cows diagnosed not pregnant at the time of pregnancy diagnosis were resynchronized on the same day using the Ovsynch-56 TAI program described and inseminated 10 days later.

Dairy 2 had distinct reproductive protocols based on parity. Cows in their first and second lactation were presynchronized with 2 injections of PGF2α 14 days apart at 50 and 64 ± 3 DIM, followed 12 days later (76 ± 3 DIM) by Ovsynch-56 TAI protocol. Cows in their third or greater lactation were presynchronized with 2 injections of PGF2α 14 days apart at 55 and 69 ± 3 DIM, followed 12 days later (81 ± 3 DIM) by Ovsynch-56 TAI protocol. Nonetheless, if cows were detected in estrus after the second PGF2α of the PreSynch AI was performed and cows were automatically removed from the synchronization of ovulation program. Pregnancy diagnosis was performed at 40 ± 3 days after AI and reconfirmation at 85 ± 3 days after AI by palpation per rectum of the gravid uterine horn. Cows diagnosed not pregnant at pregnancy diagnosis were resynchronized on the same day using the Ovsynch-56 program. Cows detected in estrus were considered non pregnant and were immediately AI.

Periparturient diseases such as abortion (gestation less than 260 days), stillbirth (born dead from a gestation of 261 days or more), dystocia (calving ease score ≥ to 2 on the 5 points scale system; 1 = calving without assistance; 2 = light help with use of obstetric chains; 3 = moderate force to deliver the calf; 4 = extreme force to deliver the calf; 5 = cesarean-section or

fetotomy), hypocalcemia (clinical diseases characterized by hypothermia and incapability to stand up ruled out other diseases), retained fetal membranes (fetal membranes retained for after at least 12 hrs after parturition), metritis (reddish, fetid fluid vaginal discharge within 2 weeks postpartum), and ketosis (ketones body detected on urine before and/or after study enrolment) were diagnosed by the farm personnel and recorded in an on-farm computer software for on-farm retrieval from both dairies.

Sample Size and Statistics

A sample size of 240 cows per group was calculated (Minitab Inc, State College, PA) for $\alpha = 0.05$ and $\beta = 0.2$, to detect differences in pregnancy per artificial insemination (P/AI) of 8% when P/AI varies from 32% to 40%.

Outcomes of interest were: ovulation to the GnRH-1 and/or GnRH-2; prevalence of clinical and subclinical endometritis at 7 weeks postpartum; pregnancy to the first service diagnosed between 32 and 42 days post service (PD1) and between 74 to 90 days post service (PD2), pregnancy loss from the first service (defined as the number of cows pregnant from the first service that were not pregnant on the pregnancy reconfirmation divided by the number of cows diagnosed pregnant on the first pregnancy diagnosis) and hazard rate of pregnancy up to 300 DIM (measured 74 to 90 days after breeding). Explanatory variables included in the model were treatment status (GnRH-treated, control), calving season (cool [November to May] vs. warm [May to September]), dairy (Dairy 1 vs. Dairy 2), parity (primiparous vs. multiparous), calving related problems (group of the following events: abortion, stillbirth, dystocia, twins and RFM), metabolic problems (hypocalcaemia and ketosis), metritis, body condition score at study enrollment date (BCS $\leq$ 2.75 vs. BCS > 2.75), ovulation to GnRH-1 or GnRH-2 (yes or no), and two way interactions between GnRH treatment and other covariates. Treatment with GnRH was the main effect of interest and was forced in all the models.

Binary outcomes were analyzed by logistic regression using the LOGISTIC procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC) and backward elimination was performed by removing explanatory variables from the model with $P > 0.05$ according to Wald-statistics criterion. Hazard of ovulation up to 70 DIM and hazard to pregnancy up to 300 DIM at PD2 were analyzed by Cox's proportional hazard model using the PHREG procedure of SAS, in which 255 cows were included in this analysis. Three models were generated; first model with all variables included, except ovulation status by 24 DIM because GnRH treatment affected ovulation by 24 DIM and early ovulation is known to affect fertility; therefore, ovulation by 24 DIM would become an intermediate variable. The second model was composed by all variables except treatment status to evaluate the effect of ovulation by 24 DIM on hazard of pregnancy. The third model included GnRH treatment, ovulation by 24 DIM, and interaction between GnRH treatment and ovulation by 24 DIM besides other covariates. Hazard ratio (HR) was characterized as the daily probability of a given event (ovulation or pregnancy). The variable time was the interval in days from calving to ovulation or to pregnancy. Ten cows (5 from each group) were excluded from the study because they were sold, died or became recumbent before 28 DIM; therefore, ovulation could not be confirmed, or cows did not receive their treatment as assigned. Hence, only 480 cows (240 per group) were included in the multivariable analysis. Cows that were sold or died after 28 DIM or did not ovulated by 70 DIM were censored in the analysis of time to ovulation. Cows that were sold or died after 28 DIM, or did not conceive by 300 DIM were censored in the analysis of time to pregnancy. For the analysis of time to pregnancy, cows were considered pregnant if they were confirmed pregnant at PD2, and 480 cows were included in the analysis. In the analysis of clinical endometritis, 435 cows were included in the analysis (217 and 215 for GnRH and control groups, respectively), and 245 cows

were included in the cytological endometritis analysis (123 and 122 for GnRH and control groups, respectively). Twenty one cows were called do not breed due different reasons and were excluded of the analysis of conception rate at PD1 and PD2, leaving 459 cows in the analysis (231 and 228 cows for GnRH and control groups, respectively). One hundred and fifty five cows conceived of the first service and were included in the analysis of pregnancy loss. Kaplan-Meier plots and median days to ovulation by 70 DIM and days to pregnancy by 300 DIM were generated using MedCalc version 12.7 for Windows (MedCalc Software, Mariakerke, Belgium). When interaction between two dichotomous variables was detected, a new variable containing all the 4 combinations (dummy variables between variables 1 and 2 for example would be: [variable 1= No, variable 2 = No]; [variable 1 = Yes, variable 2 = No]; [variable 1 = No, variable 2 = Yes] and [variable 1 = Yes, variable 2 = Yes]) was created and the model was run again including only the new variable. Univariable survival analysis was generated to evaluate time to pregnancy up to 300 DIM according to GnRH-treatment and ovulation including cows that had a CL at the first US. Statistical significance was considered when *P-value* ≤ 0.05.

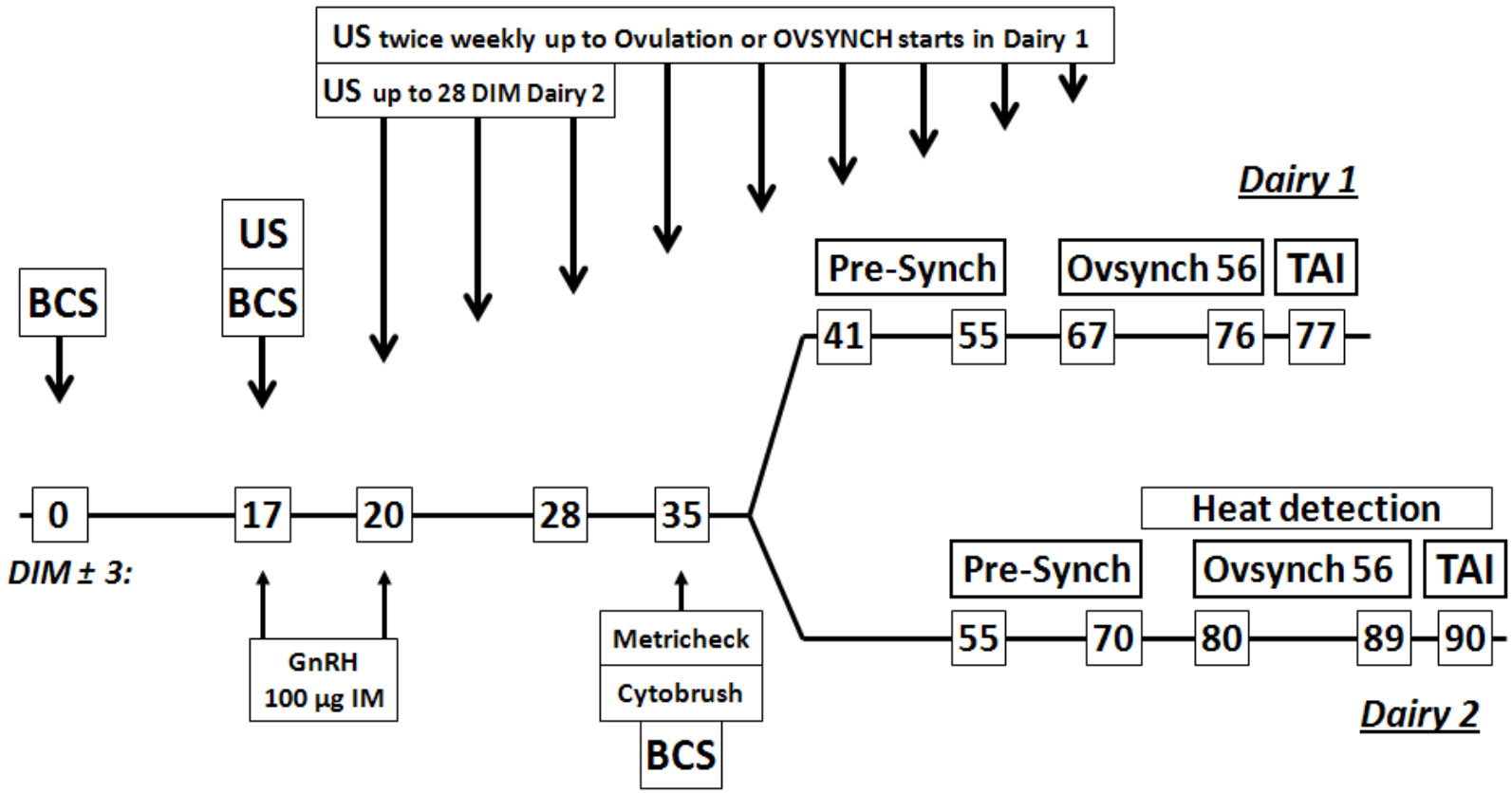

Figure 3-1. Schematic diagram of study activities. Weekly a cohort of cows without a corpus luteum at 17 ± 3 DIM was stratified by parity and randomly assigned to GnRH group (100 µg i.m. of ; Factrel® 50 µg/mL, Zoetis Ltd., Madison, NJ) or remain as control (no GnRH injection). DIM = days in milk. BCS = body condition score. US = ultrasonography. Metricheck = evaluation of the vaginal discharge using metricheck device. Cytobrush = uterine cytology. Pre-Synch = prostaglandin F2α injection 14 days apart. Ovsynch 56 = GnRH, 7days later PGF2α, 56 hrs later GnRH, 16-20 hrs later TAI. TAI = timed artificial insemination.

CHAPTER 4
RESULTS

Descriptive Statistics

Descriptive statistics is shown in Table 4-1. Of the 480 cows used in the analysis, 255 were from Dairy 1 and 225 were from Dairy 2; 216 were primiparous and 264 were multiparous; 114 had BCS ≤ 2.75 and 366 had BCS > 2.75 at 17 ± 3 DIM; 321 calved in the warm season (May to September) and 159 calved in the cool season (October to April). Prevalence of calving problems, metabolic problems and metritis were 32.9 %, 27.1 % and 20.6%, respectively. None of the variables was significantly different between GnRH-treated and control cows.

Ovulation

Treatment with GnRH significantly increased ($P < 0.001$) ovulation rates within 3.5 days from GnRH-1 or GnRH-2. Ovulation within 3.5 days of GnRH-1 was 44.6% for GnRH-treated cows and 20.9% for control cows. GnRH-treated cows had 2.9 times the odds of ovulation to GnRH-1 compared to control cows (95 % CI = 1.9 to 4.4; Table 4-2). BCS at study enrollment also affected ($P = 0.01$) ovulation within 3.5 days of GnRH-1, and 36.3% of the cows with BCS > 2.75 ovulated compared to 21.7% of the cows with BCS ≤ 2.75. Cows with BCS > 2.75 had 1.9 times the odds of ovulation within 3.5 days of GnRH-1 compared to cows with BCS ≤ 2.75 (95 % CI = 1.1 to 3.1). Ovulation within 3.5 days of GnRH-2 was 61.7 % for GnRH-treated cows and 30.5 % for control cows. Ovulation within 3.5 days of GnRH-2 was also influenced by calving season, and ovulation was higher in the warm season than in the cool season (49.5 % vs. 32.2 %; OR = 2.8; 95% CI = 1.6 to 4.9; P < 0.001). Overall ovulation by 24 ± 3 DIM (response to either GnRH-1 or GnRH-2, measured 3.5 days after treatment) was significantly higher for GnRH-treated cows than control cows (78.7% vs. 45.0%; OR = 4.7; 95% CI = 3.2 to 7.5; $P < 0.001$). Cows that calved in the warm season also had higher overall ovulation by 24 ± 3 DIM

than cows that calved in the cool season (67.3% vs. 50.9%; OR = 2.2.; 95% CI = 1.4 to 3.3; $P <$ 0.001). Median days to ovulation up to 70 DIM was decreased by 8 days ($P = 0.003$) for GnRH-treated cows compared to control cows (24 d vs. 32 d; Figure 4-1).

Uterine Health Outcomes

Administration of GnRH early postpartum did not affect the prevalence of CE (Table 4-3). Prevalence of CE was 23.9 % for GnRH-treated cows and 18.6 % for control cows. The prevalence of CE was significantly higher for cows that had calving problems (32.6 vs. 15.9%; OR = 2.2; 95% CI = 1.3 to 3.6; $P = 0.001$) and metritis (40.6 vs. 15.8%; OR = 3.1; 95% CI = 1.9 to 5.4; $P = 0.001$) compared to cows that did not have these conditions. The prevalence of CTE was also not different between GnRH-treated and control cows (30.9% vs. 32.8%; $P = 0.61$). Cows that had metritis had increased prevalence of CTE (50.7 vs. 23.5%; OR = 3.4; 95% CI = 1.9 to 5.9; $P < 0.001$).

Reproductive Outcomes

Conception to the first service diagnosed at PD1 was similar for GnRH and control groups (37.6% vs. 38.6%; OR = 0.8; 95% CI = 0.5 to 1.2; $P = 0.29$; Table 4-4). Cows that ovulated by 24 ± 3 DIM (40.9 vs. 33.4%; OR = 1.6; 95% CI = 1.1 to 3.0; $P = 0.04$), cows in Dairy 1 (45.4 vs. 30.1%; OR = 2.0; 95% CI = 1.4 to 2.9; $P < 0.001$) and cows with BCS > 2.75 at study enrollment (41.6 vs. 26.6%; OR = 1.8; 95% CI = 1.1 to 3.1; $P = 0.01$) had higher conception at first service. Table 4-5 shows conception to the first service diagnosed at PD2, in which only dairy (39.6 vs. 26.5% for Dairy 1 and Dairy 2, respectively; OR = 1.8; 95% CI = 1.2 to 2.7; $P = 0.004$) and BCS at study enrollment (36.5 vs. 22.8% for BCS > 2.75 and BCS ≤ 2.75, respectively; OR = 1.8; 95% CI = 1.1 to 3.1; $P = 0.015$) had significant effect. Pregnancy loss from the first service diagnosed at PD2 (Table 4-6) was significantly higher for control group compared to GnRH-treated group (18.1% vs. 6.8%; OR = 6.25; 95% CI = 2.1 to 20.2; $P < 0.01$);

for cows that had metritis compared to cows that did not have metritis (21.5% vs. 9.7%, OR = 3.6; 95% CI = 1.3 to 10.2;*P* = 0.02); and for cows that ovulated from G1G2 compared to cows that did not ovulate from G1G2 (14.5% vs. 8.6%, OR = 3.9; 95% CI = 1.2 to 14.2; *P* = 0.02). In the CL group (cows with detectable CL at study enrollment day), the pregnancy loss was 6.9%.

Figure 4-2 shows time to pregnancy up to 300 DIM at PD2 according to treatment status for the GnRH-treated and control groups that respectively had median days to pregnancy and proportion of cows pregnant by 300 DIM of 122 - 78.8 % and 136 - 76.3 % (univariable analysis containing only treatment status, *P* = 0.9). Yet Figure 4-3 shows similar analysis as the previous however, with the CL group included with median days to pregnancy of 120 days and 78.3 % of cows pregnant by 300 DIM (univariable analysis containing only treatment status, and with CL group included; *P* = 0.5). Figure 4-4 shows time to pregnancy up to 300 DIM at PD2 according to ovulation status for cows without CL at study enrollment that did or did not ovulate by 24 DIM. The respective median days to pregnancy and proportion of cows pregnant by 300 DIM for cows that ovulated and for cows that did not ovulate were 121 - 81.8 % and 155 - 70.5 % (univariable analysis containing only ovulation status, *P* = 0.001). In Figure 4-5, similar analysis performed in Figure 4-4 is showed, although including the CL group (cows with a detectable CL at 17 ± 3 DIM) in which the median days to pregnancy and proportion of cows pregnant by 300 DIM were similar as showed above for cows that ovulated and for cows that did not ovulate by 24 ± 3 DIM, and 120 - 78.3 % for the CL group (univariable analysis containing only ovulation status, which CL group is included; *P* = 0.003).

Hazard of pregnancy up to 300 DIM at PD2 for the first model (multivariable analysis with all variables except ovulation status by 24 DIM; treatment status composed only by GnRH-treated and control groups) was influenced only by BCS, in which cows with BCS > 2.75 had

higher hazard of pregnancy than cows with BCS ≤ 2.75 at 17 ± 3 DIM (HR= 1.3; 95% CI = 1.02 to 1.6; *P* = 0.03). Treatment status did not have effect in the hazard of pregnancy in the first model (*P* = 0.91; Table 4-7).

In the second model (composed with all variables except treatment status) only ovulation had significant effect (*P* < 0.01; Table 4-8) in hazard of pregnancy up to 300 DIM at PD2, in which cows that ovulated by 24 DIM had higher hazard of pregnancy compared to cows that did not ovulated by 24 DIM (HR =1.4, 95% CI = 1.1 to 1.8). Regarding the third model (composed with all variables), treatment status and the interaction treatment with ovulation had significant effects in the hazard of pregnancy up to 300 DIM at PD2 (*P* = 0.03 and *P* = 0.05, respectively). Ovulation had no effect on hazard of pregnancy in the third model (*P* = 0.17).Table 4-9 shows the interaction of treatment with ovulation for the third model. In which GnRH-treated cows that ovulated to G1G2 had 2 times the hazard of pregnancy compared to GnRH-treated cows that did not ovulate (95% CI = 1.3 to 2.9; *P* < 0.001), and 1.3 times the hazard of pregnancy compared to control cows that failed to ovulate by 24 DIM (95% CI = 1.0 to 1.7; *P* = 0.05). There was no difference in hazard of pregnancy for GnRH-treated cows that ovulated compared to control cows that also ovulated (*P* = 0.70). Control cows that ovulated and that did not ovulate had no difference in the hazard of pregnancy among themselves (*P* = 0.17), and both groups had higher hazard of pregnancy than GnRH-treated cows that did not ovulate by 24 DIM (HR = 2.0; 95% CI = 1.2 to 2.8; *P* = 0.002, and HR = 1.7; 95% CI = 1.03 to 2.5; *P* = 0.03, for control cows that ovulated and control cows that did not ovulate, respectively). Median days to pregnancy and proportion of cows pregnant by 300 DIM for GnRH-treated cows that ovulated, GnRH-treated cows that did not ovulate, control cows that ovulated and control cows that did not ovulated by

24 DIM were 119 d – 80.9 %, 168 d – 58.9 %, 133 d – 83.4 %, and 140 d – 74.9 %, respectively (Figure 4-6).

Table 4-1. Descriptive statistics for GnRH-treated and control groups.

Variables-level	All cows n	GnRH[1] n (%)	Control[1] n (%)	*P-value*
Location				
Dairy 1	255	127 (49.8%)	128 (50.2%)	0.91
Dairy 2	225	112 (49.8%)	113 (50.2%)	-
Parity				
Primiparous	216	110 (50.9%)	106 (49.1%)	0.71
Multiparous	264	130 (49.2%)	134 (50.8%)	-
Calving season[2]				
Warm	321	161 (50.1%)	160 (49.9%)	0.92
Cool	159	79 (49.7%)	80 (50.3%)	-
Calving problems[3]				
Yes	158	83 (52.5%)	75 (47.5%)	0.43
No	322	157 (48.7%)	165 (51.3%)	-
Metabolic problems[4]				
Yes	130	60 (46.1%)	70 (53.9%)	0.30
No	350	180 (51.4%)	170 (48.6%)	-
Metritis[5]				
Yes	99	53 (53.5%)	46 (46.5%)	0.43
No	381	187 (49.1%)	194 (50.9%)	-
BCS enrollment[6]				
≤ 2.75	114	48 (42.1%)	66 (57.9%)	0.06
> 2.75	366	192 (52.4%)	174 (47.6%)	-

[1]GnRH treated group, cows received 100µg i.m. of gonadorelin hydrolochloride at 17 ± 3 and 20 ± 3 DIM (n = 240) and control group had no hormonal treatment (n = 240).
[2]Calving season characterized as warm (May to September) or cool (November to May).
[3]Calving problems (i.e. abortion, dystocia, retain fetal membranes, twins or stillbirth).
[4]Metabolic problems (i.e. ketosis, hypocalcemia).
[5]Metritis, characterized for malodorous reddish fluid vaginal with or without fever, diagnosis following the dairy standard operation procedures.
[6]Body condition score evaluation based on Ferguson et al., 1994 (5 points scale varying from 1 = thin to 5 = fat) evaluated at study day enrollment (17 ± 3 DIM).

Table 4-2. Effects of GnRH administration at 17 and 20 ± 3 days postpartum on ovulation response in dairy cows.

Outcome variable	Outcome level	Stratum	Cows, n	%	OR[1]	95% CI[2]	*P-value*
Ovulation G1[3]	Treatment Groups	GnRH	240	44.6	2.9	1.9 to 4.4	< 0.001
		Control	240	20.9	referent	-	-
	BCS enrolment[4]	> 2.75	366	36.3	1.9	1.1 to 3.1	0.01
		≤ 2.75	114	21.7	referent	-	-
Ovulation G2[3]	Calving season[5]	Warm	208	49.5	2.6	1.5 to 4.6	< 0.001
		Cool	115	32.2	referent	-	-
	GnRH*Parity	Prim*GnRH	110	78.2	3.0	1.6 to 5.7	< 0.001
		Mult*GnRH	130	79.2	2.3	1.2 to 4.2	0.008
		Prim*Control	40	37.7	0.5	0.2 to 0.9	0.02
		Mult*Control	134	50.7	referent	-	-
Ovulation G1 & G2[3]	Treatment Groups	GnRH	240	78.7	4.7	3.2 to 7.5	< 0.001
		Control	240	45.0	referent	-	-
	Calving season	Warm	321	67.3	2.2	1.4 to 3.3	< 0.001
		Cool	159	50.9	referent	-	-

[1]OR = Odds ratio.
[2] CI = 95% confidence interval.
[3]Cows in the GnRH group received an i.m. injection of 100µg of gonadorelin hydrochloride at 17 ± 3 DIM (G1) and at 20 ± 3 DIM (G2). Ovulation to G1 or G2 was evaluated up to 3.5 days post administration.
[4]Body condition score evaluation based on Ferguson et al., 1994 (5 points scale varying from 1 = thin to 5 = fat) evaluated at study day enrolment (17 ± 3 DIM).
5Calving season characterized as warm (May to September) or cool (November to May).

Table 4-3. Effects of GnRH administration at 17 and 20 ± 3 days postpartum and other variables on prevalence of clinical[1] (CE) and cytological endometritis[2] (CTE) in dairy cows.

Outcome variable	Outcome level	Stratum	Cows, n	%	OR[3]	95% CI[4]	*P-value*
Clinical endometritis	Treatment groups	GnRH	217	23.9	1.3	0.8 to 2.1	0.23
		Control	215	18.6	referent	-	-
	Calving problems[5]	Yes	138	32.6	2.2	1.3 to 3.6	0.001
		No	294	15.9	referent	-	-
	Metritis[6]	Yes	91	40.6	3.1	1.9 to 5.4	< 0.001
		No	341	15.8	referent	-	-
Cytological endometritis	Treatment groups	GnRH	123	30.9	0.9	0.5 to 1.5	0.61
		Control	122	32.8	referent	-	-
	Metritis	Yes	75	50.7	3.4	1.9 to 5.9	< 0.001
		No	170	23.5	referent	-	-

[1]Clinical endometritis, characterized by score ≥ 2 on metricheck evaluation at 35 DIM.
[2]Cytological endometritis, characterized by presence of ≥ 10% PMNL in the uterine cytology at 35 DIM. CTE evaluated only in Dairy 1.
[3]OR = Odds ratio.
[4]CI = 95% confidence interval.
[5]Calving problems (abortion, dystocia, retain fetal membranes, twins or stillbirth).
[6]Metritis, characterized for malodorous reddish fluid vaginal with or without fever, diagnosis following the dairy standard operation procedures.

Table 4-4. Effects of GnRH administration at 17 and 20 ± 3 days postpartum in dairy cows without a CL at study enrolment on conception rate at PD1 from first service.

Outcome level	Stratum	Cows, n	%	OR[1]	95% CI[2]	*P-value*
Treatment groups	GnRH	231	37.6	0.8	0.5 to 1.2	0.29
	Control	228	38.6	referent	-	-
Dairy	1	240	45.4	2.0	1.4 to 2.9	< 0.001
	2	219	30.1	referent	-	-
Ovulation G1G2[3]	Yes	286	40.9	1.6	1.1 to 3.0	0.044
	No	173	33.4	referent	-	-
BCS enrollment[4]	> 2.75	354	41.6	1.8	1.1 to 3.1	0.014
	≤ 2.75	105	26.6	referent	-	-

[1]OR = Odds ratio.
[2] CI = 95% confidence interval.
[3]Ovulation response to G1 or G2, monitoring for ovulation up to 3.5 days post GnRH administration (i.m. injection of 100µg of gonadorelin hydrochloride at 17 ± 3 DIM and at 20 ± 3 DIM respectively).
[4]Body condition score evaluation based on Ferguson et al., 1994 (5 points scale varying from 1 = thin to 5 = fat) evaluated at study day enrolment (17 ± 3 DIM).

Table 4-5. Effects of GnRH administration at 17 and 20 ± 3 days postpartum in dairy cows without a CL at study enrolment on conception rate at PD2 from first service.

Outcome level	Stratum	Cows, n	%	OR[1]	95% CI[2]	*P-value*
Treatment groups	GnRH	231	35.0	1.1	0.7 to 1.6	0.53
	Control	228	33.5	referent	-	-
Dairy	1	240	39.6	1.8	1.2 to 2.7	0.004
	2	219	26.5	referent	-	-
BCS enrollment[3]	> 2.75	354	36.5	1.8	1.1 to 3.1	0.015
	≤ 2.75	105	22.8	referent	-	-

[1]OR = Odds ratio.
[2] CI = 95% confidence interval.
[3]Body condition score evaluation based on Ferguson et al., 1994 (5 points scale varying from 1 = thin to 5 = fat) evaluated at study day enrolment (17 ± 3 DIM).

Table 4-6. Effects of GnRH administration at 17 and 20 ± 3 days postpartum in dairy cows without a CL at study enrolment on pregnancy loss of the first service[1].

Outcome level	Stratum	Cows, n	%	OR[2]	95% CI[3]	*P-value*
Treatment groups	GnRH	87	6.8	0.16	0.05 to 0.5	< 0.01
	Control	88	18.1	referent	-	-
Metritis[4]	Yes	42	21.5	3.6	1.3 to 10.2	0.02
	No	133	9.7	referent	-	-
Ovulation G1G2[5]	Yes	117	14.5	3.9	1.2 to 14.2	0.02
	No	58	8.6	referent	-	-

[1] Calculated as [(No. of pregnant cows on PD1from first service that were not pregnant on PD2 from first service / No. of pregnant cows on PD1 from first service)*100].
[2]OR = Odds ratio.
[3] CI = 95% confidence interval.
[4]Metritis, characterized for malodorous reddish fluid vaginal with or without fever, diagnosis following the dairy standard operation procedures.
[5]Ovulation response to G1or G2, monitoring for ovulation up to 3.5 days post GnRH administration (i.m. injection of 100µg of gonadorelin hydrochloride at 17 ± 3 DIM and at 20 ± 3 DIM respectively).

Table 4-7. Effects of GnRH administration at 17 and 20 ± 3 days postpartum during the first month of lactation on time to pregnancy up to 300 DIM based on PD2 for Model 1 (Multivariable analysis with all variables included, except ovulation).

Outcome level	Stratum	Cows, n	HR[1]	95% CI[2]	*P-value*
Treatment	GnRH[3]	240	1.0	0.8 to 1.2	0.91
	Control	240	referent	-	-
BCS enrollment[4]	> 2.75	365	1.3	1.02 to 1.6	0.03
	≤ 2.75	115	referent	-	-

[1]HR = Hazard ratio.
[2] CI = 95% confidence interval.
[3]GnRH treated group, cows received 100µg i.m. of gonadorelin hydrolochloride at 17 ± 3 and 20 ± 3 DIM and control group had no hormonal treatment.
[4]Body condition score evaluation based on Ferguson et al., 1994 (5 points scale varying from 1 = thin to 5 = fat) evaluated at study day enrolment (17 ± 3 DIM).

Table 4-8. Effects of GnRH administration at 17 and 20 ± 3 days postpartum during the first month of lactation on time to pregnancy up to 300 DIM based on PD2 for Model 2 (Multivariable analysis with all variables included, except treatment status).

Outcome level	Stratum	Cows, n	HR[1]	95% CI[2]	*P-value*
Ovulation G1G2[3]	Yes	297	1.4	1.1 to 1.8	< 0.01
	No	183	referent	-	-

[1]HR = Hazard ratio.
[2] CI = 95% confidence interval.
[3]Ovulation response to G1or G2, monitoring for ovulation up to 3.5 days post GnRH administration (i.m. injection of 100µg of gonadorelin hydrochloride at 17 ± 3 DIM and at 20 ± 3 DIM respectively).

Table 4-9. Effects of GnRH administration at 17 and 20 ± 3 days postpartum during the first month of lactation on time to pregnancy up to 300 DIM based on PD2 for Model 3 (Multivariable analysis with all variables included).

Outcome level	Stratum	Cows, n	HR[1]	95% CI[2]	*P-value*
Treatment*ovulation [3]	-	-	-	-	0.05
	GnRH*Ov [4]	189	1.3	1.0 to 1.7	0.05
	GnRH*NoOv [4]	51	0.6	0.4 to 1.0	0.03
	Control*Ov	132	1.2	0.9 to 1.6	0.17
	Control*NoOv	108	referent	-	-

[1]HR = Hazard ratio.
[2] CI = 95% confidence interval.
[3]Treatment interaction with ovulation characterized in four groups: GnRH*Ov = GnRH-treated cows that ovulated by 28 DIM; GnRH*NoOv = GnRH-treated cows that did not ovulate; Control*Ov = control cows that ovulated by 28 DIM; and Control*NoOv = control cows that did not ovulate.
[4]Ovulation response to G1or G2, monitoring for ovulation up to 3.5 days post GnRH administration (i.m. injection of 100µg of gonadorelin hydrochloride at 17 ± 3 DIM and at 20 ± 3 DIM respectively).

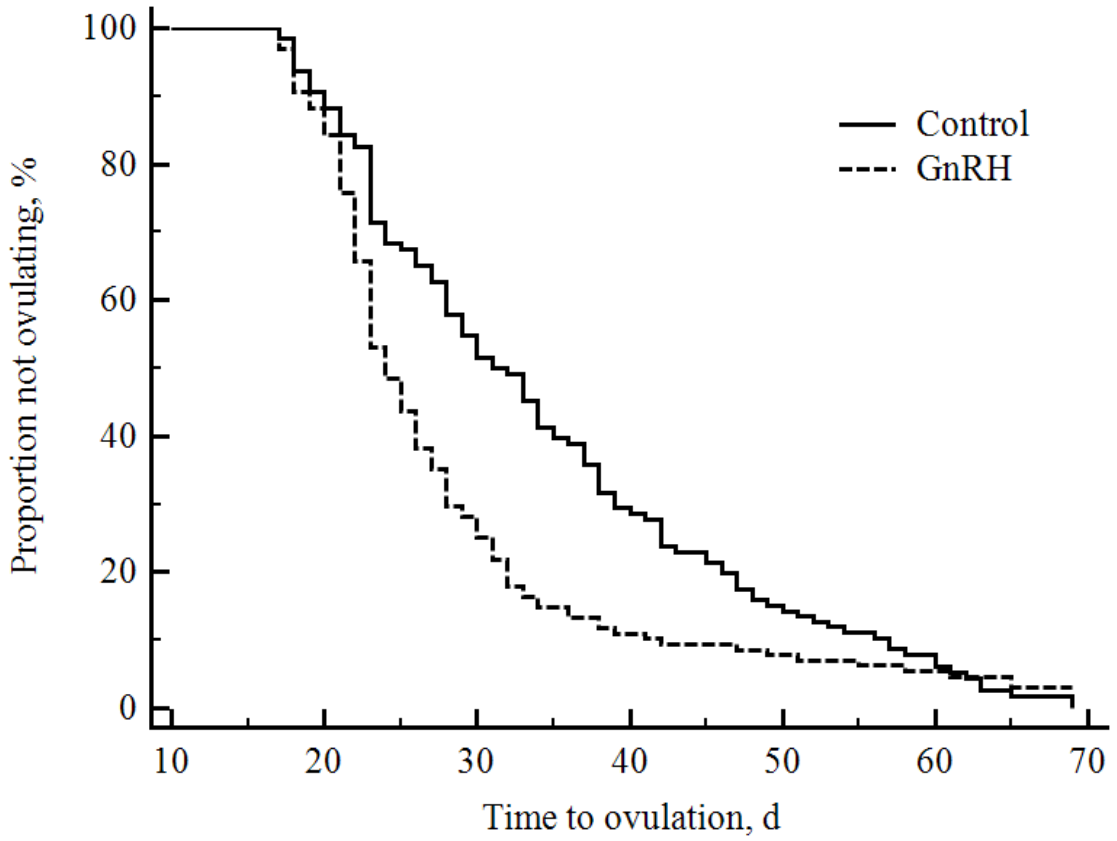

Figure 4-1. Time to ovulation up to 70 DIM for GnRH-treated group (dashed line; n = 128) and control group (solid line; n = 127) in Dairy 1. GnRH-treated group (cows received 100 µg i.m. injection of gonadorelin hydrochloride at 17 ± 3 and 20 ± 3 DIM) and control group (no further injection) had median days to ovulation and proportion of cows that ovulated by 70 DIM of 24 days, 96.8 %, and 32 days, 97.6 % respectively (P = 0.003).

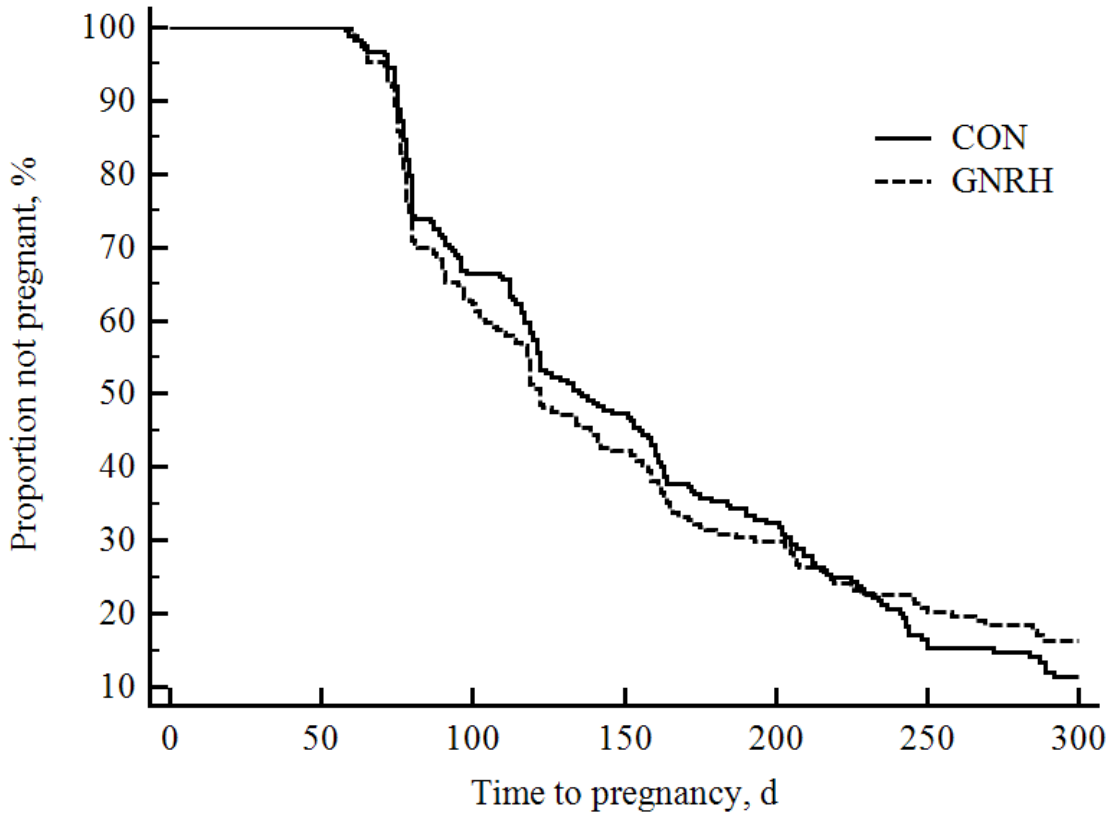

Figure 4-2. Time to pregnancy at PD2 up to 300 DIM for GnRH-treated group (GNRH; dashed line; n = 240) and control group (CON; solid line; n= 240). GnRH-treated group (cows received 100µg i.m. injection of gonadorelin hydrochloride at 17 ± 3 and 20 ± 3 DIM) and control group (no hormonal injection) had median days to pregnancy and proportion of cows pregnant by 300 DIM of 122 days - 78.8 % and 136 days - 76.3 %, respectively (Univariable survival analysis; *P* = 0.93).

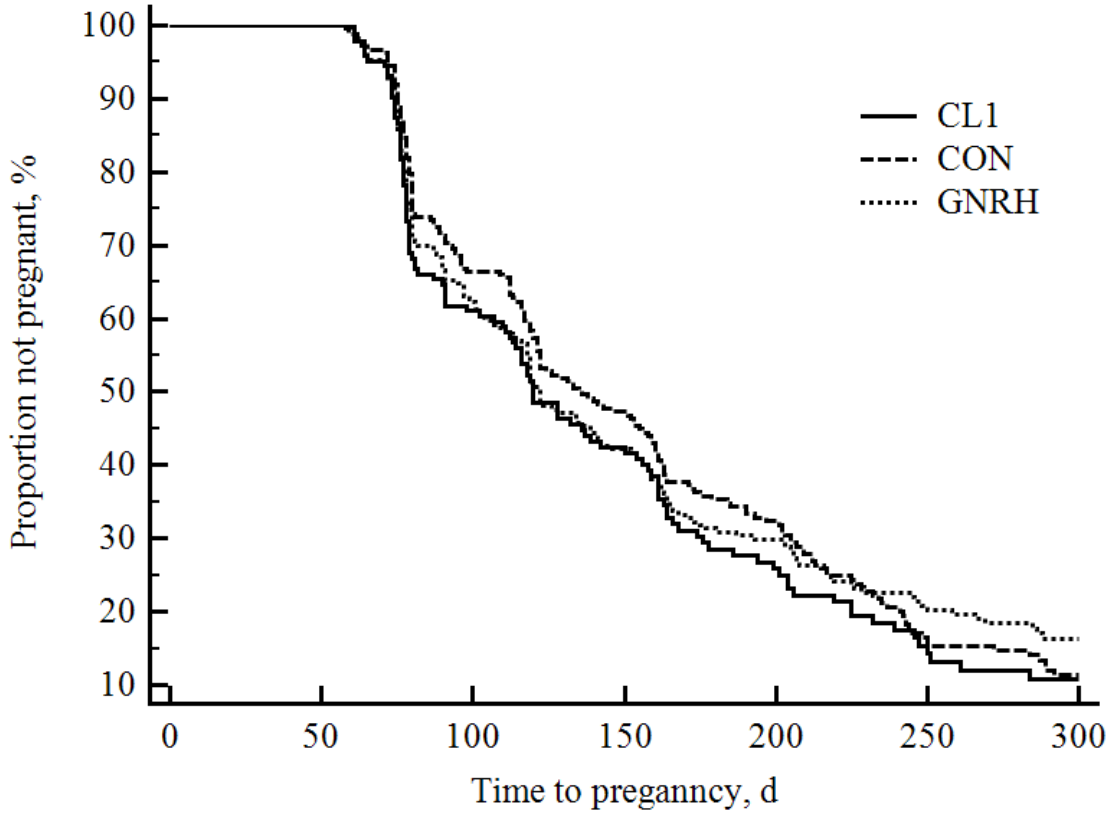

Figure 4-3. Time to pregnancy at PD2 up to 300 DIM for cows with a CL in the beginning of the study (CL1; solid line, n = 147), control group (CON; dashed line; n= 240) and GnRH-treated group (GNRH; dotted line; n = 240). Cows in the GnRH-treated group received 100µg i.m. injection of gonadorelin hydrochloride at 17 ± 3 and 20 ± 3 DIM, and control group no hormonal injection. Median days to pregnancy and proportion of cows pregnant by 300 DIM of 120 days - 78.3 %; 136 days - 76.3 % and 122 days - 78.8 % for CL group, control group and GnRH-treated group respectively (Univariable survival analysis; $P = 0.5$).

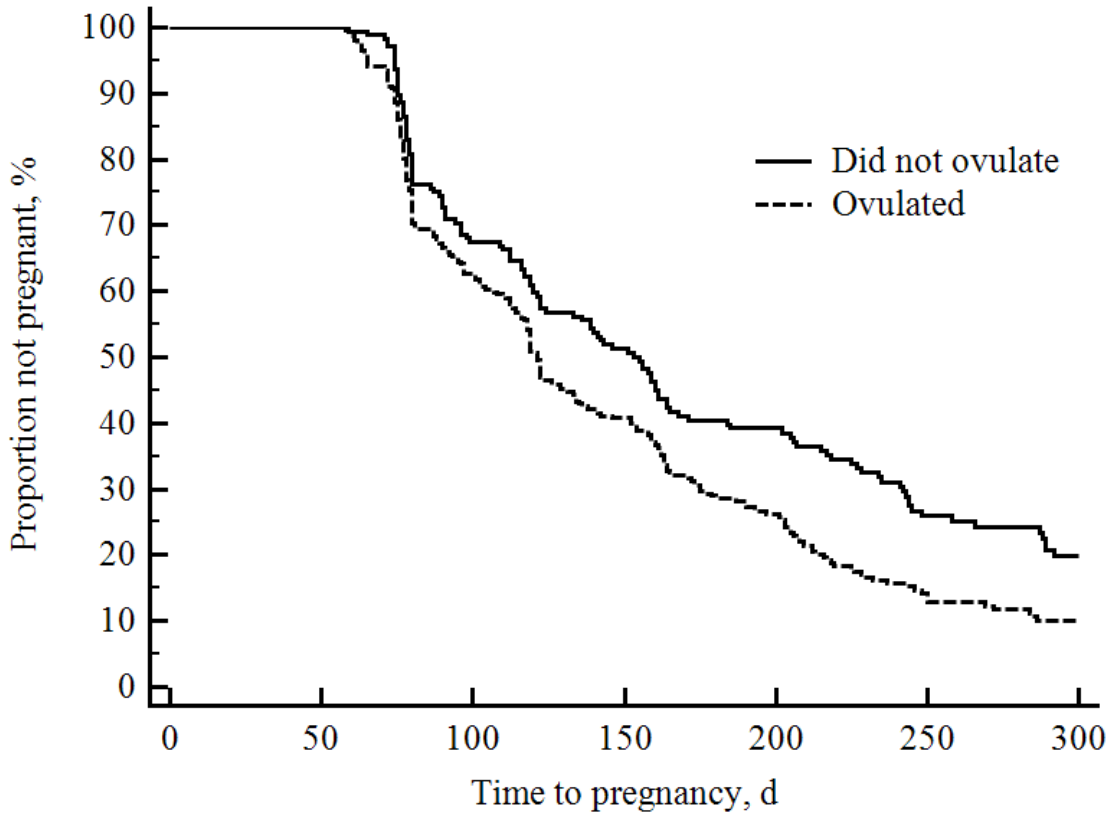

Figure 4-4. Time to pregnancy at PD2 up to 300 DIM according to ovulation status for cows that ovulated (Ovulated; dashed line; n = 297) and cows that did not ovulate (Did not ovulate; solid line; n = 183). Ovulation was characterized from cows enrolled in the study without a CL and evaluated up to 24 DIM; and ovulation group and not ovulation group had median days to pregnancy and proportion of cows pregnant by 300 DIM of 121 days - 81.9 % and 155 days - 70.5 % respectively (Univariable survival analysis; $P = 0.001$).

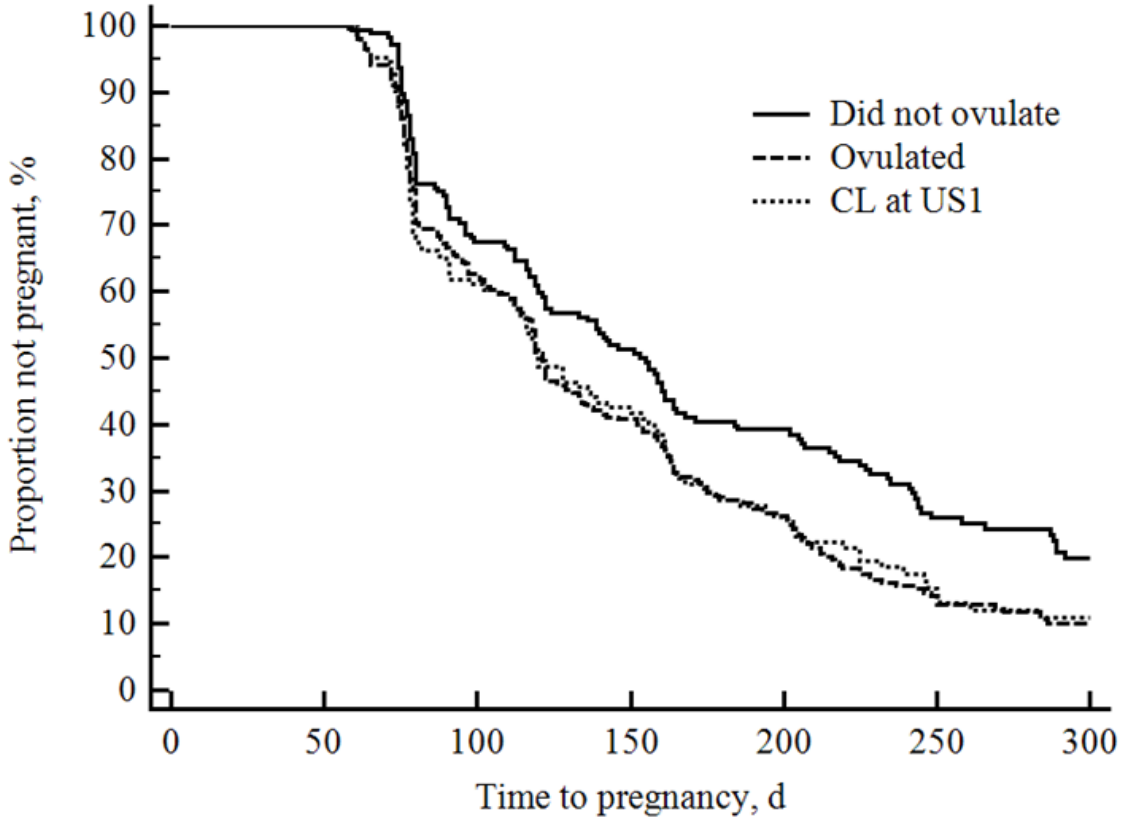

Figure 4-5. Time to pregnancy at PD2 up to 300 DIM according to ovulation status for cows that entered the study without CL and did not ovulate (Did not ovulate; solid line; n = 183), for cows that entered the study without CL and ovulated by 28 DIM (Ovulated; dashed line; n = 297) and for cows with a CL in the beginning of the study (CL1 at US1; dotted line; n = 147). Ovulation was characterized only in cows enrolled in the study without a CL, and evaluated up to 24 DIM. Median days to pregnancy and proportion of cows pregnant by 300 DIM of 155 days - 70.5 %; 121 days - 81.9 % and 120 days - 78.3 % for cows that did not ovulate by 24 DIM , cows that ovulated by 24 DIM and cows with CL at study enrollment, respectively (Univariable survival analysis; $P = 0.003$).

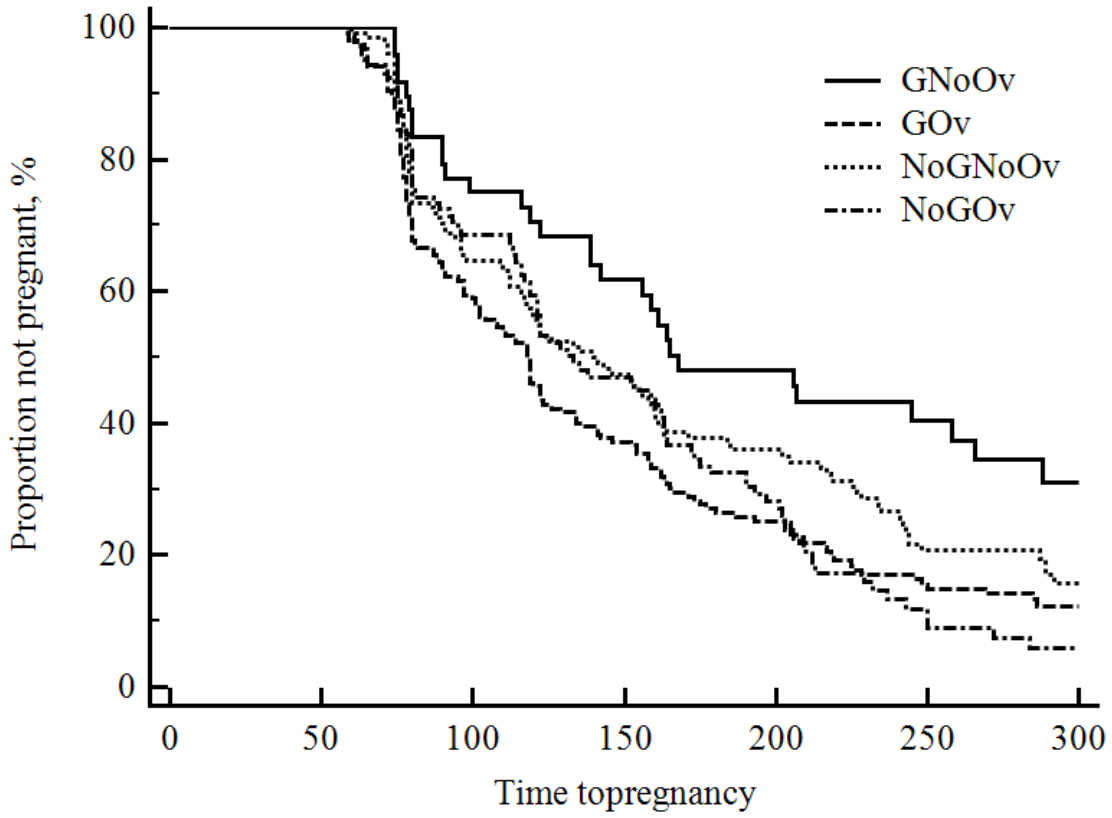

Figure 4-6. Time to pregnancy at PD2 up to 300 DIM according to treatment*ovulation status for GnRH-treated cows that ovulated (GOv; dashed line; n =189) or did not ovulate (GNoOv; solid line; n = 51) and control cows that ovulated (NoGOv; dash-dotted line; n = 108) or did not ovulate (NoGNoOv; dotted line; n = 132) (Multivariable analysis; Treatment*Ovulation; *P* = 0.045). GOv had increased hazard of pregnancy (HR) compared to GNoOv (HR = 2.0; 95% CI = 1.4 to 2.9; *P* < 0.001), or NoGNoOv (HR = 1.3; *P* = 0.05), and similar to NoGOv (HR = 1.1; *P* = 0.70). Control cows that did or that did not ovulate had similar hazard of pregnancy among themselves (HR = 1.2; *P* = 0.17) and higher hazard of pregnancy than GnRH-treated cows that did not ovulate (*P* = 0.002 and *P* = 0.033, respectively). Median days to pregnancy and proportion of cows pregnant by 300 DIM of 119 days - 80.9 %; 168 days - 58.9 %; 133 days - 83.4 %; 140 days - 74.9 % for GOv, GNoOv, NoGOv, NoGNoOv cows respectively.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSIONS

GnRH usage in dairy cows has been evident since the ‘70 and early ‘80 when it was used for the treatment of ovarian follicular cysts in dairy cows (Kittok et al., 1973; Britt et al., 1977; Troxel and Kesler, 1984) and for induction of ovulation in early postpartum (Britt et al., 1974; Fernandes et al., 1978; Kesler et al., 1978). Nowadays, GnRH is used mostly in association with prostaglandins (Richardson et al., 1983; Benmrad and Stevenson, 1986; Thatcher et al., 1993; Pursley et al., 1997) and or progestins (Thatcher et al., 1993; Lynch et al., 1999; Xu et al., 2000; Lima et al., 2011; Bisinotto et al., 2013) for the purpose of synchronization of ovulation as part of TAI protocol.

The randomized clinical trial reported herein was designed to evaluate the effect of GnRH agonist administration at 17 ± 3 and 20 ± 3 DIM on ovulation responses by the end of the fourth week postpartum, prevalence of CE and CTE at 35 DIM and reproductive outcomes from the first service and up to 300 DIM. Our results showed that both the GnRH-1 and GnRH-2 increased ovulation within 3 to 4 days of administration compared to control cows which resulted in overall greater ovulation by 24 DIM (78.7% vs. 45.0%). This findings support the thought that the pituitary gland responsiveness to GnRH (Fernandes et al., 1978; Alam and Dobson, 1987; Bosu et al., 1988) is restored within 14 days postpartum and agree with others that showed that exogenous GnRH can induce ovulation from 10 to 30 DIM (Britt et al., 1974; McDougall et al., 1995; Gümen and Seguin, 2003; Amaya-Montoya et al., 2007).

Interestingly, Benmrad and Stevenson, (1986) reported similar ovulation rate (75%) with only a single GnRH injection (200 µg) between 10 and 14 DIM while only 28% of the controls ovulated in the same period (within 3 to 5 days post treatment). Beam and Butler, (1999) reported that virtually all Holstein dairy cows have the first wave of follicle growth starting two

weeks postpartum; therefore, by administering GnRH-1 between 14 and 20 DIM, our aim was to increase ovulation of the dominate follicle of the first follicular wave postpartum. Nonetheless, at the time of GnRH-1, 23.4% of the cows already had a CL; therefore, it is possible that earlier administration of GnRH at a narrower interval (between 10 and 14 DIM) could have achieved higher ovulation rate than administration between 14 and 20 DIM. Because follicular growth is not tightly synchronized, as it can be observed by the pattern of ovulation in the control group, a two injection treatment scheme was used herein. If only one injection was administered, only 44.6% of the cows would have ovulated within 7 days of GnRH administration, which is a little over the double of the ovulation in the control group (20.9%) or an increase of 23.7 percentage points. This improvement is in agreement with a recent report where only one injection of 100 μg of GnRH was given at 29.2 ± 5.2 DIM and ovulation was improved by 17.9 percentage units (70.9% vs. 53.0%). One overt difference from the report herein and the report by Benmrad and Stevenson, (1986) is the higher dose (200 μg) of GnRH used by the latter. Nonetheless, it has been shown that only 50 μg of GnRH are needed to induce ovulation (Fricke et al., 1998); therefore, it seems plausible that two injections of GnRH are needed to achieve a greater increase in ovulation rate. The interval of 3.5 days would allow follicles that did not respond to the first GnRH to grow another 5 to 9 mm (growth of 1.5 to 2.6 mm/day; Ginther et al., 1997), which would allow them to gain ovulatory capacity if they were at least 6 mm at GnRH-1 (Sartori et al., 2001). It is possible that waiting another day or two would have increased ovulatory response even further; however, this needs to be investigated.

In a studied done by McDougall et al. (1995), ovulation rate of 90 % was achieved with the use of a synthetic GnRH agonist (250 μg) in cows with a dominant follicle (follicles of at least 10 mm in diameter) detected after 14 days post partum, while only 10% of the controls

cows (receiving only saline solution) that also had dominant follicles ovulated in the same period (up to 4 days post treatment). It is expected that administering GnRH to only cows that had a dominate follicle would increase ovulatory response; however, it is intriguing that only 10% of the controls would ovulate spontaneously. In our herds, spontaneous ovulation was high; 23.4% already had a CL between 14 and 20 DIM (day of GnRH-1), 20.9% of the control cows ovulated within 7 days of GnRH-1 and another 30.5% of the remaining anovular control cows ovulated within 7 days of GnRH-2. It is possible that differences in metabolic state among herds used herein and the herd used by McDougall et al., (1995), such as deeper NEB, could contribute to lower ovulation rate in control cows.

Body condition score at 7 weeks postpartum had a significant effect on ovulation to G1 with cows that had BCS > 2.75 having higher odds of ovulation compared to cows with BCS ≤ 2.75 at 17 ± 3 DIM. Higher BCS loss is associated to longer and pronounced negative energy balance (NEB) and it is extensively showed to delay resumption of postpartum ovarian cyclicity and ovulation (Heuwieser et al., 1994; Beam and Butler, 1999; Butler 2005; Santos et al., 2009; Kim et al., 2012). In situation of negative energy balance, high concentrations of NEFA and low IGF-1 are detected in bloodstream, NEFA can be inhibitory of granulosa cells proliferation and also increase their apoptotic rate (Jorritsma et al., 2004; Vanholder et al., 2005), IGF-1 in turn, stimulates the development of follicles maximizing steroidogenesis in which positively feeds-back in LH secretion that further stimulates ovulation. However, BCS loss are associated to NEB that compromise the pulse frequency of LH, hence compromising estradiol production, follicular selection, maturation and ovulation (Butler, 2006). Nonetheless, effort to minimize the body condition loss postpartum can minimize the negative effects of NEB on resumption of ovarian cyclicity and ovulation postpartum and subsequent pregnancy/AI.

There was an interaction between GnRH treatment and parity on ovulation to GnRH-2. This interaction was mainly because of a lower spontaneous ovulation in primiparous control cows compared to multiparous control cows. Previous showed lower postpartum resumption of ovulation for primiparous compared with multiparous (Gümen and Seguin, 2003; Santos et al., 2009). Primiparous are known to have greater incidence of metritis (Goshen and Shpigel, 2006) and to experience greater degree of negative energy balance (Wathes et al., 2007; Meikle et al., 2004), which are known to affect resumption of ovulation postpartum (Peter et al., 1989; Beam and Butler, 1999; Williams et al., 2007, 2008). Santos et al. (2009) hypothesized that primiparous cows are more sensitive to metabolic and endocrine signals in the transition period that might delay resumption of ovarian cyclicity postpartum. It is speculated in our study that the main deficiency in primiparous cows compared to multiparous was the inability to induce a GnRH/LH surge and not a lack of dominant follicles since administration of GnRH was able to induce similar ovulatory response in primiparous and multiparous but spontaneous ovulation was lower in primiparous compared to multiparous.

Season of calving had a significant effect on overall ovulation rate and cows that calved in the warm season had higher odds of ovulation than cows that calved in the cool season. These findings agree with previous reports where ovulation was increased in the warm season (Hansen and Hauser, 1983; Opsomer et al., 2000; Walsh et al., 2007; Santos et al., 2009; Kim et al., 2012). Santos et al., 2009 hypothesized that photoperiod stimulation and or nutritional changes are influencing this seasonal pattern of increased ovulation on during the warm season. Photoperiod may impact follicle development because melatonin is decreased with increased day length, and melatonin has been shown to have a negative impact on IGF-1 secretion in cows (Dahl et al., 2000, 2002). Hence, a decrease in IGF-1 would negatively impact estradiol

production by granulosa cells and impair follicle development (Beam and Butler, 1998; Beam and Butler, 1999). Furthermore, melatonin has been shown to decrease LH concentrations in ovariectomized cows (Rhodes et al., 1979). The change from negative feedback to a positive feedback of estradiol on LH is important for resumption of cyclicity (Legan et al., 1977; Schillo et al., 1982b). In ovariectomized heifers, it has been observed that release of LH induced by estradiol was greater for heifers exposed to 18 h of light and 6 h of dark than for heifers exposed to short days (Hansen et al., 1982). Furthermore, primiparous cows that calved in the winter but were exposed to 18 h of light and 6 h of dark had reduced intervals to first ovulation and to first estrus compared to cows exposed to natural day light cows; although, no difference was observed for multiparous (Hansen and Hauser, 1984).

Uterine blood flow in the warmer season of the year is decreased. It is believed that during the final trimester of the gestation, the reduction of nutrients delivered to the calf due the reduction in uterine blood flow, the conceptus adaptively increase the branching of the vessels and capillary density within the placentomes. This adaptive situation carry over effects after parturition in which the rich vascularized caruncles are capable to produce high amounts of prostaglandins early postpartum. Among these prostaglandins, PGF2α is being produced in large amounts hastening uterine involution (Lewis et al., 1984), which could explain the lower rate of metritis in cows that calved in summer observed in our study (17.4 % vs. 27.8 % for cows that calved in the warm and cool season respectively) and showed elsewhere (Benzaquen et al., 2006). The combination of these factors may support the association in which calving in warmer time of the year favor the higher overall ovulation rate achieved in the current studies and showed elsewhere (Opsomer et al., 2000; Walsh et al., 2007; Santos et al., 2009; Kim et al., 2012).

Etherington et al (1984) demonstrated that uterine health was negatively impacted by the early use of GnRH postpartum in lactating dairy cows due to an increased frequency of pyometra. However, our findings showed no statistical difference on the frequency of either clinical or cytological endometritis (a less subjective diagnosis of uterine health status done by cytology of the uterus), the proportions of this condition were similar in both group (30.9% vs. 32.8% of cytological endometritis positive diagnosis for GnRH-treated and control groups respectively, $P = 0.61$). Our results agree with Stevenson and Call (1988) where no evidence of negative effects (such as high frequency of pyometra) of GnRH administration early in lactation (between 11 to 25 DIM) on uterine health was observed. Calving related problems significantly increased the odds of having CE, and metritis increased the odds of having both CE and CTE. The occurrence of CTE in our study (31.9 %) was lower than reported by Gilbert et al. (2005), ranging from 43 % to 73 % however, in his study, sampling was performed between 40 to 60 DIM and cutoff point for CTE was lower ($\geq$ 5 % PMN in the endometrial), in which could explain the higher prevalence obtained compared to our study that used a higher cutoff point ($\geq$ 10 %). Indeed, ours results are in agreement with studies that used similar cutoff point for PMN count ($\geq$ 10 %), in which the mean prevalence of CTE was 25.9% (Cheong et al., 2011). Although Rutigliano et al. (2008) showed lower prevalence of CTE (prevalence of 18.2 % using higher cutoff point for CTE, PMN $\geq$ 18 %), together, ours findings are in agreement that cows with peripartum uterine disorders (i.e. metritis, dystocia, retained fetal membranes) have increased odds of CTE. Postpartum diseases such as dystocia, retain fetal membranes and metritis predispose cows to endometritis by reduced uterine involution and disruption of the balance between the immune response of the cow and the bacteria load present in the lumen of the uterus. Negative energy status induced from the reduction of dry matter intake on the cows

affected by those disorders (Huzzey et al., 2007) compromise the availability of energy to the neutrophils (the main leukocyte type involved in bacteria clearance in uterine infection) that impair its function, such phagocytosis and killing capacity (Cai et al., 1994; Kehrli and Goff 1989; Gilbert et al., 1993), given course for the chronic infection of the uterus post 21 days postpartum.

Pregnancy to the first service was not different for GnRH-treated and control groups. In a recently study (Jeong et al., 2013) in which GnRH was injected at 29 ± 5 DIM in health cows without a CL, GnRH increased conception rate to the first AI (38.7% vs. 29.1 %) compared to control cows even though the increase in ovulatory response reported herein (~34 percentage points) was considerably greater than what was reported in the study by Jeong et al., (2013) (~18 percentage points). The main differences from the study by Jeong et al., (2013) were the time of GnRH administration and the reproductive management of the cows. In the study herein, cows received GnRH at 17 ± 3 and 20 ± 3DIM and both farms used TAI programs as part of their reproductive management while in the study by Jeong et al., cows received GnRH at 29 ± 5 DIM and the farm relied exclusively on estrus detection for the first AI. Because GnRH was administered earlier, it is possible that control cows had enough time to resume cyclicity and reestablish fertility to the level of GnRH-treated cows. In fact, survival curves showed that by 60 DIM, the proportion of cows that were cycling was similar between GnRH-treated and control cows. When ovulation occurred spontaneously and AI was performed upon estrus detection, cows that were cycling by 21 DIM had increased fertility compared to cows that started cycling from 21 to 49 DIM (Galvão et al., 2010). Likewise, others have observed higher fertility in cows that experienced an earlier resumption of cyclicity and were submitted to AI upon estrus detection (Thatcher and Wilcox, 1973; Darwash et al., 1997). It is not clear why TAI would

mask any beneficial effect of early cyclicity because when cows are not cycling at the start of the Ovsynch program, conception rates are indeed decreased (Galvão et al., 2004; Moreira et al., 2001; Santos et al., 2004). Nonetheless, since a similar proportion of cows were cycling in the GnRH-treated and control groups approximately one week before the start of the Ovsynch program, it is possible that differences in fertility because of earlier cyclicity in the GnRH-treated group and control group were attenuated. Nonetheless, when evaluating fertility of cows that were already cycling at 17 ± 3 DIM, cows that started cycling by 24 ± 3 DIM and cows that remained anovulatory, it is clear that cows that were cycling at 17 ± 3 DIM and cows that were induced to cycle from 17 to 24 ± 3 DIM had improved fertility than cows that remained anovulatory up to 24 ± 3 DIM.

BCS at study enrollment also affected the likelihood of pregnancy to the first service, and cows with BCS > 2.75 at time of the study enrollment had 1.8 times the odds of conceiving compared to cows with BCS ≤ 2.75. This positive association of high BCS enhancing fertility can be explained by the fact in which cows in better BCS are also known to be in better metabolic status with adequate concentrations of glucose, insulin and IGF-1 in blood that increase rates of ovarian follicle growth. The consequence is the enhanced steroidogenesis with high follicular estradiol production that positively feeds back on the hypothalamus-pituitary axis up to the point to induces LH surge and ovulation (Beam and Butler, 1999). Several studies have shown improved cyclicity and fertility for cows with higher BCS (Pryce et al., 2001; review by López-Gatius et al., 2003; Butler et al., 2006; Roche et al., 2007; Santos et al., 2009). Herein, cows with BCS > 2.75 had increased ovulation within 3.5 d of GnRH-1 (36.3% vs. 21.7%), which probably contributed to improvement of fertility.

Dairy had an effect in conception rate at first service, in which Dairy 1 had higher conception rates than Dairy 2. Differences in conception rates between dairies may be because of a multitude of cow-level and herd-level factors that goes beyond the scope of this study. Our main goal in including Dairy as a fixed effect was to be able to test for interactions between GnRH-treatment and Dairy; however, one was not observed, indicating that GnRH treatment was equally effective in both dairies. Therefore, it is safe to assume that similar response to GnRH treatment may be expected in dairies with similar characteristics to the ones used herein.

One important component of the reproductive performance is the ability of the pregnant cow to maintain and carry the conceptus to term. Therefore, pregnancy loss is an important component of herd reproductive performance. In the present study, GnRH-treated cows had lower pregnancy loss compared to control cows. To the authors knowledge this is the first time that GnRH treatment early postpartum is associated to reduction in late pregnancy loss. A potential explanation for decreased pregnancy loss in the GnRH group would be the increased ovulation rate by 24 ± 3 DIM because cows that remain anovulatory by the end of the voluntary waiting period have an increased pregnancy loss (Galvão et al., 2004; Santos et al., 2004). Nonetheless, pregnancy loss was actually higher in cows that ovulated by 24 ± 3 DIM. In fact, all the pregnancy losses in the GnRH-treated group came from the cows that ovulated [8% (6/75) in GnRH-treated that ovulated and 0 (0/12) in GnRH-treated that did not ovulate], and in the control group most of the pregnancy losses also came from the cows that ovulated [26.1% (11/42) in control that ovulated and 10.8% (5/46) in control that did not ovulate]. In cows that had a CL at 17 ± 3 pregnancy loss was low compared to cows that ovulated between 17 ± 3 and 24 ± 3 DIM [6.9% (4/58) vs. 14.5 (17/117)]; therefore, at this point it is not clear why GnRH administration resulted in decreased pregnancy loss and why cows that ovulated by 24 ± 3 DIM

had increased pregnancy loss since spontaneous early ovulation did not have the same negative effect on pregnancy loss. There is always a chance for type 1 error; therefore, further research is needed to confirm the beneficial effect of GnRH administration on pregnancy loss and the negative effect of ovulation between 17 ± 3 and 24 ± 3 DIM on pregnancy loss compared to cows that had ovulated by 17 ± 3 DIM and cows that failed to ovulate by 24 ± 3 DIM.

Metritis in our study was also associated with increased incidence of pregnancy loss and interestingly there are not much relates of this association. Few studies showed results of the negative effect of metritis in pregnancy loss (Ribeiro et al., 2013, unpublished). In fact, the complete mechanism in which metritis leads to the interruption of the pregnancy is not well understood or demonstrated. In an epidemiologic study evaluating fertility of grazing dairy cows, metritis increased the odds of pregnancy loss by 4 times compared to no metritis cows (Ribeiro et al., 2013, unpublished). What it is known it is the role of metritis reducing reproductive performance (review by Fourichon et al., 2000; Williams et al., 2008). Infection of the uterus also disturb ovarian follicle growth and function, and in cows that ovulated, reduced CL size and lower progesterone production occur affecting reproduction however, the mechanism is not yet elucidated (Williams et al., 2007). Moreover, metritis being associated to development of endometritis as demonstrated in our study and elsewhere (Galvão et al., 2009, 2011). López-Gatius et al. (1996) showed association of uterine diseases such as retained fetal membranes and pyometra in pregnancy loss, in which cows that underwent to these pathologies had 1.8 and 2.6 odds of pregnancy loss compared to non-disease cows, respectively. This research group speculated that the alterations in the uterine environment caused by those diseases could be critical during the implantation of the conceptus, compromising the attachment and survival of the embryo, although it was just a hypothesis of possible mechanism and it was not proved. The

overall effect of uterine diseases such as metritis and endometritis in the cows' uterus is damage to the uterine glands and activation of the inflammatory cascade with release of pro-inflammatory cytokines. Among the cytokines, tumor necrosis factor-α (TNF- α) stimulates the release of PGF2α from the endometrium and luteal cells, hence inducing luteolysis (Skarzynski et al., 2005); therefore, if the inflammation persists the pregnancy may be lost. Although metritis occurs within the first 3 weeks postpartum (Sheldon et al., 2006) and breeding usually takes place after 60 DIM, the direct association of metritis with pregnancy loss is through its association with CE and CTE as observed herein. The inflammatory process associated with uterine diseases lead to occlusion of the endometrial glands, dilation of underlying glands with deposit of connective tissue and formation of scar tissue in the uterus, which may affect embryonic implantation and maintenance of gestation. Clinical endometritis (Galvão et al., 2009) and CTE (Lima et al., 2013) have been shown to increase the risk of pregnancy loss. In vitro studies showed that culturing embryos in a medium containing non-specific inflammatory products (Hill and Gilbert, 2008) or pro-inflammatory cytokines such as TNFα (Soto et al., 2003) impair early embryo development. More recently, it was demonstrated that expression of genes involved in maintenance of membrane stability and the cell cycle were downregulated in embryos from cows with CTE (Hoelker et al., 2012). Furthermore, embryos from cows with CTE had developmental retardation, which corroborates with the findings from previous studies (Hill and Gilbert, 2008; Soto et al., 2003).

Hazard of pregnancy by 300 DIM based on PD2 was evaluated and an interaction between GnRH treatment and ovulation from 17 ± 3 DIM to 24 ± 3 DIM was observed. The hazard of pregnancy for GnRH-treated cows that ovulated was 2 times the hazard for GnRH-treated cows that did not ovulate and 1.3 times greater than control cows that did not ovulate,

however similar to control cows that ovulated. Cows that responded and ovulated from GnRH injection are more ready to conceive then cows that did not ovulate, which agrees with previous findings that precocious resumption of ovulation postpatum positively effects fertility (Darwash et al., 1997; Santos et al., 2009; Galvão et al. 2010; Kim et al., 2012). Recently, a study performed in Korean dairy herds (Jeong et al., 2013) demonstrated increased hazard of pregnancy by 210 DIM (HR = 1.3; 95 % CI = 1.055 to 1.61; *P* = 0.01) for GnRH treated cows around one month postpartum compared to control cows. The higher conception rate obtained with the use of GnRH, shorter voluntary waiting period combined to the enrolment of only health cows in the study may explain the success in the GnRH use postpartum in the Korean study. We can speculate that this group of cows may be in a better metabolic status that leads to a higher hazard of pregnancy. The low reproductive performance of the GnRH-treated cows that failed to ovulate may be explained by the overall worse uterine health in this group. It has been hypothesized that early ovulation is a marker for uneventful transition into lactation (Galvão et al., 2010), with cows ovulating early having better uterine health (i.e decreased prevalence of CTE) than cows that fail to ovulate. Probably cows that ovulate are in a better health status, which would lead to higher IGF-1, higher LH pulse frequency and greater follicle development than cows that fail to ovulate. To help confirm our hypothesis, GnRH-treated cows that failed to ovulate had greater prevalence of CTE than cows that ovulated (43 vs. 25%). Interestingly, prevalence of metritis (22 vs. 22%) and CE (25 vs. 23%) did not differ between GnRH-treated cows that ovulated and did not ovulate. This data shows that probably there were differences in metabolic state in cows that did and did not ovulate to GnRH, independently of disease state (i.e. metritis), that later translate into improved uterine health (i.e. decreased CTE) in cows that ovulate to GnRH. In the control group, the prevalence of metritis (18 vs 20%), CE (20 vs. 17%),

or CTE (32 vs. 33%) did not differ for control cows that did and did not ovulate, respectively. It was also observed that prevalence of metritis (11%), CE (11%) and CTE (26%) for cows that had a CL at 17 ± 3 DIM were considerably lower than cows that did not have a CL, again confirming our hypothesis of an overall better health status in cows with early ovulation (Galvão et al., 2010).

In conclusion, the administrations of two doses of GnRH agonist at 17 and at 20 ± 3 DIM in lactating Holstein cows successfully induced ovulation up to 24 ± 3 DIM. GnRH treatment did not affect uterine health status and pregnancy to the first service although significantly decreased pregnancy loss to the first service. Time to pregnancy was not affected by GnRH treatment, although hazard of pregnancy was increased in cows that responded to GnRH treatment but was decreased in cows that did not respond to GnRH treatment. In summary, the use of GnRH in early lactation dairy cows lead to increased ovulation but does not affect long term fertility. The effect of GnRH administration on pregnancy loss needs further investigation.

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BIOGRAPHICAL SKETCH

João Henrique Jabur Bittar was born in Goiânia, the capital the state called Goiás, Brazil in 1980. He is the youngest of three siblings of a Syrian-Lebanese descendant father born in Brazil and his wife, who was born and raised in a rural area of Minas Gerais state. Since his childhood, João Henrique has been interested in livestock, especially farm animals, and led him to pursue a degree of Veterinary Medicine in the Federal University of Goiás in 1999. Throughout his veterinary studies, he became very active participating in several internship programs related to beef and dairy production and equine, as well as in a pharmaceutical company, which enhanced his knowledge and network of professional contacts. After graduation, he worked as a veterinarian with beef and dairy cattle in Goiás and Mato Grosso, both states considered one of the larger producers of livestock and agriculture in Brazil. In 2006, he joined the SENAR (an educational institution funded with agriculture commodity tax revenues) where his duties included development of hoof care education and training programs of equine for farmers and farmer workers in the state of Goiás. In 2007, he moved to the United States to learn more about dairy farming in this country, especially in the state of New York and to enhance his English communication skills. In 2008, he started working for a large dairy farm group also in New York that was client of the Ambulatory Veterinarian Service of Cornell University. In this position, he had the opportunity to interact with university veterinarians, residents, and interns. João's experience with dairy farmers in New York and with the Ambulatory Veterinarian Service of Cornell University inspired him to pursue advanced education in the United States. In the summer of 2011, he was offered and accepted the position of Resident in Food Animal Reproductive and Medicine at the University of Florida, College of Veterinary Medicine, Department of Large Animal Clinical Sciences. In summer 2012, he was admitted to the Masters of Science Program in Veterinary Medical Sciences at the same

University, and he is expecting to graduate in summer 2013. João Henrique's next goals are to complete his residency training by July 2014, and later to pursue a doctoral degree in the United States or go back to veterinary practice in the United States or in his home country.