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I am submitting herewith a dissertation written by Julio Omar Giordano entitled “Possible strategies to increase ovulatory follicle size and reduce ovulation time in lactating dairy cows”. I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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POSSIBLE STRATEGIES TO INCREASE
OVULATORY FOLLICLE SIZE AND REDUCE TIME
TO OVULATION IN LACTATING DAIRY COWS

A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Julio Omar Giordano
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This dissertation is dedicated to the most beautiful and important people of my life, Soledad and Julio Agustín. Sole, only you know how important is for me to have your support and love to accomplish my goals.

I also wish to dedicate this dissertation to my family in Argentina, in particular to my father Omar and my mother Maria Teresa. Papa you are the first and most knowledgeable teacher I ever had. You extended your passion for hard work and the veterinary profession to me. Mama you were the best source of support for life and continuation of my education.
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I have no words to thank Raúl and Eugenia Almeida as well as the rest of the Almeida family who gave me everything they could and made my life in Tennessee much easier and fun. Finally I have to thank Soledad for her patience, caring, and being such a beautiful person.
Specific objectives of this study were to examine growth response of the dominant follicle (DF) after administration of Folltropin-V (FSH and LH) at onset of luteolysis and investigate use of human chorionic gonadotropin (hCG) for decreasing ovulation time in lactating Holstein cows. On day 8 or 9 of a synchronized cycle, cows \( (n = 35) \) received an EAZI-BREED CIDR plus 100 μg of GnRH. CIDRs were removed 7 days later and cows were administered 500 μg cloprostenol. Concurrently, cows were randomly allocated to receive either 80 mg Folltropin-V (FSH, \( n = 19 \)) or 4 mL of sterile saline (SAL, \( n = 16 \)). Forty-nine hours later, cows that had received Folltropin-V or Saline were randomly subdivided to receive either 100 μg dose of GnRH or 3000 IU of hCG. Ultrasonography was performed to assess growth of the ovulatory follicle and confirm ovulation. Data were analyzed using the MIXED procedure of SAS. Size of the ovulatory follicle at time of GnRH/hCG administration was not different between Folltropin-V or Saline groups (17.1 ± 0.7 vs. 17.7 ± 0.6 mm, respectively; \( P = 0.521 \)). Total growth of the ovulatory follicle from CIDR removal to GnRH/hCG administration did not differ between FSH (3.2 ± 0.6 mm) and Saline-treated cows (3.3 ± 0.4 mm; \( P = 0.891 \)). When assessed from time of CIDR removal, ovulation occurred at a similar time for Folltropin-V (76.9 ± 0.7 h) and Saline-treated cows (78.0 ± 0.5 h; \( P = 0.196 \)). When calculated from time of LH or hCG peak to ovulation, FSH-hCG cows presented the shortest time to ovulation (19.9 ± 0.4 h; \( P = 0.066 \)). Whereas, from GnRH/hCG administration to
ovulation, FSH-GnRH presented the earliest ovulation (25.0 ± 1.2 h; \( P = 0.013 \)).

In conclusion, Folltropin-V did not increase size of the ovulatory follicle at the time of GnRH/hCG administration. However, earlier ovulation occurred when combined with hCG as measured \textit{from time of gonadotropin peak}. Conversely, when ovulation time is calculated \textit{from GnRH/hCG administration}, FSH-GnRH produced the earliest ovulation.
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AI = artificial insemination  
BCS = body condition score  
BM = basement membrane  
BS = blood sample  
BV = blood vessel  
BW = body weight  
cAMP = cyclic adenosine monophosphate  
CIDR = controlled internal drug release  
CIDR-r = controlled internal drug release-removal  
CL = corpus luteum  
COC = cumulus-oocyte complex  
COX-2 = cyclooxygenase-2  
CR = conception rate  
CREB = cAMP regulatory element-binding protein  
CT = connective tissue  
CV = coefficient of variation  
DAG = diacylglycerol  
DF = dominant follicle  
DIM = days in milk  
cCG = equine chorionic gonadotropin  
ECM = extracellular matrix  
ET = embryo transfer  
FSH = follicle stimulating hormone  
F1 = largest follicle present on both ovaries  
F2 = second largest follicle present on both ovaries  
GC = granulosa cells  
GDP = guanosine diphosphate  
GnRH = gonadotropin releasing hormone  
GPCR = G protein-coupled receptor  
GTP = guanosine triphosphate  
hCG = human chorionic gonadotropin  
HS = heat stress  
IACUC = Institutional Animal Care and Use Committee  
I\textsuperscript{125}-hCG = iodinated human chorionic gonadotropin  
IP\textsubscript{2} = inositol biphosphate  
IP\textsubscript{3} = inositol triphosphate  
IU = international unit  
Kg = kilogram  
LH = luteinizing hormone  
LHR = luteinizing hormone receptor  
LSM = least squares means  
MAPK = mitogen-activated protein kinase
ME = mature equivalent
mRNA = messenger ribonucleic acid
MMP = matrix metalloproteinase
MMP-2 = matrix metalloproteinase-2
MMP-13 = matrix metalloproteinase-13
MMP-14 = matrix metalloproteinase-14
NRC = National Research Council
OE = ovarian epithelium
OT = oxytocin
PA = plasminogen activator
PAI-1 = plasminogen activator inhibitor-1
PAI-2 = plasminogen activator inhibitor-2
PG = prostaglandins
PGE₂ = prostaglandin E₂
PGES = prostaglandin E synthase
PGF₂α = prostaglandin F₂α
PGFS = prostaglandin F synthase
PGH-2 = prostaglandin H₂
PGHS-2 = prostaglandin G/H-2 synthase
PKA = protein kinase A
PKC = protein kinase C
PLC = phospholipase C
PR = pregnancy rate
RIA = radioimmunoassay
SEM = standard error of the mean
TAI = timed artificial insemination
TC = theca cells
TIMP = tissue inhibitor of metalloproteinase
TIMP-2 = tissue inhibitor of metalloproteinase-2
TSH = thyroid stimulating hormone
tPA = tissue plasminogen activator
uPA = urokinase plasminogen activator
uPAR = urokinase plasminogen activator receptor
US = ultrasonography
CHAPTER 1
INTRODUCTION

Environmental heat stress (HS) is a severe problem affecting approximately 60% of the world’s dairy cattle population (reviewed by Wolfenson et al., 2000). For the United States dairy industry alone, the economic losses associated with low milk yield and reduced reproduction approaches $897 million dollars per year (St-Pierre et al., 2003).

Summer HS negatively alters fertility of dairy herds (Erb et al., 1940; Ingraham et al., 1976; Gwazdauskas et al., 1981; Cavestany et al., 1985; López-Gatius 2003; de Vries et al., 2005b; de Vries and Risco 2005) when lactating cows are either acutely or chronically exposed to high ambient temperature and relative humidity. Consequently pregnancy rates of dairy herds dramatically decrease during the summer season in regions affected by HS (de Vries and Risco 2005). Previous research indicates that HS may mediate its negative effects on reproduction by altering hormonal concentrations (Gilad et al., 1993; Wolfenson et al., 1997; Roth et al., 2001; Wolfenson et al., 1993), uterine environment (Roman-Ponce et al., 1978), follicular dynamics (Badinga et al., 1993; Wolfenson et al., 1995; Wilson et al., 1998a,b), decreasing size of the dominant follicle (DF) (Badinga et al., 1933; Wilson et al., 1998a,b; Cunha et al., 2007) and/or oocyte quality (Edwards and Hansen 1996; Al-Katanani et al., 2002; Edwards et al., 2005). Prominent changes of the maturing oocyte in response to elevated temperatures have been shown both in vivo (Putney et al., 1989b) and in vitro

1
(Edwards and Hansen 1996; Ju et al., 1999; Edwards et al., 2005) indicating that the female gamete is very sensitive to HS. Indeed, exposure of dairy cattle to heat stress conditions during estrus compromised subsequent embryonic development suggesting that the major effects of hyperthermia occur when oocytes are completing the process of maturation within the Graafian follicle (Putney et al., 1989b). Further, in vitro studies have shown that the ability of the oocyte to complete the process of nuclear (i.e., progression to metaphase II) and cytoplasmic (i.e., translocation of cortical granules to the periphery of the cytoplasm) maturation is not affected. However, exposure of the oocyte to physiologically-relevant elevated temperatures hastens the timing required for the completion of the process (Edwards et al., 2005). Oocytes exposed to 41°C during in vitro maturation (IVM) reached nuclear and cytoplasmic maturation earlier, and when fertilized (IVF) within 4 to 8 h prior to the usual 24 h post maturation produced blastocyst development similar to those in the non heat-stressed control group (Edwards et al., 2005; Schrock et al., 2007). Taken together, these observations suggest that HS hastens oocyte maturation resulting in fertilization of an otherwise “aged” oocyte if IVF is performed at the usual 24 h post-maturation period.

If as previously reported the time of ovulation in cattle is not modified by HS (Gwazdauskas et al., 1981; White et al., 2002), and elevated temperatures in vivo alter oocyte maturation in a similar manner as in vitro, then by the time the oocyte is ovulated in a heat stressed cow it may already be “aged”. Under these hypothetical circumstances, it is unlikely that the oocyte will develop into a
normal embryo and establish a pregnancy. Previous efforts of others have clearly documented that oocyte aging before fertilization has a negative consequence on subsequent embryonic development (reviewed by Fissore et al., 2002).

With this in mind therapeutic strategies aimed to induce earlier ovulation may be necessary to ensure the release of a mature “non-aged” oocyte in cows undergoing HS. It was hypothesized that one possible strategy to reduce time from onset of oocyte maturation (triggered by the gonadotropin preovulatory surge in vivo) to ovulation may be to induce ovulation of larger follicles. A possible approach to accomplish this goal may be to induce supplemental growth of the DF during its final growth phase in order to have a larger preovulatory follicle at the time of an exogenously induced gonadotropin surge. Supplemental growth of the DF may be possibly stimulated by the administration of a hormone preparation (Folltropin-V) containing FSH and LH, hormones that under normal physiological conditions are responsible for stimulating growth of ovarian follicles (reviewed by Lucy 2007; reviewed by Crowe 1999). Then, possible larger ovulatory follicles may be more sensitive to the gonadotropin surge and respond with early ovulation. Another possible advantage of using gonadotropins in heat stressed-cows may be to offset the negative effect of HS to reduce DF size as previously reported by others (Badinga et al., 1993; Wilson et al., 1998a,b; Cunha et al., 2007).

In cattle, the two most utilized hormones to induce ovulation are GnRH which acts indirectly by increasing LH release from the pituitary gland resulting in a LH surge and, human Chorionic Gonadotropin (hCG) which acts directly on
the ovary by binding LH receptors (reviewed by De Rensis and Peters, 1999). Thus, besides testing the growth response of the DF to Folltropin-V (FSH and LH) administration, this experiment was also intended to test if the administration of hCG would have an effect in the timing of ovulation. Unlike GnRH, the use of hCG to induce ovulation in heat stressed cows may have some advantages including; a mechanism of action independent of the pituitary gland which might be affected by the effects of elevated temperatures in lactating dairy cows (Gilad et al., 1993), a prolonged plasma half-life (Schmitt et al., 1996), greater affinity for LH receptors (Henderson et al., 1984), and the capability to remain bound to LH receptors for longer periods of time than LH (Mock and Niswender 1983; Mock et al., 1983; Henderson et al., 1984).

Specific objectives of this study were: 1) to examine the growth response of the DF after administration of Folltropin-V (FSH and LH) at the onset of luteolysis, 2) to investigate if the administration of hCG may decrease the time to ovulation when compared to a GnRH induced LH surge and 3) to examine if the combination of Folltropin-V (FSH and LH) with either hCG or GnRH may decrease the time to ovulation in lactating dairy cows.
2.1. General Effects of Heat Stress on Dairy Production Systems

Environmental heat stress (HS) is a severe problem affecting approximately 60% of the world’s dairy cattle population (reviewed by Wolfenson et al., 2000). Dairy farms located in the Southern region of the United States are systematically affected by HS during the warm season (reviewed by Jordan 2003). However, the negative impact of HS in dairy cattle has been demonstrated in locations as far north as Wisconsin (Sartori et al., 2002; Oseni et al., 2003). Dairy production systems are seriously impacted by the economic losses associated with this phenomenon. Decreased milk yield (Maust et al., 1972; West et al., 2003) and impaired reproduction (Thatcher 1974; Cavestany et al., 1985) account for the majority of losses associated with heat stress. Additionally, incidence of other herd health problems including higher somatic cell counts (Igono et al., 1988), retained placenta (Dubois and Williams 1980), and metabolic disorders are aggravated during HS conditions.

Armstrong (1994) defined HS as “any combination of environmental conditions causing the effective temperature of the environment to be higher than the animal’s thermoneutral zone”. Lactating dairy cows exposed to high ambient temperatures and relative humidity (RH), typical of the warm season, present difficulties dissipating heat produced by physical activity and metabolic
processes. The extra heat load produced by milk synthesis impairs their ability to cope with HS. Consequently, high producing cows are more severely affected than cows with lower production (Berman et al., 1985). Even more evident are the differences when comparing the amount of heat produced by lactating or non-lactating dairy cows. Purwanto et al. (1990) reported a 48.5% increase in heat produced by cows yielding 31.6 kg/d of milk when compared to dry cows under the same environmental conditions.

Other factors common to current production systems can affect performance of dairy cows. For example, since bST was proven to increase milk production in heat-stressed cows (Mohammed and Johnson 1985; Elvinger et al., 1992; Settivari et al., 2007), its use is widespread in dairy farms located in regions affected by HS. Unfortunately, while bST increases milk yield, it also further elevates heat production (Mohammed and Johnson 1985; Elvinger et al., 1992) exacerbating the heat load of lactating cows.

Failure to efficiently eliminate the increased heat load disrupts the animal’s homeostatic equilibrium evoking a variety of physiological responses to maintain comfort. As body core temperature rises, the cow attempts to eliminate excess heat load by increasing respiration rate (Elvinger et al., 1992), and water consumption (Sanchez et al., 1994; Ronchi et al., 2001); however, feed intake (West 1994) and physical activity are markedly reduced (Thatcher et al., 1974; West 1994). An immediate consequence is a reduction in the amount of milk produced, the extent of which is usually correlated to the degree of HS. Many previous studies evaluated milk production in cows undergoing thermal stress
(Johnson et al., 1962; Ingraham 1979; Ravagnolo et al., 2000, West et al., 2003) reporting variable results. Even though slight differences are found among the studies, all data reviewed reported a decrease in production. The differences could be attributed to the variation in environmental and management conditions characteristic of each study as well as differences in the approach to quantify HS.

Temperature humidity index (THI) is a method usually employed to predict the degree of HS. It is calculated by combining ambient temperature and relative humidity (RH) into one value (NOAA, 1976). According to this index, HS in dairy cattle starts at a THI of 72, which is also the end of the comfort zone for cows (reviewed by Armstrong 1994). A THI value of 72 is obtained with a temperature of 72°F at 100% humidity, 77°F at 50% humidity, or 82°F at 20% humidity. As seen, different combinations of temperature and RH, easily reached in areas of dairy production, induce HS in lactating dairy cows. In this regard, utilizing weather information and production records from dairy farms in Georgia, Ravagnolo et al. (2000) estimated a decrease of 0.2 kg of milk, 0.009 kg of protein and 0.012 kg of fat produced by each unit of increment in the THI above 72. Furthermore, a loss of 165 kg/cow/year in milk was calculated after having more than one third of the year with THI ≥ 72.
2.2. Impact of Heat Stress on Economics of Dairy Production Systems

The estimated annual losses by livestock industries in the United States associated with summer HS ranges between $1.69 to 2.36 billion (St-Pierre *et al.*, 2003). Considering the dairy industry alone, it represents $897 to 1.5 billion dollars per year of the total loss. Therefore, the dairy industry ranks first in the list of food producing animals affected by HS (St-Pierre *et al.*, 2003). Decreased milk production and reproductive inefficiency of dairy herds are responsible for most of the economic losses; however, other causes such as higher incidence of mastitis (Giesecke 1985) and increased mortality (Hahn 1985) further affect profitability of the industry.

Thermal stress drastically decreases pregnancy rate resulting in an increase in days open for the herd (Oseni *et al.*, 2003; St-Pierre *et al.*, 2003). If pregnancy is delayed, then cows will produce milk during prolonged periods of time in the decreasing area of the lactation curve when efficiency of production is medium or low. Moreover, cows becoming pregnant in late lactation are at higher risk of being culled from the herd for other reasons including mastitis, lameness, and low production (Gröhn *et al.*, 1998; Booth *et al.*, 2004). Considering that dairy cows in the US have 3 lactations in average (Hare *et al.*, 2006), it is clear that the utmost economic benefit is obtained when they have as many lactation peaks as possible since during this period, cows produce at their maximum efficiency (Britt *et al.*, 2003).
Using meteorological data from more than 100 years and data on animal response to HS from the literature, St-Pierre et al. (2003) created specific models to assess the economic losses associated with HS by livestock industries in the United States. For this analysis, productive and reproductive parameters usually employed to evaluate the economic efficiency of dairy farms were selected. The results of this observational study indicate that number of days open in herds from the Southeast were higher than in herds located in the Northern states of the country.

Realistic economic estimations of diminished reproductive performance are hard to calculate given the number of different factors affecting the system. For example, variable results were obtained in studies (Plaizier et al., 1997; French and Nebel, 2003) aimed at calculating the cost of cows having extra days open. The variability can be attributed to the different conditions considered in the estimation. However, it is generally the rule that there is an implied cost when cows remain open for extended periods after the voluntary waiting period (VWP). The magnitude of this cost increases with longer days in milk because of lower milk production during the current lactation as well as in the lifetime of the cow. According to de Vries et al. (2005a) under Florida conditions, the cost of each day open after the VWP ranges from $0.81 to $13.33. The lowest values representing the losses during early lactation; whereas, the highest values represent late lactation when the risk of being culled for other reasons is much higher.

Taken together, the fact that HS increases number of days open and the extra cost associated, clearly stresses the value of attaining an early pregnancy for
profitability of commercial dairy farms. Achievement of acceptable pregnancy rates during the summer months would have a tremendous positive impact on the economy of the industry by maximizing net return per cow per year.

2.3. General Effects of Heat Stress on Reproduction

In mammals, lactation is a physiologic process critical for the survival of the species. Through secretion of milk, the dam provides the offspring with all the nutrients required for growth and development. Establishment of a pregnancy is the prerequisite for mammary gland development and milk secretion, unless they are artificially induced by exogenous hormones (Harness et al., 1978; Sawyer et al., 1986). After calving, the regular stimuli produced either by suckling in beef cows or milking in dairy cows is critical to maintain lactation. In dairy cows, the amount of milk produced follows a curvilinear pattern increasing from calving up to approximately 6 to 9 weeks when the maximum peak of production occurs and then decreases (Nebel and McGilliard 1993). Following a variable period of time specific to every production scheme, this carefully orchestrated process is interrupted either when weaning occurs in beef cows or the cessation of lactation, known as the dry period, is forced in dairy cows. Of the two viable alternatives previously described to induce milk production, exogenous hormone administration or pregnancy and calving, it is clear that the natural strategy has prevailed over the years. Consequently, the entire dairy industry relies upon cows becoming pregnant to have lactation. Ideally, all the cows in a herd become
pregnant; however, in the real world, this condition is not always the rule and dairy producers struggle with the poor reproductive efficiency of modern dairy cattle.

Over the last fifty years, intensification of the dairy industry through changes in nutritional and health management programs, coupled with intensive genetic selection towards high productive potential, prompted a marked increase in individual cow milk production (Nebel and McGilliard 1993; Lof et al., 2007). However, over the same period of time, all parameters utilized to monitor reproductive performance of dairy herds evidenced a consistent decline in fertility. For instance, conception rate (proportion of services that result in pregnancy; Morton et al., 2007) for first service in lactating dairy cows in New York decreased from approximately 65% in 1951 to 40% in 1996 (reviewed by Butler 1998). Furthermore, Washburn et al. (2002) found over 35% decrease in conception rates from the late 1970s to the late 1990s in dairy herds located in 10 southeastern states of the United States. This trend is not only observed in the United States but also in other countries where first-service conception rates have been reported to decrease over time (Australia, Macmillan et al., 1996; Ireland, Roche 2000; United Kingdom, Royal et al., 2000).

Summer HS has long been recognized to negatively alter fertility of dairy herds (Erb et al., 1940; Ingraham et al., 1976; Roman-Ponce et al., 1977; Cavestany et al., 1985) when lactating cows are either acutely or chronically exposed to high ambient temperature and RH. For instance, pregnancy rate (PR; cumulative number of conceptions/cumulative number of eligible cows in a 21 d
which is a common parameter used to measure reproductive performance of dairy herds (de Vries and Risco 2005; Ferguson and Galligan 1999) decreases dramatically during the summer months in regions affected by HS. Pregnancy rates during an eight year period in dairy herds of Florida and Georgia using artificial insemination (AI), natural service (NS) or combination of both breeding systems were 17.9% during winter whereas, during summer PR was 9.0% which represent a large decrease in PR (de Vries et al., 2005b). Another report including data from approximately 2.8 million DHIA lactation records during > 25 years period indicates an 11% difference in PR between the winter and summer season (de Vries and Risco 2005). Thus, environmental HS is of major concern to the modern dairy industry given its contribution to poor reproductive performance of herds. In the Southeast where herds are growing in cow numbers and the system is being intensified, the losses associated with this phenomenon are concerning (Washburn et al., 2002).

Most research suggests that detrimental effects of HS in livestock species are mediated by maternal hyperthermia (sheep, Alliston et al., 1961; swine, Edwards et al., 1968; beef, Dunlap and Vincent 1971; dairy, Putney et al., 1988); which in turn, alters essential mechanisms for the establishment and maintenance of pregnancy. Elevated rectal and uterine temperatures during a period of time as early as 35 to 42 d prior to expected ovulation (Al-Katanani et al., 2002; Morton et al., 2007) and, including late gestation (Collier et al., 1982) were linked to reproductive dysfunction. However, the major reproductive losses are observed when maternal hyperthermia occurs around the time of estrus and during early
pregnancy (Ulberg and Burfening 1967; Putney et al., 1988, 1989b; Ealy et al., 1993).

Failure of cows to establish and maintain pregnancy when exposed to high ambient temperatures and relative humidity was consistently associated with alterations in mechanisms involved in this strictly organized physiologic process. Previous research indicates that HS mediates its effect on reproduction by several means including:

- possible dysfunction of the hypothalamic-pituitary axis (Gilad et al., 1993),
- aberrant follicular dominance pattern (Badinga et al., 1993; Wolfenson et al., 1995; Roth et al., 2000), and decreased follicle size (Badinga et al., 1993; Wilson et al., 1998a,b; Cunha et al., 2007)
- diminished expression of estrus (Gwazdauskas et al., 1981; Younas et al., 1993),
- modified steroidogenesis in ovarian follicles (Wolfenson et al., 1997; Roth et al., 2001) and corpus luteum (CL) (Wolfenson et al., 1993; Howell et al., 1994),
- alterations of oocyte quality (Edwards and Hansen 1996; Zeron et al., 2001; Edwards et al., 2005),
- inhibits embryo development (Putney et al., 1988; Monty and Racowsky 1987),
- reduced cellular function (Edwards and Hansen 1996) and uterine blood flow (Roman-Ponce et al., 1978).
These alterations frequently take place concurrently with other effects of HS such as decreased feed intake and negative energy balance (reviewed by de Rensis and Scaramuzzi 2003) contributing in an additive or synergistic fashion to the already established problem.

2.4. Brief Overview of Normal Reproductive Physiology in the Cow

The estrous cycle comprises the time elapsed between consecutive ovulations. In the cow, the duration ranges from 17 to 23 d (average 21 d; Ireland and Roche 1983). A series of hormones and growth factors secreted by the hypothalamic-pituitary-gonadal axis and the uterus are responsible for regulation of the estrous cycle. The anterior pituitary gland contributes by producing two gonadotropins known as follicle stimulating hormone (FSH) and luteinizing hormone (LH) which act in conjunction to control growth and development of ovarian follicles (reviewed by Crowe 1999). These two hormones are synthesized and secreted in response to hypothalamic-derived gonadotropin releasing hormone (GnRH). While a pulse of GnRH is typically associated with a pulse of LH, in the case of FSH, synthesis but not secretion is linked to GnRH. Instead, FSH secretion is closely regulated by a different feedback mechanism including estradiol-17β and other glycoproteins (reviewed by Welt et al., 2002).

In cows, follicles grow in a wave-like manner (Savio et al., 1988; Sirois and Fortune 1988; Ginther et al., 1989; Knopf et al., 1989). During the cycle, emergence of a follicular wave is associated with FSH while LH stimulates final
follicular growth and triggers the process of ovulation (reviewed by Moore and Thatcher 2006). Progesterone and estradiol-17β are key regulators of gonadotropin secretion given their influence in the secretory activity of the hypothalamus and pituitary gland. Inhibin, activin, and follistatin are a group of associated glycoproteins implicated in the regulation of FSH secretion (Phillips 2005). Other molecular components produced by the hypothalamic-pituitary-gonadal axis and the uterus are linked to different events of the estrous cycle; however, discussion of their functions and mechanism of action goes beyond the scope of this review.

The day of estrus, when the cow is receptive to be mounted, is defined as the beginning of the cycle or day 0. Ovulation occurs in approximately 24 to 30 h after the onset of estrus (Rajamahendran et al., 1989; Walker et al., 1996; Bloch et al., 2006) resulting in release of the oocyte and formation of the CL in the remaining follicular tissue. This transitory gland will secrete progesterone until day 16 to 18 when uterine PGF$_2\alpha$ is released (McCracken et al., 1981). In response to prostaglandin, the CL regresses establishing the end of the luteal phase. However, if an embryo is present it will send the adequate signal to the uterus to evade luteolysis and maintain a functional CL (Bazer et al., 1991; Thatcher et al., 1995). Upon luteolysis, progesterone concentration drops to basal levels releasing the block to LH secretion. As a result, faster pulsatility of LH occurs that stimulates the dominant follicle(s) (DF) to grow to preovulatory size (12 to 22 mm; reviewed by Crowe, 1999). Luteinizing hormone not only stimulates follicular growth, but also enhances the DF secretory capacity resulting
in high amounts of estradiol secretion. Estradiol-17\(\beta\) concentrations in blood circulation increase until the stimulus of estradiol on the hypothalamus and pituitary is sufficient to trigger estrus behavior and the preovulatory LH surge (reviewed by Clarke 1989; reviewed by Allrich 1994). At this time, the cow is receptive to be mounted by the bull if natural service is utilized or be artificially inseminated.

In cattle, the pattern of follicular development follows a wave-like manner with the presence of 2 or 3 waves (95% of the cases) of growth and atresia (Ireland and Roche 1987; Sirois and Fortune 1988). The number of waves in each estrous cycle is highly correlated to the duration of the cycle since cows having shorter interestrual periods will usually have 2 waves; whereas, those having longer estrous cycles will usually follow a 3 wave pattern (Townson et al., 2002). Each wave is characterized by recruitment of a growing pool of follicles ~3 to 4 mm in size. This cohort of follicles undergoes a common growth phase until one is selected as dominant while the rest are considered subordinates (reviewed by Crowe 1999). Under some circumstances, two follicles will be selected as dominant leading to double ovulation if these follicles correspond to the ovulatory wave of the cycle. While subordinate follicles discontinue their growth and undergo atresia, the dominant follicle, which is ~8 mm in diameter at selection, continues increasing in size in a process called deviation (Ginther et al., 1996; Ginther 2000; Kulick et al., 2001). When dominance of a follicle(s) coincides with high levels of progesterone, characteristic of mid-luteal phase, the hormonal environment is inadequate to sustain either further growth or ovulation with the
consequent atresia of the dominant follicle. Conversely, by the end of luteal phase when luteolysis is produced, the hormonal milieu is characterized by low levels of progesterone and high estradiol-17β concentrations which induce high LH pulsatility favoring final growth of the DF to preovulatory size (12-22 mm). At this point in time the DF produces sufficient estradiol-17β to exert a positive feedback at the pituitary level and induce the preovulatory LH surge which then triggers the process ovulation and luteinization (reviewed by Smith et al., 1994).

Once the ovulatory process begins, granulosa and theca cells, which represent the two primary types of cells forming a follicle, undergo a series of profound morphological and biochemical transformations known as luteinization (reviewed by Smith et al., 1994). A significant change in hormone production occurs during this phase. There is a switch in steroid production from estradiol-17β to progesterone as well as intrafollicular secretion of local acting hormones, growth factors, cytokines, and enzymes necessary for follicular wall breakdown (reviewed by Espey 1994). Immediately after follicular rupture, a cumulus oocyte complex (COC; oocyte with its surrounding cummulus cells) is released and the follicular cavity is filled with blood to form what is called the corpus hemorrhagicum which will later become the CL (reviewed by Stocco et al., 2006). The formation of the CL is critical for maintenance of pregnancy if successful fertilization and embryo development have occurred (Mann et al., 1999; Okuda et al., 2002).
2.5. Possible Mechanisms for Decreased Reproductive Efficiency during Heat Stress

Elucidation of mechanisms associated with reproductive inefficiency of dairy cows suffering HS has never been a simple task. Therefore, a considerable amount of in vivo and in vitro models have been utilized in an effort to decipher basic mechanisms behind HS-derived infertility. Despite a general consensus on most of the subjects studied, often times the results obtained in a particular study are inconsistent with those generated by others. Disparity in the results may be attributed to the great variation in the experimental designs employed and conditions underlying each particular study. For instance, results obtained in studies using acute vs. chronic HS, lactating vs. non-lactating cows, heifers vs. cows or different blood sampling frequencies are very difficult to compare because of disparity in the physiological status of each particular type of animal or the nature and severity of HS.

In the following section, the author describes at the best of his knowledge how HS may be disrupting reproduction in dairy cows in the most updated and detailed manner possible.
2.5.1. Response of Hypothalamic-Pituitary-Gonadal Axis under Heat Stress Conditions

Alterations in hormonal secretion have been long implicated as a possible cause of infertility in cows suffering HS (Thatcher 1974). Therefore, several studies have been designed to evaluate how HS modifies hormone secretion and its consequences. Clarification of the endocrine mechanisms involved in reproductive failure would not only be important to better understand the physiology of cows suffering HS but also to develop therapeutic strategies capable of overcoming poor fertility associated with HS.

Madan and Johnson (1973) reported that heifers suffering HS, after placement in a controlled environment with temperatures of 33.5°C and 55% RH, had lower basal LH concentration during the estrous cycle than their non-HS counterparts. Under the same conditions, those heifers undergoing thermal stress presented lower LH concentrations during the preovulatory peak. Wise et al. (1988) reported that non-cooled lactating dairy cows during the summer had lower LH pulsatility (pulse frequency) in the early stages of the estrous cycle; however, LH pulsatility was similar for cooled and non-cooled cows when evaluated during mid cycle. Later research performed by Gilad et al. (1993) suggested that impaired gonadotropin secretion in chronic heat stressed cows may be linked to estradiol concentrations. In their experiment, basal and peak levels of FSH and LH were measured in lactating cows during mid cycle before and after administration of GnRH to test the responsiveness of the hypothalamic-pituitary-
gonadal axis. Chronic HS decreased mean levels and pulse amplitude of tonic LH secretion and diminished GnRH-induced LH release by 27%. It should be noted that under conditions of this study, effects of HS were only evident in cows having low estradiol concentrations in contrast to cows presenting high concentrations of estradiol. Conversely, results of other studies disagree with Gilad et al. (1993) reporting either no differences (Gwazdauskas et al., 1981; Rosemberg et al., 1982; Younas et al., 1993; Ronchi et al., 2001) or in some cases increased (Roma-Ponce et al., 1981) basal and preovulatory surge concentrations of LH.

For FSH, Gilad et al. (1993) reported reduced plasma concentrations after GnRH administration but only in cows with low levels of estradiol (as with LH) when acute HS was induced during the winter. Chronic HS during the summer lowered FSH concentrations as well but just in one of the two analyzed periods. In contrast, Roth et al. (2000) demonstrated that plasma concentrations of FSH prior to emergence of the second follicular wave and during the preovulatory surge were higher for HS cows than in cooled cows not treated with GnRH. This was accompanied by lower levels of inhibin during the estrous cycle of the HS group. Conversely, in a study to characterize the effect of HS on Holstein heifers in a controlled environment, it was reported that baseline levels, pulse frequency and amplitude of FSH were unchanged by thermal stress (Ronchi et al., 2001).

Similar controversy exists on effects of HS on steroidogenesis in dairy cows. Some studies reported no differences (Roman-Ponce et al., 1981; Wise et al., 1988; Badinga et al., 1993; Roth et al., 2000; Roth et al., 2001; Ronchi et al.,
decreased (Gwazdauskas *et al.*, 1981; Wilson *et al.*, 1998a,b) or even increased (Rosemberg *et al.*, 1982; Folman *et al.*, 1983) estradiol follicular synthesis and blood concentrations. The great variation in experimental designs and conditions in which these experiments were performed might be responsible for the disparity in the results. For instance, Wolfenson *et al.* (1995) reported that plasma estradiol concentrations in heat stressed cows were higher at the beginning of the estrous cycle, lower during dominance of the first wave dominant follicle, higher at the time of second wave dominant follicle dominance, and the same at the preovulatory surge. Wilson *et al.* (1998a,b) observed lower estradiol concentrations in both heat stressed cows and heifers starting the day of luteolysis in a set of two experiments placing the animals in environmental chambers. Additionally, in vitro studies carried out to clarify the reasons for lower estradiol concentrations under in vivo HS conditions documented decreased steroidogenic activity in theca cells of cows exposed to HS during variable periods of time (Wolfenson *et al.*, 1997; Roth *et al.*, 2001). It was concluded that the possible mechanism mediating deficient estradiol production was damage to theca cells. Lower androstenedione (substrate for estradiol synthesis) concentrations in follicular fluid of heat stressed cows suggested that theca cells affected by hyperthermia are unable to maintain androstenedione secretion at normal rates in contrast to granulosa cells which in the same study showed similar metabolic and secretory capacity regardless of treatment.

Progesterone production by the CL is important for normal cyclicity as well as for establishment and maintenance of pregnancy. Lower levels in plasma
(Rosemberg et al., 1982; Folman et al., 1983; Howell et al., 1994; Ronchi et al., 2001) and decreased in vitro production (Wolfenson et al., 1993) of progesterone have been reported for cows suffering HS. Conversely, other studies found no differences in progesterone concentrations in non cooled cows during the summer (Wise et al., 1988; Younas et al. 1993; Wolfenson et al., 1995). Despite controversy in results from some experiments, it is usually considered that exposure to summer HS suppresses progesterone production. Inadequate levels of progesterone before and after insemination may be detrimental for fertility of dairy cows.

2.5.2. Follicular Dynamics in Cows Undergoing Heat Stress

Disturbances of follicular dynamics are prominent during periods of HS. The major effects of hyperthermia on follicular growth and development include a reduced capability of the DF to exert dominance over subordinate follicles (Badinga et al., 1993; Wolfenson et al., 1995; Roth et al., 2000; Guzeloglu et al., 2001) and decreased size of the DF (Badinga et al., 1993; Wilson et al., 1998a,b; Cunha et al., 2007).

Impaired DF dominance is reflected by 1) inefficiency of the DF to suppress growth of the subordinate follicles, as evidenced by higher numbers of large-sized follicles (≥ 10 mm) after deviation and 2) earlier emergence (2 to 4 d) of the subsequent follicular wave (Wolfenson et al., 1995; Roth et al., 2000). Increased number of large-sized follicles after deviation suggests alterations of the
hormonal environment in a particular way that supports growth of subordinate follicles for longer periods of time. Roth et al. (2000) suggested that the reason for this anomalous growth pattern was the higher basal and peak FSH concentrations observed in heat stressed cows. Consistent with this finding is the concerning lowered plasma inhibin concentrations reported in the same study since this hormone is one of the major suppressors of FSH secretion in bovine. Therefore, it was concluded that impaired dominance was a consequence of hyperthermia impeding normal secretion of hormones (inhibin and estradiol) related to FSH regulation by follicular cells (Wolfenson et al., 1993; Roth et al., 2000). Another feature of disturbed follicular dynamics in heat stressed cows is the emergence of the second follicular wave 2 to 4 d earlier than in non heat stressed cows (Wolfenson et al., 1995; Roth et al., 2000). An end result of earlier wave emergence is the growth of the dominant follicle for an extra period of time after recruitment. Based on this assumption, Wolfenson et al. (1995) suggested that in cows presenting estrous cycles with two follicular waves, the oocyte may be “older” or “aged” at the time of ovulation. It is uncertain whether this mechanism is responsible or not for the infertility syndrome observed in dairy cows during the summer HS. If so, it would be through a direct effect on follicular cells and the environment on the oocyte.

Another particular aspect of follicular dynamics in lactating dairy cows undergoing HS is the presence of DF of smaller size. Badinga et al. (1993) reported that size of the DF was bigger and contained more follicular fluid in cows maintained under a shade management system when compared to cows with
no shade. These observations are in agreement with those of Wilson et al. (1998a, b) who reported a decrease in size of the DF for heifers and lactating dairy cows under artificially induced HS conditions. Furthermore, a recent study conducted in Wisconsin where HS during summer is not as extreme as in the southern United States suggests that size of the DF follicle is affected by HS. Results of this study indicate that cows ovulated smaller follicles during the summer season when compared to the winter season (Cunha et al., 2007).

2.5.3. Estrus Behavior in Heat Stressed Cows

It has been shown that HS alters expression of estrus by decreasing the proportion of cows showing signs of heat (Younas et al., 1993). In addition, both duration and intensity of estrus are also reduced (Gangwar et al., 1965; Madan and Johnson 1973; Gwazdauskas et al., 1981; Wolfenson et al., 1988). Indeed, Nebel et al. (1997) reported that the number of mounts during estrus in lactating cows during summer was one-half the number of mounts during winter. In contrasts, others had reported no seasonal effects on expression of estrus (Badinga et al., 1994); however; as most of the studies show, it seems clear that cows undergoing HS have a decreased estrous expression during the warm season.

Estrous behavior depends upon circulating estradiol-17β acting on the hypothalamus. During the preovulatory period, the dominant follicle plays a critical role in the initiation of estrus by the secretion of high amounts of estradiol-17β. When concentrations of this steroid hormone increase in blood, a
surge is produced triggering estrus behavior (reviewed by Allrich 1994). In the bovine, signs of heat include increased motor activity, mounting of herdmates, sniffing other cow’s vulvar area and standing when mounted (Van Eerdenburg et al., 2002). Associated with these behavioral changes is an augmentation in the production of metabolic heat. Therefore, cows under environmental conditions unfavorable for heat dissipation attempt to decrease their physical activity in order to decrease production of excess heat. It is then reasonable to hypothesize that the most likely cause of altered estrus expression is the reduction in motor activity seen in dairy cows undergoing thermal stress. Another explanation could be the lower estradiol concentrations during the preovulatory phase reported by Gwazdauskas et al. (1981). However, this mechanism seemed to be unlikely since estradiol still produced the ovulatory LH surge and ovulation in all animals studied.

As reproductive performance of dairy herds using artificial insemination (AI) depends upon efficient and accurate detection of estrus, altered expression of estrus causes difficulties in detection. Consequently, cows undergoing HS are either less likely to be detected when showing estrus resulting in fewer cows submitted for breeding or inseminated at the inappropriate time. Under these circumstances, maximizing the number of cows artificially inseminated requires either improvement in efficiency of the estrous detection techniques, which are labor and time consuming, or the use of timed artificial insemination (TAI) protocols, which allow breeding 100% of the available cows at the same time without detecting estrus.
2.5.4. Ovulation in Heat Stressed Cows

Direct (confirmation of ovulation by rectal palpation or ultrasonography) and indirect (presence of at least one CL) evidence from several reports indicates that the proportion of cows ovulating during summer or under controlled HS conditions are similar than for non HS cows (Gwazdauskas et al., 1981; Putney et al., 1989b; Wolfenson et al., 1995; White et al., 2002; Bloch et al., 2006). Conversely, others had reported that ovulation rate confirmed by the presence of one or more CL’s 11 d after AI is affected during the warm season in high producing lactating dairy cows (López-Gatius et al., 2004; López-Gatius et al., 2005). In one study (López-Gatius et al., 2004) ovulation rate during a well defined warm season was significantly lower than during cool season (70.5 vs. 89.7%, respectively) whereas, in a subsequent study ovulation rate was again significantly lower during the warm than in the cool season (87.6 and 96.9%, respectively). Discrepancies among the results of those studies claiming no differences in ovulation rate (Gwazdauskas et al., 1981; Putney et al., 1989b; Wolfenson et al., 1995; White et al., 2002; Bloch et al., 2006) and, the two most recent studies showing decreased ovulation rate in lactating cows during HS (López-Gatius et al., 2004; López-Gatius et al., 2005) limit further conclusions about ovulation rate under HS conditions.

Regarding timing of ovulation, few studies have evaluated ovulation time in HS cattle. For instance, Gwazdauskas et al. (1981) assessed hormonal patterns and ovulation in Holstein heifers placed in environmental chambers to induce
hyperthermia. The results of this study, in which ovulation was assessed by rectal palpation, suggest no alteration in timing of ovulation in heat stressed heifers. Despite observing a decrease in the duration of estrus (21.0 ± 3.8 vs. 16.0 ± 3.7 h for control and HS, respectively) the time interval from the LH surge to ovulation was similar for control and HS heifers. Further, Wolfenson et al. (1995) reported that in dairy cows undergoing HS emergence of the second-wave/DF occurs 2 to 4 d earlier; however, they did not observe differences in duration of estrus and time of ovulation. A recent study evaluated the timing from the onset of estrus to ovulation in high yielding dairy cows during spring, summer and fall in Israel (Bloch et al., 2006). During summer, maximum and minimum air temperature and RH were 32.7 and 22.5°C, 69 and 53% respectively. A sprinkling and ventilation system was used to cool the cows; however, because of the high ambient temperatures and RH it is logical to consider that this cows were under conditions of thermal stress. The results of this study indicate that, neither the ability of cows to ovulate nor time of ovulation was different during summer since ovulation was confirmed in 100% of the animals included in the experiment and, data from the three (spring, summer and fall) seasons were pooled together suggesting no differences in ovulation time.

No other experiments have been conducted with either heifers or lactating dairy cows to evaluate timing of ovulation under HS conditions. Therefore, it will be assumed that the timing required for the process is unchanged by environmentally induced hyperthermia. This assumption is based on the few experiments discussed above and another study utilizing mature beef cows in
Oklahoma (White et al., 2002). The authors evaluated the time elapsed between onset of estrus and ovulation during the summer, winter and spring. No differences were found among the three seasons indicating that, at least for this animal model which greatly differs from a lactating dairy cow, high ambient temperature during the summer months did not affect the average time at which ovulation occurred.

2.5.5. Effects of Heat Stress on Oocytes

Alterations of embryonic development caused by the effect of elevated temperatures on the oocyte have been shown both in vivo (Putney et al., 1989b) and in vitro (Edwards and Hansen 1996; Ju et al., 1999; Edwards et al., 2005) indicating that the female gamete during maturation is very sensitive to HS. The extent of the damage produced by hyperthermia is quite variable depending upon temperature and duration of the exposure (Edwards and Hansen 1997; Edwards et al., 2005)

Oocytes seem to be affected during both the early stages of follicular development and the periovulatory period (when they undergo the process of maturation). Effects of HS during early stages of follicular development were reported by Al-katanani et al. (2002) who performed a set of two in vitro experiments to compare the developmental potential of vitro matured (IVM) and fertilized (IVF) oocytes collected from antral follicles in dairy cattle during either the warm (cooled vs. not for 42 d) or cool season. It was concluded that 1) oocyte
(non preovulatory oocytes) competence in Holstein cows declines during the summer and 2) cooling the cows for a period of 42 d prior to oocyte collection does not alleviate seasonal effect. In support to the data of Al-katanani *et al.* (2002), other in vitro experiments have shown lower embryo development when oocytes obtained from antral follicles were directly exposed to HS (Payton *et al.*, 2004) and when oocytes are obtained during the hot *vs.* the cool season (Rocha *et al*., 1998; Rutledge *et al*., 1999).

In bovine, compelling evidence indicates that the major effects of hyperthermia occur after the onset of estrus when the oocyte is undergoing the process of maturation. In this regard, Putney *et al.* (1989b) reported that early embryonic development was dramatically affected in dairy heifers acutely exposed to HS during the periovulatory period. Embryos collected from Holstein heifers placed in environmental chambers under conditions sufficient to increase rectal temperature to > 41.0°C for the first 10 h after the onset of estrus were retarded and/or arrested at the 8- to 16- cell stage. In addition, when subjected to morphological analysis, most of these embryos were classified as fair to poor quality. Taken together, these observations indicate that maternal hyperthermia during estrus, which coincides with oocyte maturation in vivo, alters the female gamete when it is preparing for fertilization.

The findings of Putney *et al.* (1989b) in vivo are supported by later research performed in vitro which indicated that exposure of oocytes at 41°C during maturation decreased the proportion of embryos developing to the blastocyst stage (Edwards and Hansen 1996, 1997). Continued efforts to elucidate
the possible mechanisms responsible for impaired embryonic development of
heat-stressed oocytes suggested that the ability of the oocyte to complete the
process of nuclear (i.e., progression to metaphase II) and cytoplasmic (i.e.,
translocation of cortical granules to the periphery of the cytoplasm) maturation in
vitro is not affected by exposure of the oocyte to temperatures easily reached by
cows undergoing HS. Ova cultured under hyperthermic conditions (41°C)
completed nuclear and cytoplasmic maturation in a similar fashion than the non
heat stressed counterparts. However, while hyperthermia did not affect the
capability of the oocyte to mature, it did alter the timing required for the
completion of the process. In this study, oocytes exposed to 41°C for the first 12 h
of IVM reached nuclear and cytoplasmic maturation 4 to 6 h earlier than the non
heat stressed controls. These results prompted an additional experiment to test if
earlier IVF may improve blastocyst development in heat stressed oocytes.
Interestingly, IVF 5 h earlier than the usual 24 h used for IVM/IVF produced
blastocyst development similar to those in the non heat stressed control group. In
agreement with this study, Schrock et al. (2007) reported that earlier IVF at 16, 18
and 20 h was beneficial to increase development of heat stressed oocytes.
Development to the blastocyst stage was higher for the heat-stressed group IVF at
18 and 20 h when compared to IVF at 24 h post maturation. Moreover, the stage
of development and quality scores of the embryos that did developed to the
blastocyst stage were similar for those derived from heat-stressed and non-heat
stressed oocytes. It is worth noting that even though a clear improvement in
developmental rates was found in oocytes undergoing HS (close to control values,
≥ 20%), earlier IVF did not completely eliminate the harmful effects of hyperthermia. Based on the results of this set of experiments (Edwards et al., 2005; Schrock et al., 2007), the authors suggested that the major effect of elevated temperature is to hasten oocyte maturation resulting in the fertilization of an “aged” oocyte if IVF is performed 24 h post-maturation.

If in vivo effects of hyperthermia are similar to those observed in vitro by Edwards et al. (2005) and Schrock et al. (2007), then premature aging of the oocyte when contained within the follicle is of concern given the short fertile lifespan of matured oocytes (8 to 10 h; Hunter 1985). Since the time elapsed from the LH surge to ovulation is not modified by HS (Gwazdauskas et al., 1981; Wolfenson et al., 1995; White et al., 2002; Bloch et al., 2006) and hyperthermia induces premature aging of the oocyte, then it is logical to speculate that by the time of their release from the follicle, oocytes may be “aged” and consequently less competent. Under these hypothetic circumstances, it is unlikely that the oocyte will develop into a normal embryo and establish a pregnancy.

### 2.6. Strategies to Improve Reproductive Efficiency in Heat Stressed Cows

#### 2.6.1. Modification of the Cow Microenvironment

The impact of providing cows with shade and cooling to alleviate the effect of summer HS in reproduction has been extensively evaluated (reviewed by Jordan et al., 2003). The two most common methods of environmental
management used to mitigate effects of HS include protecting cattle from direct solar radiation with supplemental shade or favor evaporative cooling through forced ventilation and sprinklers (Armstrong 1994; Collier et al., 2006). Many studies were conducted to evaluate if providing cows with shade and cooling during the summer months improved reproduction in hot environments. For instance, conception rates (CR) of cows exposed to a shade system during the summer in Florida were significantly higher than cows maintained in uncovered facilities (38.2 vs. 14.3%, respectively) indicating that decreasing direct solar radiation on the cow alleviates HS and helps to improve fertility (Roman-Ponce et al., 1981). On the other hand, favoring evaporative cooling through forced ventilation and sprinkling resulted in a higher proportion of cows expressing estrus and less were considered as anestrus (Her et al., 1988). In this study, despite the better cyclicity and estrous detection in the cooled group, the CR was not different between groups, suggesting that cooling cows with the system used was not sufficient to mitigate the negative impact of HS. Conversely, other reports have shown improvements in reproductive performance when comparing the use of evaporative cooling with shade to alleviate HS. Lactating Holstein cows receiving different cooling treatments (sprinklers and forced ventilation or shade) during the preovulatory period and early embryonic development had significant differences in PR (Ealy et al., 1994). In this study, it was concluded that pregnancy rates may be further improved with evaporative cooling than with shade since the PR were 16% and 6.2%, respectively (Ealy et al., 1994).
2.6.2. *Nutritional Strategies to Improve Reproduction in Heat Stressed Cows*

Under conditions of heat stress regulatory mechanisms are activated in lactating dairy cows to decrease dry matter intake in an attempt to lower the amount of metabolic heat produced (reviewed by De Rensis and Scaramuzzi 2003). A direct consequence is to exacerbate the negative energy balance associated with the high levels of milk production. Therefore, it is necessary to modify the cow’s diet in order to reestablish adequate amounts of energy intake. Since higher proportions of fiber in the diet of cattle generate extra amounts of metabolic heat (Reynolds *et al*., 1991) a possible strategy to increase energy intake while limiting the amount of heat produced is to decrease fiber and increase the amount of high energy compounds such as concentrates and fat in the diet (Mody *et al*., 1967; Beede and Collier 1986; Knap and Grummer 1991). The benefit of diets with low fiber to high concentrate ratios for dairy cows under HS may be to improve the general comfort of the cow as a result of lower heat production and therefore improve fertility (West 2003).

Others attempted to improve lactating dairy cow’s fertility during the summer through supplementation with antioxidants (Ealy *et al*., 1994; Aréchiga *et al*., 1998) since reports of in vitro studies using murine and bovine embryos showed a positive effect on embryo development when supplementing culture media with antioxidants (Ealy *et al*., 1992; Malayer *et al*., 1992; Aréchiga *et al*., 1994). The reason for improved development seemed to be a reduction of heat shock effects on the embryo. Therefore, it was speculated that administering
antioxidant substances such as Vitamin E or β-carotene (Vitamin A precursor), to lactating dairy cows would have the same benefits as in vitro. For instance, Ealy et al. (1994) provided a diet supplemented with Vitamin E to meet the requirements of lactating dairy cows and administered 3000 IU i.m. at the time of AI. No differences were found in pregnancy rate between supplemented and control groups suggesting that Vitamin E was not effective in alleviating the effects of HS or, the dose and time of administration were inadequate. In contrast, inclusion of β-carotene for long periods of time (≥ 90 d) seemed to have a mild beneficial effect on reproduction (Aréchiga et al., 1998). In this study, the percentage of pregnant cows at 120 d was significantly higher in the supplemented group. The poor improvement in reproductive efficiency observed in these experiments after supplementing HS dairy cattle with Vitamin E or β-carotene suggests that in vivo modification of the antioxidant status of the cow does not entirely solve the problem of summer infertility.

2.6.3. Use of Reproductive Management and Biotechnologies to Ameliorate Reproductive Performance under Heat Stress

2.6.3.1. Use of Natural Service

In order to avoid estrous detection and improve fertility, some dairy producers tend to increase the use of natural service during the summer months (reviewed by Hansen and Aréchiga 1999; reviewed by de Rensis and Scaramuzzi
However, this breeding system does not overcome the negative impact of heat stress on the cow. Record analysis of an 8-year period indicated that PR of dairy herds during the summer months in Florida and Georgia, using natural service as the only breeding system, was almost half of the PR obtained during the winter months (9.8% vs. 18%, respectively; de Vries et al., 2005b). These results are not surprising since bull fertility also declines under HS conditions (Fricke 2003).

Semen quality of ejaculates obtained in periods of thermal stress are characterized by decreased sperm concentration, lower sperm motility, and increased number of morphologically abnormal sperm (Fricke 2003). Moreover, the quality of the semen is not similar to pre-stress conditions after at least two months of exposure to conditions of HS (Fricke 2003). In addition to the incapability of the bull to improve PR, the loss of genetic progress (reviewed by Hansen and Aréchiga 1999), the cost associated with maintenance of the bull(s), and the risk for the farm personnel (Fricke 2003) are other drawbacks of implementing natural service as breeding systems in dairy herds. As a possible solution, the use of AI has been proposed to bypass the negative impact of HS on bull fertility (reviewed by Hansen and Aréchiga 1999) and avoid other costs and risks associated with maintenance of bulls. However, bypassing negative effects of HS on the quality of the semen through the use of AI after estrus detection is not a viable alternative to ameliorate reproductive efficiency of dairy cows. Indeed, several researchers reported diminished PR using AI in regions affected by HS (Schmitt et al., 1996; de Vries et al., 2005b) which is not
surprising since chances of obtaining a pregnancy depend upon adequate estrus
detection and conception rate which are usually impaired during HS (Ingraham et al., 1976; Younas et al., 1993; Nebel et al., 1997).

2.6.3.2. Use of Gonadotropin Releasing Hormone at the Time of Estrus

Administration of GnRH at or around the onset of estrus was proposed as a possible strategy to help solve the infertility problem of lactating dairy cows in hot climates. Ullah et al. (1996) reported higher CR in cows treated with GnRH after visual observation of estrus under conditions of HS. In this experiment, the difference in CR observed was attributed to the higher concentrations of progesterone in treated cows as a result of GnRH injection. Other researchers reported similar benefits of administering GnRH at or around the time of the onset of estrus. Overall CR in lactating dairy cows in Israel increased from 35.1% to 51.6% when GnRH was given within 3 h of the onset of estrus during the summer months (Kaim et al., 2003). However, in this study, statistical differences were only observed in primiparous cows, while no significant differences were observed in multiparous cows. Based on results of this, and a previous experiment in which GnRH was given at the time of onset of estrus (Kaim et al., 2003), it was suggested that the beneficial effects of this practice consist in reduction of the time interval (~1.5 h) from the onset of estrus to the preovulatory LH surge. Moreover, GnRH-treated cows had higher LH surges resulting in higher overall concentrations of LH. The authors concluded that GnRH at the time of onset of
estrus may have improved fertility in dairy cows during the summer by several mechanisms including increased height of the LH surge and overall concentration of LH, preventing delayed ovulation, and possibly by increasing progesterone secretion by the CL. Therefore, administration of GnRH or its analogs at the time of onset of estrus seems to be a promising alternative to improve fertility under HS. Indeed, Edwards et al. (2005) hypothesized that administering GnRH at the onset of estrus may improve fertility by reducing the time interval from estrus to ovulation therefore maximizing the chances of fertilization of the oocyte during its fertile lifespan. However, application of this protocol in commercial dairy farms is far from being extensively adopted because of impractically of estrus detection and labor demands.

### 2.6.3.3. Synchronization of Ovulation and Timed Artificial Insemination (TAI)

Synchronization of ovulation protocols consist in the administration of a series of hormones at scheduled times to manipulate growth of ovarian follicles, regression of the CL, and ovulation. The ultimate purpose of these protocols is to synchronize the time of ovulation and inseminate cows at a fixed time eliminating the need for estrus detection. Ovsynch, which consist in the application of an initial dose of GnRH at random stages of the estrous cycle, PGF$_{2\alpha}$ 7 d later followed by another dose of GnRH at 48 h and AI 16 h later (Pursley et al., 1995), is the most widely used protocol in commercial dairy herds (Caraviello et al., 2006). With protocols like ovsynch it is possible to obtain conception rates similar
to those observed in cows bred after estrus detection with the advantage that ovsynch allows AI of as many cows as desired at the same time without estrus detection (Pursley et al., 1995; Pursley et al., 1997; Stevenson et al., 1999; Peters and Pursley 2003).

As several reports showed similar CR for cows bred by TAI or by AI after estrus detection, researchers speculated that their use might be beneficial for dairy cattle under conditions of HS. The major benefit of using these protocols would be the opportunity to inseminate all available cows without detecting estrus (Aréchiga et al., 1998), since expression of estrous behavior is of shorter duration (Younas et al., 1993) and less intensity (Gangwar et al., 1965; Madan and Johnson, 1973; Gwazdauskas et al., 1981) in heat-stressed lactating dairy cows. In this regard, Aréchiga et al. (1998) evaluated the use of a TAI protocol as a strategy to improve PR of lactating cows in hot environments. In their study, they reported a higher cumulative PR at 90 d postpartum for the TAI group compared to AI after detection of estrous during the hot season. Similar improvements in PR with TAI during summer were reported by de la Sota et al. (1997). However, despite improvements in PR at 90 d in both studies, CR at first service was not different for TAI and control cows, indicating that the most probable cause of the improved PR with TAI was the higher AI submission rates. In contrast others have observed no differences in cumulative PR (90 and 135 d postpartum) of cows bred using AI after estrus detection or TAI during the winter and summer (de Rensis et al., 2002). Pregnancy rates of TAI cows during summer were similar
to those of the winter but did not significantly differ from the PR of cows AI after estrus detection (de Rensis et al., 2002).

In conclusion, the use of synchronization of ovulation and TAI protocols has some benefits for dairy reproduction during the summer. With their application, higher cumulative pregnancy rates can be achieved and days open can be reduced because of the higher AI submission rates. However, in spite of the beneficial effects associated with TAI, the current protocols can not mitigate effects of HS on CR.

2.6.3.4. Induction of Accessory Corpus Luteum

Increasing progesterone concentrations after insemination of lactating dairy cows might help to establish and maintain pregnancy during its early stages when the majority of the losses occur (reviewed by Santos et al., 2004). Because in HS dairy cows the majority of embryonic losses take place within 7 d after breeding (Putney et al., 1988; Ealy et al., 1993) a positive effect of increased progesterone concentrations might be only observed when induced during this critical period (Schmitt et al., 1996). In this regard, several researchers were successful in increasing blood progesterone concentrations in dairy cattle after AI by inducing formation of accessory CL (Helmer and Britt 1986; Price and Webb 1989; Walton et al., 1990; Fricke et al., 1993; Schmitt et al., 1996; Diaz et al., 1998; Santos et al., 2001). Accessory CL can be induced by administering an ovulatory dose of GnRH or hCG between days 5 and 11 of the estrous cycle.
following AI. The resulting increased progesterone concentrations might benefit embryonic development in cows undergoing HS. Indeed, Schmitt et al. (1996) evaluated if the injection of hCG, GnRH or combinations of both 5 d after AI would increase CR in heifers and lactating cows during periods of HS. Despite the good ovulatory response and CL formation (91% GnRH and 93% hCG), CR of the treated group was not different from controls for neither heifers nor cows. Conversely, a more recent study reported a tendency to have better conception rates in cows undergoing mild heat stress treated with GnRH either on day 5 or 11 after TAI (Willard et al., 2003). Since induction of accessory CL was not consistently effective in increasing PR under experimental conditions, whether or not this strategy may be a possible solution to the HS-induced infertility under commercial conditions is uncertain.

2.6.3.5. Embryo Transfer

The use of embryo transfer (ET) to circumvent the negative impact of HS on reproduction of dairy cows has been proposed by several researchers (Putney et al., 1989a; Drost et al., 1999; reviewed Hansen and Aréchiga 1999; reviewed by Jordan 2003). Transferring D7 embryos into recipient cows might bypass negative effects of uterine hyperthermia on the early embryo (Putney et al., 1989a; reviewed by Hansen and Aréchiga 1999). Putney et al. (1989a) evaluated the potential advantage of using ET instead of AI to improve PR during the summer months in a subtropical environment. The percentage of cows pregnant
with ET at 40 d was greater than in the AI group (29.2 vs. 13.5%, respectively), despite an estimated 18.4% pregnancy loss in the ET group after pregnancy determination by blood progesterone 21d after estrus (Putney et al. 1989a). Later research suggested that the outcome of an ET program also depends upon the source of embryos (in vivo vs. in vitro) and if embryos are transferred fresh or frozen (Drost et al., 1999; Ambrose et al., 1999; Al-katanani et al., 2002). In this regard, Drost et al. (1999) compared the PR of AI with the PR of an ET program after transfer of frozen embryos either collected from superovulated donors or produced by IVF. No differences among groups were evident when pregnancy was estimated through progesterone determination at 21 d. However, at 42 d, a significant difference was observed between the group receiving embryos from superovulated donors (35.4% CR) when compared to the group receiving in vitro produced embryos (18.8% CR).

Other researchers attempted to improve results obtained with transfer of in vitro produced embryos since they represent a more economical source for transfer in commercial operations. The best results were obtained when embryos produced by this technique are transferred fresh to recipient cows (Ambrose et al., 1999; Al-katanani et al., 2002). Further, the recent study of Franco et al. (2005) reported a PR at 67 d of 57% when in vitro produced embryos were transferred fresh to lactating crossbred dairy cattle. This PR is higher than the previously reported for heat stressed cows receiving in vivo derived embryos (35.4%; Drost et al., 1999). However, limited conclusions can be drawn from this study given the low number of transferred embryos (n = 14).
Finally, in spite of the apparent benefits of using ET to mitigate effects of HS on lactating dairy cows, this technology has not been extensively adopted at the commercial level. Its application requires the involvement of highly experienced technicians and the use of considerable amounts of supplies which usually implies a high costs for commercial producers (reviewed by Rutledge 2001). On the other hand, the best results are obtained with fresh transferred embryos; thus, requiring collection of the embryos at the time of transfer when the donor cows will also most likely be affected by HS (reviewed by Rutledge 2001).

2.7. Ovulation Mechanism and Acquisition of Ovulatory Capacity

2.7.1. Endocrine Mechanisms Responsible for Triggering Ovulation

The process of ovulation involves an intricate series of biochemical and physiological events that ultimately lead to the rupture of the preovulatory follicle, the release of a mature fertilizable oocyte (reviewed by LeMaire 1989; reviewed by Richards et al., 1998; reviewed by Sirois et al., 2004), and the transformation of the ovulatory follicle into the CL. Luteinizing hormone, via direct activation of receptors on the ovarian DF, is considered to be the physiological trigger of the process (reviewed by LeMaire 1989; reviewed by Richards et al., 1998). Thus, a prerequisite for ovulation to occur is a massive release of LH from the pituitary gland which can be detected in the peripheral circulation and is known as the “LH surge” (Henricks et al., 1970; Chenault et al,
In the cow, this surge takes place prior to, or at the onset of estrus (Henricks et al., 1970; Chenault et al., 1975; Lemon et al., 1975; Walton et al., 1987). Ovulation occurs ~ 25 h (range 22 to 33 h) thereafter (Kruip et al., 1983; Rajamahendran et al., 1989; Kaim et al., 2003; Hockett et al., 2005; Starbuck et al., 2006). Unlike concentrations observed during the luteal phase, which only support follicular growth, concentrations of LH in blood reached during the surge are sufficient to induce the plethora of biochemical mechanisms responsible for follicular wall breakdown and resumption of oocyte maturation.

During the luteal phase, high progesterone concentrations maintain a low GnRH pulse frequency and amplitude (Karsch et al., 1987). During the follicular phase, the progesterone suppression is removed allowing an increased GnRH pulsatility which consequently increases the frequency and amplitude of LH pulses (Hunter 2003). The DF responds to this higher LH pulsatility by increasing in size and producing greater amounts of estradiol-17β (reviewed by Allrich 1994) which ultimately exert a positive feedback on the hypothalamus and the pituitary gland (Chenault et al., 1975; reviewed by Allrich 1994). As the DF continues growing to ovulatory size, estradiol concentrations reach threshold level acting upon the hypothalamus and pituitary gland. At the hypothalamic level, estradiol-17β is responsible for increased GnRH pulsatility and the induction of estrus behavior (reviewed by Allrich 1994). At the pituitary level, estradiol-17β promotes responsiveness of the gonadotropes (cells responsible for FSH and LH synthesis and secretion) of the adenohypophysis to GnRH through expression of its receptors (Gregg and Nett 1989) and the mobilization of LH granules towards...
the plasma membrane (Hunter 2003). Thus, the role of estradiol in the induction of the LH surge is to increase the sensitivity of the gonadotropes to GnRH before it stimulates the pulsatile secretion of GnRH necessary for the occurrence of the LH surge.

Upon release from hypothalamic neurons, GnRH is transported via the hypophyseal portal system to the pituitary gland where it binds to its receptor in the cell membrane of gonadotropes (reviewed by Evans 1999). GnRH, as many other peptides hormones, interacts through specific amino acid residues present in its molecule with the NH$_2$-terminal domain of a specific G-protein coupled receptor known as GnRH type-1 (reviewed by Millar 2005). Formation of the GnRH-receptor complex is followed by a receptor conformational change and activation of G-proteins and non-G-proteins intracellular signaling pathways (reviewed by Millar 2005). It is suggested from studies using mice αT3-1 cells (Sundaresan $et$ $al.$, 1996; Kaiser $et$ $al.$, 1997) that GnRH binding results in activation of heterotrimeric GTP-binding G proteins of the G$_q$/G$_{11}$ families which then activate membrane phospholipase C (PLC). Through enzymatic breakdown of inositol biphosphate (IP$_2$) present in the plasma membrane, PLC produces inositol triphosphate (IP$_3$) and diacylglycerol (DAG) which lead to the activation of protein kinase C (PKC). This protein kinase (activates/deactivates other proteins by phosphorylation) stimulates the mitogen-activated protein kinases (MAPK’s) cascade which, in turn, regulates expression of genes required for synthesis of the alpha and beta peptide chains that make up FSH and LH. In regard to secretion, the two gonadotropins seem to be differentially regulated.
since each GnRH pulse is followed by a LH pulse but not FSH. Secretion of FSH is carefully regulated by other factors including inhibin, activin, follistatin, and estradiol-17β (reviewed by Millar 2005).

Once gonadotropins are secreted by the pituitary gland, they reach blood circulation and are taken up preferentially by ovarian tissues (Hunter 2003), favored by a marked increase in the permeability of the thecal vascular wreath (Halterman and Murdoch 1986). Once LH reaches the follicular interstitial space, it is then transported and accumulated in the follicular fluid (Fortune and Hansel 1985) of the ovulatory follicle where it binds to LH receptors of the granulosa and theca cell layers (Hunter 2003).

2.7.2. Effects of Luteinizing Hormone on Cells of the Ovulatory Follicle

Luteinizing hormone is a heterodimeric glycoprotein composed of two different polypeptide chains, α and β subunits, linked by non covalent bonds (Callesen et al., 1987; Cambarnous 1988; Stockell Hartree and Renwick 1992). This glycoprotein along with FSH, TSH (thyroid stimulating hormone) and the chorionic gonadotropins (eCG; equine chorionic gonadotropin and hCG; human chorionic gonadotropin) represents a family of hormones characterized by sharing the same α subunit but differing in the amino acid composition of their β subunit (Pierce and Parsons 1981) which is responsible for hormone receptor specificity (Cambarnous 1988; Stockell Hartree and Renwick 1992). Binding of the hormone
to its receptor depends on the presence of the complete heterodimer, since individual subunit lacks binding ability (Catt and Dufau 1973).

The LH receptor (LHR) is located on the cell plasma membrane given that its ligands are high molecular weight proteins incapable of traversing lipid bilayers (Catt and Dufau 1976). This receptor belongs to the G-protein-coupled-receptors (GPCR) superfamily (Kroeze et al., 2003; Vassilatis et al., 2003) and is activated by both LH and hCG, which bind the receptor with high affinity and specificity (Hearn and Gomme 2000). Nevertheless, there are some evident differences in hormone receptor interaction between LH and hCG. In vitro studies utilizing bovine follicular (theca) cells cultures indicate that physicochemical differences between hCG and LH may be responsible for the observed greater affinity of hCG for the LHR in these cells (Henderson et al., 1984). Furthermore, the binding of hCG to LHR presents greater stability than that of LH, consequently hCG remains bound to the receptor for longer periods of time than LH (Mock and Niswender 1983; Mock et al., 1983; Henderson et al., 1984). These marked differences in binding ability to the LHR and duration of hormone/receptor interaction between hCG and LH, do not necessarily imply functional differences since the ability to stimulate androgens synthesis by thecal cells was shown to be similar for both hormones (Henderson et al., 1984). On the other hand, contrary to the observations that hCG has greater affinity for the LHR in thecal cells, an in vitro study using bovine cumulus oocyte complexes (COC’s) indicated that LH presents nine fold greater binding capacity to LHR than hCG (Baltar et al., 2000). The reason(s) for the discrepancy in the results of binding
ability of LH and hCG when using either theca cells or COC’s is uncertain but differences between theca and cumulus (granulosa type) cells may be implied.

Structurally, the LHR is similar to all members of the GPCR superfamily as evident by seven transmembrane helices; three extracellular and three intracellular loops, and in most of them, an intracellular C-terminal tail (reviewed by Dufau 1998). Luteinizing hormone receptors are present in both theca and granulosa cells of the Graafian follicle. Theca cells first express LHR shortly after formation of the follicular antrum (~ 3 to 4 mm); whereas, granulosa cells begin expressing LHR when the follicle is ~ 9 mm in diameter as evidenced in both types of cells by the presence of LHR mRNA (Xu et al., 1995). Data from tests of hCG binding to intact follicles in pigs (Channing et al., 1981) and rats (Lawrence et al., 1980; Peng et al., 1991) indicate that within granulosa cells, the mural granulosa subpopulation (adjacent to the basement membrane) has a higher number of LHR than those of the cumulus cells (surrounding the oocyte). An immediate consequence of this marked difference is a divergent response of the two different sublineages to the preovulatory LH surge with the mural granulosa cells producing the greater response to LH stimulation (Russell and Robker 2007).

Upon binding of ligands (LH or hCG), the hormone-receptor interaction induces conformational changes of intracellular loops and the carboxy-terminal domain of the receptor, which then interacts with, and activates G protein systems (Strader et al., 1994; reviewed by Dufau 1998). Luteinizing hormone through its membrane receptors activates $G_{\alpha S}$ proteins which are heterotrimers composed of an $\alpha$ subunit and a $\beta\gamma$ complex bound to GDP (guanosine diphosphate) in the
inactive state. Once activated, G\(_{\alpha}\) proteins exchange GDP for GTP (guanosine triphosphate) decreasing \(\alpha\) subunit affinity for the \(\beta\gamma\) complex. Consequently, the heterotrimer dissociates and the \(\alpha\) subunit migrates within the cell to activate adenylate cyclase which results in a large intracellular increase in the production of the second messenger cAMP (cyclic adenosine monophosphate). Subsequently, the downstream regulatory activity of cAMP consists in the activation of protein kinase A (PKA; Marsh 1976). Thereafter, PKA phosphorylates other regulatory proteins such as cAMP regulatory element-binding protein (CREB; Mukherjee \textit{et al}., 1996; Salvador \textit{et al}., 2002) and the MAPK’s pathway (Das \textit{et al}., 1996; Salvador \textit{et al}., 2002), which then modulate the transcription of genes associated with ovulation.

2.7.3. Series of Events Leading to Rupture of the Ovulatory Follicle and Oocyte Release

As discussed above, the initiation signal for ovulation is the LH surge through activation of LH receptors on the plasma membrane of follicular and ovarian cells. Downstream of LH receptors, several pathways are stimulated triggering the process of follicle wall rupture (Figure 1) and initiation of oocyte maturation which culminates in the release of a viable female gamete ready for fertilization. A complex series of biochemical mechanisms are required to break down the multiple layers forming the follicular wall and the ovarian tissue surrounding the ovulatory follicle. The end result of this degradation process is
Figure 1. Proposed model of ovulation based on the literature reviewed. Many steps of the process occur within the cells of the follicle wall and then end products are released into the follicular antrum (cavity). However, for simplification, all processes were drawn within the follicular antrum (cavity). LH/hCG = luteinizing hormone/human Chorionic Gonadotropin, CT = connective tissue, BV = blood vessel, BM = basement membrane, OE = ovarian epithelium, TC = theca cells layer, GC = granulosa cells layer, P₄ = progesterone, P₄R = progesterone receptor, E₂ = estradiol-17β, COX-2 = cyclooxygenase 2, AA = arachidonic acid, PGH₂ = prostaglandin H₂, PGES = prostaglandin E synthase, PGFS = prostaglandin F synthase, PGE₂ = prostaglandin E₂, PGF₂α = prostaglandin F₂ alpha, uPA = urokinase plasminogen activator, tPA = tissue plasminogen activator, uPAR = urokinase plasminogen activator receptor, MMP-14 = matrix metalloproteinase 14, MMP-13 = matrix metalloproteinase 13, TIMP-2 = tissue inhibitor of the matrix metalloproteinase 2, ECM = extracellular matrix. See the text for explanation of the process. Relevant references utilized for development of proposed model of ovulation: Dieleman and Blankenstein 1985; Murdoch et al., 1986; Liu 1999; Komar et al., 2001; Bakke et al., 2002; Cassar et al., 2002; Dow et al., 2002; Jo et al., 2002; Jo and Fortune 2003; Li et al., 2004; Bakke et al., 2006; Bridges et al., 2006; Li et al., 2006; Bridges and Fortune 2007; Li et al., 2007.
the rupture of the follicular wall allowing release of the oocyte and the cells comprising the COC (cumulus oocytes complex).

During the final stages of follicular growth and prior to the preovulatory gonadotropin surge, estradiol is the primary steroid produced by the preovulatory follicle (Dieleman and Blankenstein 1984; Sirois 1994). Once the LH surge has occurred, there is a shift in the type of steroids synthesized by the follicle with estradiol-$17\beta$ concentrations decreasing while progesterone concentrations increase (Murdoch and Dunn 1982; Dieleman and Blankenstein 1985; Murdoch et al., 1986; Sirois 1994; Komar et al., 2001; Jo and Fortune 2003; Li et al., 2007). In the bovine, concentrations of progesterone present a peak by 2 to 3.5 h after a GnRH injection (Jo and Fortune 2003) then decrease, to finally present another peak at 24 h post GnRH (Jo and Fortune 2003; Li et al., 2007). Komar et al. (2001) presented compelling evidence of redundant mechanisms regulating the switch in steroid hormones production. They showed that a coordinated decrease in the expression of two key regulatory enzymes in estradiol production, P450 17α-hydroxylase (converts pregnenolone to dehydroepiandrosterone) and P450 aromatase (converts androstenedione to estradiol), would be responsible for the lowered estradiol concentrations in follicular fluid. Besides this increased progesterone production by the follicle during the periovulatory period, there is a dramatic biphasic increase in the abundance of progesterone receptor mRNA in granulosa cells of bovine pre-ovulatory follicles. Progesterone receptor mRNA levels peak within 6 h of the LH surge, return to pre-LH peak levels at 12 h
(Cassar et al., 2002; Jo et al., 2002) to finally increase 24 h after the gonadotropin surge (Jo et al., 2002; Figure 1).

Despite the demonstration of a pattern of progesterone synthesis and progesterone receptor expression in preovulatory follicles, the downstream pathway(s) activated by the hormone-receptor complex needs further clarification. In sheep, it has been proposed that the function of progesterone in ovulation is to facilitate collagenolytic activity in the follicular wall through activation of the enzyme PGE$_2$-9-ketoreductase responsible for the conversion of PGE$_2$ into PGF$_{2\alpha}$, a process previously implicated in the rupture of the follicle wall and ovulation (Murdoch et al., 1986; Murdoch and Farris 1988). This relationship between progesterone/progesterone receptor and prostaglandins was also demonstrated in cattle recently by Bridges et al. (2006) who performed a series of experiments in vivo and in vitro to test the possible regulation of prostaglandin production by progesterone. Interestingly, they provided evidence that the rise in intrafollicular prostaglandins (PGE$_2$ and PGF$_{2\alpha}$), characteristic of the periovulatory period, may be mediated by progesterone via upregulation of COX-2 (rate limiting enzyme for PG’s biosynthesis) in granulosa cells and not by activation of PGE$_2$-9-ketoreductase as shown in sheep.

Conversely, data from a recent experiment using dairy cows suggested that progesterone may not be required for the occurrence of ovulation (Li et al., 2007). In this study, intrafollicular injection of a 3β-hydroxy steroid dehydrogenase (3β-HSD; enzyme required for progesterone synthesis) inhibitor suppressed the progesterone rise as well as PG’s production in the ovulatory
follicle; whereas, it did not block ovulation in any of the treated animals (Li et al., 2007). These results support the speculation that PG’s production during the preovulatory period is linked to that of progesterone; however, the results also suggest that neither progesterone nor PG’s may have a role in ovulation. Another finding of this study was that the proteolytic activity necessary for the completion of ovulation was not affected in cows with low intrafollicular concentrations of progesterone and PG’s (Li et al., 2007).

The role of PGE$_2$ and PGF$_{2\alpha}$ as necessary mediators of the ovulatory cascade has been recognized for more than 30 years (reviewed by Sirois et al. 2004). As of today, different approaches have been utilized to elucidate function of PG’s in ovulation. Early evidence on this matter emerged from experiments using specific inhibitors of PG’s synthesis to inhibit ovulation in rabbits (Armstrong and Grinwich 1972). Subsequently, the possible implication of prostaglandins on ovulation was studied and confirmed in numerous species including bovine (De Silva and Reeves 1985; Sirois 1994; Filion et al., 2001; Bridges et al., 2006). Later research using gonadotropins to induce ovulation demonstrated the presence of a gonadotropin dependent rise in follicular prostaglandins in different species (rat, Wong and Richards 1992; bovine, Sirois 1994). Across species studied so far, the time elapsed from the gonadotropin administration to induction of prostaglandin synthesis is quite variable; however, the time course from induction of prostaglandins synthesis to ovulation is very conserved (~10 hours in the rat, cow and mare; rat, Wong and Richards 1992; bovine, Sirois 1994; mare, Sirois and Doré 1997). This similarity in the time
elapsed from the prostaglandin rise to ovulation suggest that these hormones may be the molecular component setting the time of ovulation.

It has been clearly established that the key regulatory step in the PG’s biosynthetic pathway is the selective induction of the rate-limiting enzyme prostaglandin endoperoxide synthase-2 (PGHS-2), also known as cyclooxygenase-2 (COX-2), responsible for conversion of the PG’s precursor arachidonic acid (present in the plasma membrane of almost all cells) into the substrate PGH$_2$, necessary for both PGF$_{2\alpha}$ and PGE$_2$ production (DeWitt et al., 1993). As previously discussed, Bridges et al. (2006) presented supportive evidence that progesterone acts through its intracellular receptor to regulate COX-2 mRNA expression and hence protein synthesis in bovine preovulatory follicles. In addition, progesterone may also increase PG’s production by an indirect mechanism consisting in the activation of the progesterone receptor to increase oxytocin (OT) production, which also has the capacity to induce PG’s synthesis in preovulatory follicles (Bridges and Fortune 2007; Figure 1). This is in contrast to what has been shown in rat granulosa cells cultured in vitro, where LH directly induces COX-2 mRNA and protein production by modulation of cAMP dependent kinases such as PKA, and is clearly supported by PKC and tyrosine kinase(s) (Morris and Richards 1993). Consistent with results obtained in bovine by Bridges et al. (2006), other researchers have shown a drastic depletion in follicular fluid PG’s concentration in bovine preovulatory follicles treated with an inhibitor of progesterone synthesis (Li et al., 2007). Interestingly, results of these two studies disagree in the proposed mechanism behind the low PG’s
concentration. While Bridges et al. (2006) theorizes that the lack of progesterone results in no COX-2 mRNA expression, Li et al. (2007) suggests that increased prostaglandin dehydrogenase (PGDH) expression in progesterone-depleted follicles degrades PGE$_2$ and PGF$_{2\alpha}$ faster than expected in follicular fluid, as this enzyme is responsible for PG’s metabolization (Tai et al., 2002). In regard to timing of COX-2 expression, bovine granulosa cells first express mRNA by 18 h after an ovulatory dose of hCG and reach maximum levels by 24 h (Sirois 1994). Accompanying this timely regulated expression of COX-2 in granulosa cells of preovulatory follicles, the gonadotropin surge induces a parallel expression of PGE synthase (PGES) necessary for the final conversion of PGH$_2$ into PGE$_2$ (Filion et al., 2001). Accordingly, concentrations of PGE$_2$ in follicular fluid increase dramatically by 24 to 26 h after an endogenous (Acosta et al., 2000) or exogenously induced gonadotropin surge (Sirois 1994; Bridges et al., 2006). On the other hand, the pattern of PGF$_{2\alpha}$ production during the preovulatory period resembles that of PGE$_2$ (Sirois 1994; Acosta et al., 2000; Bridges et al., 2006); however, whether or not PGF synthase (similar function as PGES) is activated and responsible for the rise in PGF$_{2\alpha}$ has not been investigated in bovine. On this regard, Murdoch and Farris (1988) suggested that in sheep, PGF$_{2\alpha}$ production in the ovulatory follicle is at the expense of PGE$_2$ by a progesterone-mediated mechanism consisting in activation of the PGE$_2$-9-ketoreductase in granulosa cells. This enzyme catalyzes the conversion of PGE$_2$ into PGF$_{2\alpha}$, even favoring a greater accumulation of PGF$_{2\alpha}$ by the end the preovulatory period (Murdoch and Farris 1988).
Although regulation of PG’s secretion in follicular fluid has been extensively studied and clarified, the specific role of these hormones in the ovulatory cascade remains obscure. The fact that PGE\textsubscript{2} and PGF\textsubscript{2\alpha} exert opposing physiological effects in vivo makes it complicated to determine their function in ovulation (Murdoch et al., 1986). Prostaglandins have been implicated in induction of collagenolytic activity necessary for follicular rupture in sheep (Murdoch et al., 1986) and rat (Reich et al., 1991). In contrast, it seems unlikely that PG’s exert the same function in bovine, since preovulatory follicles depleted of PG’s ovulated normally (Li et al., 2007). Furthermore, expression of mRNA and proteins related to degradation of the follicle wall was not affected (Li et al., 2007). Despite the uncertainty of the function of PG’s in ovulation, the pattern of expression and multiple sites where different PG’s receptors are found in preovulatory follicles suggests involvement of PGE\textsubscript{2} and PGF\textsubscript{2\alpha} in multiple mechanisms of the ovulatory cascade in bovine (Bridges and Fortune 2007).

Mechanical rupture of the follicular wall and surrounding ovarian tissues is a critical step towards completion of ovulation. Degradation of the collagenous layers of theca externa, tunica albuginea and surface epithelium at the apex of the ovulatory follicle is required for follicular rupture (Bakke et al., 2002). In addition, localized degradation and remodeling of the structural components forming the basement membrane (collagen type IV, laminin, fibronectin, and proteoglycans), separating granulosa from theca cells, and disruption of cell layers forming the follicle wall require activation of multiple mechanisms, that are
responsible for downstream induction of specific enzyme systems as depicted in Figure 1.

The plasminogen activator (PA) system and matrix metalloproteinase (MMP’s) system, as well as their specific inhibitors, are two major families of proteolytic enzymes implicated in the rupture of the ovulatory follicle (Smith et al., 2002; Murdoch and Gottsch 2003). The PA system consists of multiple elements including the zymogen plasminogen, which can be catalytically converted to plasmin by either the urokinase (uPA) or tissue plasminogen activators (tPA; Tsafriri and Reich 1999; Murdoch and Gottsch 2003). Plasmin is a proteolytic enzyme capable of degrading type IV collagen, fibronectin, laminin, elastin, and proteoglycans forming the follicular extracellular matrix (ECM) by hydrolysis of target peptide bonds (reviewed by Liu 1999). Nevertheless, like with any other enzyme system, generation of plasmin by PA is rigorously regulated at several levels from gene transcription to inhibition of enzymatic activity (Collen 1999). In the ECM environment, activity of the PA system is limited by the action of specific inhibitors (PA inhibitor type-1, (PAI-1) and the PA inhibitor type-2 (PAI-2)) produced in tissues to help control plasmin degradation (Saksela and Rifkin 1988; Ny et al., 1993; Dow et al., 2002a). Another mechanism suggested to regulate the PA system is the presence of specific surface receptors for uPA in some cells. These receptors, also known as uPAR, bind uPA to the plasma membrane favoring catalytic activation of cell bound plasminogen. In this manner proteolytic activity can be localized to the cell surface and immediate vicinity (Blasi et al., 1994; Ny et al., 2002). In cattle, there
is compelling evidence that the gonadotropin surge induces expression and activity of both the PA and MMP’s systems. Dow et al. (2002b) reported an increase in expression of mRNA for tPA, uPA and uPAR along with an increased plasmin activity in preovulatory follicles after the gonadotropin surge. The pattern of mRNA expression and activity of respective proteins (tPA, uPA, uPAR and plasmin) in the different regions (base vs. apex) of the follicle as well as in the follicular fluid suggest that the PA system mediates rupture of the follicle wall. In addition, a relevant finding of the series of experiments performed by Dow et al. (2002b) is that induction of tPA, uPA and uPAR mRNA expression by the gonadotropin surge increases with time elapsed from the surge, with the most dramatic increase in the relative amount of uPA and uPAR towards the end of the ovulatory process. Interestingly, Dow and coworkers (2002b) also reported an increased enzymatic activity of tPA and uPA in the follicle wall and a steady increase in plasmin activity in follicular fluid from 0 h to 24 h following the LH surge.

On the other hand the MMP’s system is another proposed mediator of the ovulatory process. MMP’s are a large protein superfamily of more than 26 metal-dependent enzymes capable of degrading and remodeling components of the ECM similar to the PA system. All members of the MMP family contain zinc binding sites, are synthesized as latent proenzymes, and can be inhibited by agents known as tissue-inhibitors of metalloproteinase (TIMPs).

Even when the presence of members of the MMP’s family and their inhibitors has been demonstrated in cattle preovulatory follicles (Bakke et al.,
2002; Bakke et al., 2004; Li et al., 2004), the dynamics of their expression and function during ovulation are far from being completely understood. MMP-14, also known as membrane type 1 MMP, has been implicated in activation of other MMP’s (MMP-2 and MMP-13) as well as in focalizing their activities on the cell surface through formation of a complex with TIMP-2 (tissue metalloproteinase inhibitor-2; Strongin et al., 1995). MMP-14 is also known for its self capacity to degrade components of the ECM (Knauper et al., 1996; Ohuchi et al., 1997). In an attempt to clarify the role of MMP-14 in ovulation in bovine, Bakke et al. (2002) evaluated MMP-14, MMP-2 and TIMP-2 mRNA expression and activity in response to the LH surge. They observed a significant increase in MMP-14 and TIMP-2 mRNA’s abundance in preovulatory follicles following the LH surge. Likewise, MMP-2 mRNA abundance was unchanged suggesting that its expression is independent of MMP-14 in contrast to previous speculations. Based on the results of this study, it seems more likely that MMP-14, by a mechanism other than MMP-2 activation, and TIMP-2 may have a role in the regulation of the ovulatory follicle rupture (Bakke et al., 2002). A similar experiment performed recently by the same lab produced comparable results in regard to the abundance of mRNA for MMP-14 and TIMP-2 in preovulatory follicles (Li et al., 2006). In addition, a novel finding of this study was that MMP-13 mRNA abundance increased in a similar fashion to that of MMP-14 suggesting a likely regulation of MMP-13 by MMP-14 as previously proposed (Figure 1).

As the factors required for PA and MMP’s systems activation located downstream gonadotropin receptors are still unknown, researchers attempted to
relate the prostanoids rise in follicular fluid of preovulatory follicles to expression of PA and MMP’s systems (Li et al., 2006). Their approach consisted in assessing the possible involvement of prostaglandins in the activation of the PA and MMP systems by measuring PA and MMP’s mRNA abundance after inhibiting prostaglandin production (Li et al., 2006). Interestingly, results of their study lead to the suggestion of a potential role of prostaglandins in the maintenance of tPA mRNA expression and plasmin activity in response to the LH surge. Conversely, the expression of most MMP’s measured was not affected when PG’s production was inhibited suggesting that the modulation of MMP’s system is independent from PG’s.

In sheep, a different pathway involving direct action of LH on the surface ovarian epithelial cells has been proposed to activate PA and MMP’s systems (Murdoch and Gottsch 2003). According to this model, activation of LH receptors induces expression of uPA which later converts plasminogen to plasmin. In response to plasmin activation, collagenase becomes functional beginning with degradation of the ECM; whereas, in the vascular endothelium, plasmin stimulates release of tumor necrosis factor-α (TNF-α), which is able to activate the MMP’s system stimulating further proteolysis of the ECM (Murdoch and Gottsch 2003).

In conclusion, results of several years of intensive research in an effort to decipher the molecular mechanisms of ovulation have lead to a better, yet incomplete understanding of the factors that are up and down regulated by the follicular cells in response to the LH surge. How these factors interconnect and their very specific function in the ovulatory process remain unclear.
2.7.4. Acquisition of Ovulatory Capacity in Bovine Follicles

As follicles grow in size, cells of the granulosa and theca layers undergo changes reflecting the state of follicle maturation which ultimately has an effect on the potential response of the follicle to hormonal stimulation. Theca cells first express LHR shortly after formation of the follicular antrum (~ 3 to 4 mm); whereas, granulosa cells begin expressing LHR when the follicle is ~ 9 mm in diameter (Xu et al., 1995). Accordingly, in dairy cows it has been shown that follicles are capable of ovulating when stimulated by LH approximately 1 day after diameter deviation when follicles are ~ 10 mm (Sartori et al., 2001). Following acquisition of ovulatory capacity, there is a clear relationship between size of the follicle and response to LH since the dose necessary to induce ovulation of the majority of 10 mm follicles was 6 times higher than the necessary dose to ovulate 12 mm follicles (Sartori et al., 2001). Evidence from binding of I\(^{125}\)-hCG to LH/hCG receptors in bovine dominant follicles suggests, that as the DF increases in size from early to late phase of the first follicular wave, it acquires a greater number of receptors for LH, primarily in granulosa cells (Stewart et al., 1996). In addition, potentially ovulatory follicles collected after luteolysis had greater I\(^{125}\)-hCG binding capacity (indicator of LH receptors number) as size increased (Ireland and Roche 1982; Ireland and Roche 1983). In growing follicles, FSH and estradiol-17\(\beta\) synergize to induce the synthesis of LHR in granulosa cells until later stages of follicular development which may explain the larger number of LHR in larger follicles as they produce greater
amounts of estradiol-17β (reviewed by Richards 1980; Kessel et al., 1985). Therefore, the number of LHR’s on the surface of granulosa cells appears to increase as follicle size increases (Kawate et al., 1989; Stewart et al., 1996; Nogueira et al., 2007) in preparation to respond to the preovulatory LH surge.

On the other hand, FSH receptors are present in follicles of a wider range of sizes (Kawate et al., 1990; Xu et al., 1995). For instance, Xu et al. (1995) documented the presence of FSH receptors in bovine follicles of the first follicular wave from 0.5 to 14 mm with significant differences in number of receptors among follicles of different sizes. As with LH, the number of FSH receptors also increases with size of the follicle during the early phases of follicle growth (Kawate et al., 1990; Bao and Garverick 1998; Garverick et al., 2002). However, the number of FSH receptors decreases after luteolysis unlike number of LHR’s which continue increasing during this phase (Ireland and Roche 1983). This difference in receptor dynamics may reflect the different hormonal requirements of the follicle to continue growing until preovulatory size, since final growth of the DF depends more in LH than in FSH (reviewed by Crowe 1999).

2.8. Summary

The literature reviewed indicates that in spite of several years of continuing efforts to decipher the causes of heat stress-derived infertility in lactating dairy cows, there is not one obvious and single mechanism responsible for this phenomenon. However, a growing body of evidence from in vitro studies
suggests that a major effect of HS is to hasten the process of oocyte maturation (Edwards et al., 2005; Schrock et al., 2007), and a possible strategy to circumvent its negative effects is to fertilize oocytes earlier to avoid the fertilization of an otherwise “aged” oocyte. As a matter of fact, performing IVF of oocytes matured at physiologically relevant elevated temperatures between 4 to 8 h earlier than the usual 24 h post maturation have proven beneficial (Edwards et al., 2005; Schrock et al., 2007). Proportion of heat stressed oocytes developing to the blastocyst stage was similar than for non heat stressed controls. Thus, if effects of HS in vivo parallel those observed in vitro, earlier release of the oocyte from the ovulatory follicle may assure the fertilization of a “non-aged” fertile oocyte. As a result, probability of conception and normal embryonic development may be increased. As of today, there are no available therapeutic strategies to induce earlier ovulation in lactating dairy cows. Therefore, we hypothesize that through the administration of Folltropin-V (FSH and LH) at the time of induced luteolysis; additional growth of the preovulatory follicle may be induced resulting in a larger follicle at the time ovulatory treatment with hCG or GnRH (induces a LH surge). Larger follicles may then be more sensitive to the ovulatory treatment and respond with early ovulation. In addition we also tested if administration of hCG would have an impact in the timing of ovulation. If hCG proves to be beneficial in inducing earlier ovulation in this study it may also have other possible advantages in heat stressed cows. Unlike GnRH which depends on pituitary LH release to induce ovulation, mechanism that may be impaired in cows undergoing heat stress (Gilad et al., 1993), hCG triggers ovulation by a direct action on the ovarian cells.
Furthermore, most research suggest that hCG presents a higher affinity for LH receptors, binds LH receptors for longer periods of time than LH, and has a prolonged plasma half-life when compared to LH.

The study described herein was performed under non-heat stress conditions because it was only intended to test the feasibility of inducing earlier ovulation in lactating dairy cows.
CHAPTER 3
EXPERIMENTAL PROCEDURES

3.1. Animals

Lactating primiparous (n = 17) and multiparous (n = 18; range 1 to 6 lactations) Holstein cows were used for conducting this experiment performed in six different replicates from October 2006 to May 2007. Cows were between two to seven years of age, weighed 485 to 870 kg (x = 642.5 kg), and ranged from 65 to 115 days in milk (DIM; x = 90 days). Average daily milk production calculated as milk mature equivalent (M. E.) was 43.9 kg (range 29.9 to 59.0 kg). All cows were housed in free stalls at the dairy unit of the East Tennessee Research and Education Center in Knoxville, Tennessee. During the course of the experiment, cows were milked twice daily at 0730 am and 0730 pm, fed ad-libitum a total mixed ration (TMR) and had open access to fresh water. The diet supplied to the animals was formulated to meet or exceed nutritional requirements of National Research Council (NRC 2001). Components of the diet were as follow: ~ 57% corn silage, ~ 34.75% dairy meal feed (U.T. Knox #0204 Dairy Meal Feed, Tennessee Farmers Cooperative, Lavergne, TN, USA; Table 1) and 8.25% alfalfa hay. In this herd, cows and replacement heifers calve year round with the majority calving from August to January. All procedures applied to the animals were approved by the Institutional Animal Care and Use Committee (IACUC).
Table 1. Description of components of dairy meal feed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protein</td>
<td>19.50 %</td>
</tr>
<tr>
<td>Crude fat</td>
<td>7.50 %</td>
</tr>
<tr>
<td>Crude fiber</td>
<td>13.00 %</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>17.50 %</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.70 %</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.50 %</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.30 ppm</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>5,000 IU/lb</td>
</tr>
</tbody>
</table>
3.2. Pre-Synchronization

The estrous cycle of 67 cows was pre-synchronized with two injections of 35 mg PGF$_{2\alpha}$ i.m. (Dinoprost tromethamine; Lutalyse, Pfizer Animal Health, Kalamazoo, MI, USA) given 14 days apart (Figure 2). Following the second injection of PGF$_{2\alpha}$, a patch containing a pressure sensing system for the detection of estrus (Heat-Watch®, CowChips LLC, Denver, CO, USA) was placed on the tailhead of each cow. When an animal was mounted, this device responded to the pressure by sending a signal to a computer system which recorded cow number, date, time and duration of the mount. A cow was considered to be in heat when 3 mounts of at least 2-second duration during a 4 h period were recorded. In addition to the Heat-Watch system, estrous activity was detected by visual observation by dairy unit personnel during 30 minutes twice a day. For replicate #5 and 6, estrus was only visually detected with the aid of Estrus Alert patches (Western Point, Inc. Merrifield, MN, USA) because unavailability of the Heat Watch system. Of the total number of animals included in the pre-synchronization, the estrus response was 52.2% (35/67).

3.3. Ovulation Protocol

Cows detected in estrus within 72 h ($n = 35$) of the second PGF$_{2\alpha}$ were utilized for the experiment (Figure 2). Eight to nine days after detection of estrus
Figure 2. Schematic representation of timeline for hormone administration during pre-synchronization and synchronization of ovulation. Estrous cycle of cows was presynchronized with two injections of PGF$_{2\alpha}$ given 14 days apart (Day -25 or -26 and -11 or -12) and estrus activity monitored with the Heat-Watch system for 3 days after second PGF$_{2\alpha}$. Eight to nine days after completion of estrus detection period, cows observed in estrus received a CIDR device and GnRH followed by PGF$_{2\alpha}$ seven days later. At the time of PGF$_{2\alpha}$ administration CIDR’s were removed and the cows randomly allocated to either receive Folltropin-V (FSH/LH) or Saline (SAL) treatment. Two days later cows within Folltropin-V and Saline groups were randomly allocated to either receive Cystorelyn (GnRH) or Chorulon (hCG) treatment to induce ovulation.
(within 72 h after second PGF$_{2\alpha}$ dose), all cows ($n = 35$) received an intravaginal device containing 1.38 g of progesterone (Day 0; EAZI-BREED CIDR; Pfizer Animal Health, New York, NY, USA) plus a 100 $\mu$g dose of a GnRH analogue i.m. (Gonadorelin; Cystorelin, Merial Limited, Inselin, NJ, USA). On Day 7, CIDR devices were removed (CIDR-r) and cows were administered a 500 $\mu$g luteolytic dose of PGF$_{2\alpha}$ i.m. (Cloprostenol; Estrumate, Schering-Plough Animal Health, Union, NJ, USA).

### 3.4. Application of Treatments

Cows were randomly allocated into two treatment groups to receive either 80 mg of Folltropin-V (FSH group; $n = 19$; Folltropin-V, Bioniche Animal Health, Belleville, Ontario, Canada) or 4 mL of sterile saline solution (SAL group; $n = 16$; Physiological Saline Solution, Butler Animal Health Supply, Dublin, OH, USA). Forty-nine hours thereafter (Day 9), cows within the Folltropin-V and Saline groups which had not ovulated were randomly subdivided to receive either a 100 $\mu$g dose of GnRH i.m (GnRH group; Cystorelin) or 3000 IU of human Chorionic Gonadotropin i.m. (hCG group; Chorulon, Intervet, Millsboro, DE, USA) generating 4 treatment combinations (FSH/GnRH, $n = 6$; FSH/hCG, $n = 8$; SAL/GnRH, $n = 8$; and SAL/hCG, $n = 8$; Figure 2). A subgroup of cows ($n = 5$) presented ovulation prior to the administration of GnRH/hCG on Day 9 and were considered a different group denominated FSH-EARLY. These animals were not included in the statistical analysis after CIDR removal.
3.5. Assessment of Ovarian Activity

Transrectal ultrasonography was carried out with a real-time B-mode ultrasound scanner equipped with a 7.5-MHz transrectal probe (Aloka SSD-500V; Aloka Company Ltd, Wallingford, CT, USA). Still images of ovarian structures present at the time of ultrasound examination were obtained and measured with ultrasound device internal calipers. Following measurement, images were individually recorded on floppy discs using an external MaviCap digital still image capture adaptor (Sony® Incorporated, model MVL-FDR1, Tokyo, Japan) for subsequent evaluation and record keeping. To minimize duration of examinations, the screen of the ultrasound was split in half in order to allow the technician to identify other structures present on the ovary, while previously identified structures were measured.

In each scan, the location and size of the F1 (largest follicle present on both ovaries) and F2 (second largest follicle on both ovaries) follicles were determined by ovarian mapping and by measuring the largest cross sectional diameter. Location and size (height and width) of the corpus luteum (CL), and any cavity present, were measured and recorded. Some animals presented “cyst like” structures on their ovaries, characterized by having fluid filled cavities larger than 25 mm and with walls of variable thickness. These structures did not show a growing pattern characteristic of healthy follicles and did not respond with either ovulation or luteinization to any of the hormonal treatments given during the synchronization of the ovulation protocol. However, in most of the animals
presenting these structures, neither ovulation nor formation of a CL from healthy follicles was impaired.

Ovarian ultrasonography was performed at the time of the first and second injections of PGF$_{2\alpha}$ during the pre-synchronization of the estrous cycle to confirm cyclicity by the presence of a CL (Figure 3). Other structures present on the ovaries such as follicles and cysts were recorded at this time. To evaluate the initiation of a new follicular wave after beginning of synchronization of the ovulation protocol, the presence of the F1 was determined on Day 0 by transrectal ultrasonography and then again on Day 2 (48 h later) to confirm ovulation or luteinization. Growth of the F1 and F2 of the new follicular wave was monitored by transrectal ultrasonography performed daily from Day 2 to Day 6, every 12 h on Day 7, and every 6 h on Day 8 and part of Day 9. Final growth of the ovulatory follicle, ovulatory size, and ovulation time was confirmed by scanning of the ovaries every 4 h beginning at the time of ovulation treatment (Day 9; GnRH/hCG administration). Scans were performed until ovulation occurred or 48 h after the cows received the ovulation treatment, whichever occurred first (Figure 3).

Ovulation was defined as disappearance of the dominant follicle at the time of an ultrasound examination compared with the previous ultrasound examination (Roelofs et al., 2004). Time of ovulation was calculated as the midpoint between the last time the ovulatory follicle was observed on the ovary and the first time it was not present. Seven to eight days after ovulation was
Figure 3. Ovarian ultrasonography (A) and blood sampling (B) timeline during the experimental protocol. Ovarian ultrasonography (US) was performed at the time of PGF$_2\alpha$ injections of presynchronization to confirm cyclicity. On Day 0 of the synchronization of ovulation protocol, US was performed to confirm the presence of a large dominant follicle (F1) and then every 24 h from Day 2 to 6 to confirm either ovulation or luteinization of the F1 and growth of the new follicular wave. On Day 7, US was performed every 12 h and then every 6 h on Day 8. Finally, US was performed every 4 h until ovulation was confirmed or 48 h following GnRH/hCG treatment whichever occurred first. Blood samples were taken by tail venipuncture on Day 0, then every 24 h from Day 2 to 6, and every 12 h on Day 7 and 8. Beginning on Day 9 at 6am, blood samples were obtained 1 h until Day 10 at 4pm. Jugular catheters placed the previous days were utilized to obtain blood samples during the high frequency period. US = ultrasonography, BS = blood sample.
observed, ovarian ultrasonography was carried out in all cows to confirm ovulation and formation of CL on the respective ovary where the ovulatory follicle was previously identified. In those animals where ovulation was not observed within the 48 h of ovulation treatment, ultrasonography was performed to evaluate status of the ovary.

3.6. **Blood Sample Collection**

Blood samples were obtained for subsequent quantification of progesterone, estradiol-17β, LH, FSH and hCG concentrations by radioimmunoassay (RIA). On Day 0, and then from Day 2 to Day 6 of the ovulation protocol, blood samples (~ 9 mL) were collected daily by tail venipuncture of the coccygeal veins using Monovette® tubes (Sarstedt, Inc. Newton, NC, USA) coinciding with the time of ultrasonography. On Day 7 and 8, samples were drawn every 12 h (Figure 3). Beginning on Day 9 at 0600 am, samples were obtained every 1 h until Day 10 at 0400 pm using Monovette® tubes attached to indwelling jugular catheters as described by Hockett *et al.* (2000). After collection, samples were immediately stored on ice for at least 30 min and then transported to the laboratory, set to room temperature and centrifuged at 2500 rpm at 4°C for 25 min. After centrifugation, plasma was harvested in 5 mL glass tubes and stored at -20°C until assayed.

Placement of jugular catheters was performed in the morning and afternoon of Day 8. The right jugular area of each cow was prepared by clipping
and subsequent disinfection with Clorexhexidine solution (Nolvosan 2%, Fort Dodge Animal Health, Overland Park, KS, USA). A metallic needle (12 gauge x 2.75 inches) was placed in the jugular vein to allow subsequent introduction of approximately 20 cm of a 12 gauge Microtub plastic catheter (ID 0.040 inch X OD 0.070 inch; Saint-Gobain Performance Plastic Inc, Akron, OH, USA). The remaining catheter (~ 60 cm) was directed towards the top of the neck in order to place a syringe adapter in the tip of the catheter between the shoulders of the cow for easy access during blood collection. After placement in the jugular vein, catheters were secured by placing stitches on the skin at different levels of the neck region. To further secure placement of the catheter line, Elastikon® wrap (Johnson & Johnson Consumer Products Company, Skillman, NJ, USA) was placed around the neck and covered with Vet wrap (3M Animal Care Products, Saint Paul, MN, USA). Heparin solution (50 IU/mL; Heparin sodium injection- USP, Baxter Health Care Corporation, Deerfield, IL, USA) was utilized to fill the catheter line to prevent blood from clotting between consecutive blood samplings. During the high frequency collection period (every 1h), samples were obtained using jugular catheters. The procedure consisted of removal of heparin solution contained in the catheter line along with 3 to 4 ml of blood using a 12 cc syringe to ensure subsequent drawing of sample blood. Thereafter, a Monovette® tube was connected to the catheter line and a blood sample (~ 7 to 9 mL) was collected followed by filling the catheter with 2 to 3 mL of heparin solution (50 IU/mL). Once obtained, blood samples were handled as previously described.
3.7. Hormone Assays

A commercial solid-phase radioimmunoassay kit (Coat-A-Count®, Siemens Medical Solutions Diagnostic, Los Angeles, CA, USA) was used to determine plasma concentrations of progesterone as described by Seals et al. (1998). Samples analyzed for progesterone were collected at each ultrasonography session beginning at time of CIDR insertion (Day 0 of experimental protocol) until 72 h post CIDR removal (Day 10). Progesterone was measured in one assay with a sensitivity of 0.02 ng/mL and with an intraassay coefficient of variation (CV) of 3.78%.

Concentrations of estradiol-17β in blood plasma were determined by RIA as described in Moura and Erickson (1997) utilizing antibody for estradiol-17β provided by Manson (Lilly Research Laboratories, Indianapolis, IN, USA). Samples analyzed for estradiol-17β were collected at each ultrasonography session. For this assay, estradiol-17β was extracted from plasma with ethyl acetate (Honeywell, Burdick & Jackson, Muskegon, MI, USA) and then evaluated by RIA. The sensitivity of the assay was 0.15 pg/mL and intra and interassay CV was 14% and 8%, respectively.

Concentrations of FSH were measured in a single RIA as previously described by Moura and Erickson (1997). Concentrations of FSH were measured in all samples collected from two days prior to CIDR removal (Day 5) to 48 h thereafter (Day 9). Bovine FSH-β antibodies and standards hormone preparations of bFSH were provided by the National Hormone and Peptide Program (Harbor-
UCLA Medical Center, Torrance, CA, USA). The assay sensitivity was 0.05 ng/mL and the intraassay CV for the single assay performed was 5.1%.

Concentrations of LH in plasma were determined by RIA following the procedures described by Moura and Erickson (1997). Bovine LH standards and anti bovine LH serum were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA). Samples utilized for LH determination were obtained from 48 h prior to CIDR removal (Day 5) to the end of the sampling period (Day 10) resulting in a total of 39 samples drawn during a 136-h period. The sensitivity of the assay was 0.03 ng/mL. The intraassay CV for this assay was 6.25% whereas the interassay CV was 1.13%.

An estimation of plasma concentrations of hCG was performed by using an immunoradiometric assay kit (IRMA; Coat-A-Count®, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Since the kit is intended for determination of hCG in human blood, the assay was validated in our laboratory. Linearity of the kit was determined by estimating concentrations of hCG in serial dilutions of bovine plasma samples. Spiking and recovery studies showed that the observed concentration was on average 94% of the expected value. Data from the manufacturer indicate that the antibody used in the assay kit is highly specific for hCG, with low crossreactivity to other glycoprotein hormones. In our assay, there were no indications of crossreactivity between the hCG antibody and bovine FSH and LH. Concentrations of hCG were estimated in every other sample taken starting 2 h prior to GnRH/hCG injection (0700 am of Day 9) until the end of the sampling period (0400 pm on Day 10). A total of 17 samples per cow were
analyzed during a 33 h period. The sensitivity of the single assay performed was 5 mIU/mL and the intraassay CV was 4.75%.

### 3.8. Statistical Analyses

Experimental data collected throughout the study were divided into three different analyses for evaluation of treatment differences. Four cows were not included in the analysis since they failed to ovulate during the 48 h period following GnRH/hCG administration. Two cows did not present ovulation and initiation of a new follicular wave, and one cow exhibited estrus 96 h after administration of PGF$_{2\alpha}$. The last cow presented two dominant follicles and failed to ovulate.

#### 3.8.1. Analysis of Animal and Production Traits

Age, parity, body weight (BW), body condition score (BCS), days in milk (DIM), and milk yield were analyzed as a randomized block design, blocking on replicate, using Proc Mixed of SAS 9.1 (2003). Differences between levels of first (FSH vs. SAL) and second treatment factors (GnRH vs. hCG) as well as their interaction (FSH-GnRH, FSH-hCG, SAL-GnRH, and SAL-hCG) were analyzed with a factorial model that included first treatment factor (FSH vs. SAL), second treatment factor (GnRH vs. hCG) and their interaction (combinations) as fixed effects. Replicates were included as random effects.
Data are presented as least squares means (LSM) ± standard error of the mean (±SEM). The Tukey-Kramer test was used to identify treatment differences among least squares means.

3.8.2. Analysis of Hormone Concentrations

Data of hormone concentrations across the experimental period were analyzed as a randomized block design and repeated measures, blocking on replicate, using Proc Mixed of SAS 9.1 (2003). For analysis of hormone concentrations the models utilized varied with the time of variable (hormone concentration) measurement. When the variable of interest was measured prior to administration of GnRH/hCG (Day 9), only analysis of differences between levels of first treatment factor were performed (FSH vs. SAL). When the variable of interest was measured after administration of GnRH/hCG (Day 9) only, a factorial model including main effects of first and second treatment factors, as well as first and second treatment factors interaction (combinations) was utilized for the analysis.

Analysis of differences in hormone concentrations for first treatment factor (FSH vs. SAL) for those cases in which the variable was measured prior to the administration of GnRH/hCG included the effects of treatment as fixed effects. Time of blood sampling was used as a repeated measures factor with an autoregressive covariance structure. Replicate and cow (replicate x treatment) were included as random effects.
Differences between levels of first (FSH vs. SAL) and second treatment factors (GnRH vs. hCG) as well as their interaction (combinations) for those cases in which the variable was measured following GnRH/hCG administration, were analyzed with a factorial model that included first treatment factor (FSH vs. SAL), second treatment factor (GnRH vs. hCG) and their interaction as fixed effects. Time of blood sampling was used as a repeated measures factor with an autoregressive covariance structure. Replicate and cow (replicate x first treatment factor x second treatment factor) were included as random effects. Parity, body weight (BW), body condition score (BCS), days in milk (DIM), and milk yield were included as covariates. A backwards selection procedure was used to eliminate covariates not meeting the significance value ($P < 0.05$) required to stay in the model.

Concentrations of progesterone were analyzed for the whole sampling period (Day 0 to 9) as well as from time of CIDR insertion (Day 0) to CIDR removal (Day 7). The slope of progesterone concentration decrease was calculated by multisource regression using Proc Mixed of SAS 9.1 (2003). The model for estimation of progesterone concentration decrease included linear and quadratic effects of time and first treatment factor (FSH vs. SAL). Contrasts were used to assess differences among levels of treatments.

Estradiol-17β concentrations were analyzed in two different periods including: from the approximate time of new follicular wave initiation (Day 2) to CIDR removal (Day 7), and from CIDR removal (Day 7) to GnRH/hCG administration (Day 9). The slope of estradiol-17β decrease was calculated by
multisource regression using Proc Mixed of SAS 9.1 (2003). The model for estimation of estradiol-17\(\beta\) concentration decrease included linear and quadratic effects of time, first treatment factor (FSH vs. SAL), second treatment factor (GnRH vs. HCG), and their interaction (combinations). Contrasts were used to assess differences among levels of treatments main effects and their interaction.

Concentrations of FSH were analyzed from 2 days prior to CIDR removal and Folltropin-V administration (Day 5) to 48 h thereafter (Day 9). Concentrations of LH were analyzed in two different periods: from 2 days prior to CIDR removal (Day 5) and Folltropin-V administration (Day 7) to GnRH/hCG administration (Day 9), and from 3 h prior to GnRH/hCG administration to 31 h thereafter (Day 9). Data of hCG concentrations were analyzed from 2 h prior to GnRH/hCG administration (Day 9) to 30 h thereafter (Day 10).

Maximum plasma concentrations and time to the maximum concentration of estradiol-17\(\beta\) after CIDR removal (Day 7) were analyzed by a model similar to that utilized for analysis of hormone concentrations across the experimental period except that time was removed as a repeated measures factor. A similar analysis was performed for LH and hCG except that maximum concentration and time to the maximum concentration were measured in relation to CIDR removal and/or GnRH/hCG administration.

Data are presented as least squares means (LSM) ± standard error of the mean (±SEM). The Tukey-Kramer test was used to identify treatment differences among least squares means.
3.8.3. Follicle Development and Ovulation

Data on follicle size, growth, and timing of ovulation were arranged in a randomized block design, blocking on replicate, using the Proc Mixed of SAS 9.1 (2003). For this analysis the models utilized varied with the time of variable (follicle size, growth, and timing of ovulation) measurement. When the variable of interest was measured prior to application of GnRH/hCG (Day 9), only the analysis of differences between first treatment factor levels was performed (FSH vs. SAL). Conversely, when the variable of interest was measured following administration of GnRH/hCG (Day 9), only the factorial model including main effects of first and second treatment factors, as well as first and second treatment factors interaction (combinations) were utilized for the analysis.

Analyses for investigating differences in follicle size and follicle growth for first treatment factor (FSH vs. SAL) were performed with a model including effects of treatment as fixed effects and replicates as random effects. Differences in follicle size, growth, and timing of ovulation for first (FSH vs. SAL) and second (GnRH vs. hCG) treatment factors, as well as their interaction (combinations) were analyzed with a factorial model that included first treatment factor (FSH vs. SAL), second treatment factor (GnRH vs. hCG) and their interaction (combinations) in the fixed effects. Replicates were included as random effects. Parity, body weight (BW), body condition score (BCS), days in milk (DIM), and milk yield were included as covariates. A backwards selection
procedure was used to eliminate covariates not meeting the significance value ($P < 0.05$) required to stay in the model.

Finally a multiple linear regression model using Proc Reg of SAS 9.1 (2003) was developed to determine the relationship of all variables to the time of ovulation. Originally the model included ovulation time as dependent variable and, size of the ovulatory follicle at time of GnRH/hCG administration, ovulatory size, time from GnRH/hCG administration to plasma maximum concentration (peak) of LH/hCG, maximum LH or hCG concentrations, percentage of the maximum LH or hCG peak concentration, time from CIDR removal to maximum estradiol-17β concentrations and maximum estradiol concentrations. A backwards selection procedure was used to eliminate independent variables not meeting the significance value ($P < 0.05$) required to stay in the model. Cows ovulating earlier where excluded from the analysis since information on several of the variables could not be recorded.

Data are presented as least squares means (LSM) ± standard error of the mean (±SEM). The Tukey-Kramer test was used to identify treatment differences among least squares means.
CHAPTER 4
RESULTS

4.1. Animal and Production Traits

Table 2 presents a description of age, parity, body weight (BW), body condition score (BCS), days in milk (DIM) and milk production of cows included in the present study which ovulated within the expected period of time following induction of ovulation with either GnRH or hCG sorted by treatment and their combinations.

4.2. Hormone Concentrations

4.2.3. Progesterone

There were not significant differences ($P = 0.253$) in progesterone concentrations during the whole sampling period in cows assigned to Folltropin-V or Saline treatment groups. However, during the same period there was an effect of time ($P < 0.0001$). Progesterone concentrations significantly increased from $4.1 \pm 0.3$ ng/mL on Day 0 (CIDR insertion) to $6.9 \pm 0.3$ ng/mL on Day 2, then significantly decreased to $1.2 \pm 0.3$ ng/mL on Day 8. Subluteal ($< 1$ ng/mL) concentrations of progesterone were reached between 24 to 36 h after CIDR removal and PGF$_{2\alpha}$ administration in both treatment groups (FSH and SAL).
Table 2. Animal and production traits of cows included in the experiment that ovulated within the expected period of time following induction of ovulation (GnRH/hCG).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Trait^1</th>
<th>Age (months)</th>
<th>Parity (#)</th>
<th>BW^2 (kg)</th>
<th>BCS^3</th>
<th>DIM^4</th>
<th>Milk yield (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Trt. Factor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td></td>
<td>36.1</td>
<td>1.8</td>
<td>640.3</td>
<td>2.9</td>
<td>78.9</td>
<td>45.8</td>
</tr>
<tr>
<td>SAL</td>
<td></td>
<td>38.9</td>
<td>2.1</td>
<td>641.7</td>
<td>2.7</td>
<td>80.8</td>
<td>43.2</td>
</tr>
<tr>
<td>SEM^5</td>
<td></td>
<td>4.6</td>
<td>0.4</td>
<td>40.7</td>
<td>0.2</td>
<td>4.0</td>
<td>2.1</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>0.665</td>
<td>0.485</td>
<td>0.975</td>
<td>0.320</td>
<td>0.722</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnRH</td>
<td></td>
<td>39.0</td>
<td>2.0</td>
<td>645.8</td>
<td>2.8</td>
<td>76.9</td>
<td>44.4</td>
</tr>
<tr>
<td>hCG</td>
<td></td>
<td>36.0</td>
<td>1.8</td>
<td>636.2</td>
<td>2.8</td>
<td>82.8</td>
<td>44.6</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>4.6</td>
<td>0.4</td>
<td>40.5</td>
<td>0.2</td>
<td>4.0</td>
<td>2.1</td>
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<tr>
<td>P-value</td>
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<td>0.644</td>
<td>0.639</td>
<td>0.821</td>
<td>0.804</td>
<td>0.268</td>
<td>0.955</td>
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<td><strong>Combinations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH-GnRH</td>
<td></td>
<td>38.2</td>
<td>1.9</td>
<td>634.1</td>
<td>2.8</td>
<td>70.5</td>
<td>44.1</td>
</tr>
<tr>
<td>FSH-hCG</td>
<td></td>
<td>34.0</td>
<td>1.6</td>
<td>646.5</td>
<td>2.9</td>
<td>87.4</td>
<td>47.5</td>
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<tr>
<td>SAL-GnRH</td>
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<td>39.7</td>
<td>2.2</td>
<td>657.5</td>
<td>2.7</td>
<td>83.4</td>
<td>44.8</td>
</tr>
<tr>
<td>SAL-hCG</td>
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<td>38.0</td>
<td>2.0</td>
<td>625.9</td>
<td>2.7</td>
<td>78.3</td>
<td>41.6</td>
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<td>SEM</td>
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<td>0.2</td>
<td>5.3</td>
<td>2.8</td>
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<tr>
<td>P-value</td>
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<td>0.841</td>
<td>0.815</td>
<td>0.597</td>
<td>0.804</td>
<td>0.045</td>
<td>0.244</td>
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<tr>
<td>Tukey test</td>
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<td>NS^6</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

^1All values are reported as least square means.
^2BW = body weight, ^3BCS = body condition score, ^4DIM = days in milk
^5SEM = pooled standard error of the mean.
^6NS = not significant
No effect of treatment-by-time interaction \((P = 0.205)\) was observed in concentrations of progesterone (Figure 4). Concentrations of progesterone from time of CIDR insertion (Day 0 of the experimental protocol) until CIDR removal (Day 7) did not differ \((P = 0.353)\) between cows receiving Folltropin-V or Saline treatment \((7.7 \pm 0.5 \text{ and } 7.0 \pm 0.5 \text{ ng/mL for FSH and SAL, respectively})\). An effect of time \((P < 0.001)\) was also observed with concentrations of progesterone increasing significantly from Day 0 to Day 2 \((4.1 \pm 0.4 \text{ and } 6.9 \pm 0.4 \text{ ng/mL, respectively})\). No effect of treatment-by-time interaction \((P = 0.109)\) was observed during this period. The slope of progesterone concentration decrease after CIDR removal and PGF\(_{2\alpha}\) administration was not different \((P = 0.279)\) for Folltropin-V and Saline treated cows \((1.2 \pm 0.1 \text{ and } 1.4 \pm 0.1 \text{ ng/mL/12 h for FSH and SAL, respectively})\).

4.2.2. Estradiol-17\(\beta\)

Concentrations estradiol-17\(\beta\) from the approximate time of initiation of the new follicular wave (Day 2) to the time of CIDR removal (Day 7), did not differ between Folltropin-V and Saline treatment groups \((P = 0.178)\) and there was not an effect of time \((P = 0.627)\) and treatment-by-time interaction \((P = 0.655; \text{Figure 5})\). Further assessment of estradiol-17\(\beta\) concentrations indicated that there was an effect of treatment \((P = 0.018)\) and time \((P < 0.0001)\) from time of CIDR removal (Day 7) to GnRH/hCG (Day 9). Mean concentrations of estradiol-17\(\beta\) were higher in Saline than in Folltropin-V treated cows \((4.6 \pm 0.3 \text{ and...} \text{(Figure 5)})\).
Figure 4. Concentrations of progesterone (P4) during whole sampling period. CIDR-in = time of CIDR insertion and GnRH administration, CIDR-r = time of CIDR removal and PGF$_2$a administration. FSH = Folltropin-V, SAL = Saline treated group. Days indicate day of the experimental protocol.
Figure 5. Estradiol-17β (E2) concentrations from approximate time of initiation of new follicular wave (Day 2) to time of CIDR removal (Day 7). NFW = approximate time of initiation of new follicular wave, CIDR-r = time of CIDR removal. FSH = Folltropin-V treated cows, SAL = Saline treated cows. Days indicate day of the experimental protocol.
3.5 ± 0.3 pg/mL for SAL and FSH groups, respectively) and significantly increased towards the time of GnRH/hCG administration (Figure 6).

Maximum values for estradiol-17β concentrations significantly differed between Folltropin-V and Saline treated groups ($P = 0.009$) whereas, there was not and effect of treatment with GnRH/hCG ($P = 0.255$) or interaction between first (FSH vs. SAL) and second treatment factors (GnRH vs. hCG; $P = 0.916$; Table 3). Time to maximum plasma concentrations of estradiol-17β after CIDR removal did not differ between Folltropin-V and Saline groups ($P = 0.371$) but, there was a weak tendency to differ for GnRH and hCG treatment groups ($P = 0.084$) and the interaction of first (FSH vs. SAL) and second (GnRH vs. hCG) treatment factors ($P = 0.084$; Table 3).

Analysis of the slope of estradiol-17β concentrations decrease after maximum values were reached showed significant differences among treatment combinations for the linear and quadratic slopes of decrease. Cows administered the FSH-hCG combination presented the slowest rate of estradiol-17β decrease (1.2 ± 0.6 pg/mL/4 h) which was different to the other three treatment combinations (FSH-GnRH, 3.5 ± 1.0; SAL-GnRH, 5.5 ± 0.6; and SAL-hCG, 4.0 ± 0.6 pg/mL/4 h; Figure 7).
Figure 6. Estradiol-17β (E2) concentrations after CIDR removal and Folltropin-V/Saline administration (Day 7) until time of GnRH or hCG administration (Day 9). CIDR-r = time of CIDR removal and Folltropin-V/Saline administration, GnRH/hCG = time of GnRH/hCG administration. FSH = Folltropin-V treated cows, SAL = Saline treated cows. Mean concentrations of E2 were significantly higher for Saline than for Folltropin-V treated cows during the represented period ($P = 0.018$).
Table 3. Maximum estradiol-17β plasma concentration and time to maximum concentration after CIDR removal.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Maximum E2 concentration (pg/mL)(^1)</th>
<th>Time to maximum E2 concentration (h)(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Trt. Factor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>10</td>
<td>5.4(^b)</td>
<td>50.0</td>
</tr>
<tr>
<td>SAL</td>
<td>16</td>
<td>7.3(^a)</td>
<td>51.0</td>
</tr>
<tr>
<td>SEM(^2)</td>
<td></td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td>0.009</td>
<td>0.371</td>
</tr>
</tbody>
</table>

| **Second Trt. Factor** |     |                                        |                                          |
| GNRRH               | 11  | 6.8                                    | 49.5                                     |
| hCG                 | 15  | 6.0                                    | 51.5                                     |
| SEM                 |     | 0.5                                    | 0.8                                      |
| P-value             |     | 0.255                                  | 0.084                                    |

| **Combinations**    |     |                                        |                                          |
| FSH-GnRH            | 3   | 5.9                                    | 48.0                                     |
| FSH-hCG             | 7   | 5.2                                    | 52.0                                     |
| SAL-GnRH            | 8   | 7.7                                    | 51.0                                     |
| SAL-hCG             | 8   | 6.8                                    | 51.0                                     |
| SEM                 |     | 0.7                                    | 1.1                                      |
| P-value             |     | 0.916                                  | 0.084                                    |

\(^{a,b}\)Least square means differ among treatments within a column.

\(^1\)Maximum E2 concentration and time to maximum E2 concentration are reported as least square means.

\(^2\)SEM = pooled standard error of the mean.
Figure 7. Concentrations of estradiol-17β from time of GnRH/hCG (Day 9) to the end of sampling period (Day 10). GnRH/hCG = time of GnRH/hCG administration, End sampling = end of sampling period for E2. FSH-GnRH = FSH-GnRH treated group, FSH-hCG = FSH-hCG treated group, SAL-GnRH = SAL-GnRH treated cows, SAL-hCG = SAL-hCG treated cows.
4.2.3. **Follicle Stimulating Hormone**

Concentrations of FSH from 48 h prior to Folltropin-V administration (Day 5) to the end of sampling period for FSH (Day 9) did not differ between Folltropin-V and Saline treatment groups (1.2 ± 0.2 and 1.2 ± 0.1 ng/mL; \( P = 0.930 \)) and there was not an effect of time (\( P = 0.192 \)) and treatment-by-time interaction (\( P = 0.347 \)). Further analysis of FSH concentrations after Folltropin-V administration (Day 7) to the end of sampling period for FSH (Day 9) showed no differences in FSH concentrations between Folltropin-V and Saline treated cows (\( P = 0.847 \)) and no effect of time (\( P = 0.157 \)) and treatment-by-time interaction (\( P = 0.253 \); Figure 8).

4.2.4. **Luteinizing Hormone**

Concentrations of LH from Day 5 to Day 9 of the experimental protocol (prior to GnRH/hCG administration) were not different in Folltropin-V and Saline treated cows (0.6 ± 0.05 and 0.6 ± 0.04 ng/mL, respectively; \( P = 0.351 \)) but were affected by time (\( P < 0.0001 \)) resulting in concentrations of LH significantly increasing towards the time of GnRH/hCG administration.

Maximum LH concentrations in hCG-treated cows were not analyzed since LH concentrations in this group of cows remained below 2 ng/mL during the entire sampling period (Figure 9). Analysis of plasma LH concentrations from 3 h prior to GnRH/hCG administration (Day 9) to 31 h post injection (Day 10)
Figure 8. Concentrations of FSH after Folltropin-V injection coincident with time of CIDR removal (Day 7) to the end of sampling period (Day 9) for Folltropin-V vs. Saline treated cows. CIDR-r = time of CIDR removal and Folltropin-V/Saline administration, GnRH/hCG = time of GnRH/hCG administration. FSH = Folltropin-V treated group, SAL = Saline treated group.
showed a significant 3-way interaction among first treatment factor (FSH vs. SAL), second treatment factor (GnRH vs. hCG) and time ($P = 0.008$). This interaction indicates that there were significant differences in concentrations of LH between Folltropin-V and Saline as well as GnRH and hCG groups which varied with time. This interaction is due to the large differences in LH concentrations between GnRH and hCG treated cows within Folltropin-V and Saline groups after GnRH/hCG administration. Only cows within Folltropin-V and Saline groups receiving GnRH had an observable LH surge producing significant differences in LH concentrations within 1 to 4 h after GnRH administration. Prior to, and 4 h after GnRH administration, LH concentrations were not significantly different among cows receiving GnRH or hCG within each of the first treatment factor levels (FSH and SAL). In addition, differences between levels of primary treatment factor are due to higher maximum concentrations of LH in SAL-GnRH than in FSH-GnRH treated cows during the surge after GnRH administration (8.0 ± 0.4 and 10.2 ± 0.2 ng/mL for FSH-GnRH and SAL-GnRH, respectively). Luteinizing hormone (LH) concentrations in cows receiving hCG were consistently low (0.8 ± 0.06 ng/mL; Figure 9) throughout the sampling period (3 h prior to GnRH hCG injection to 31 h later) and did not present a clear LH surge.

The average time to the LH maximum concentration (peak) after CIDR removal in all GnRH treated cows was 50.8 ± 0.2 h. When analyzed from GnRH administration, the average time to the peak was 1.8 ± 0.2 h (Table 4 and Figure 9). No significant differences were observed in time to LH peak after CIDR
**Figure 9.** Overall plasma concentrations of LH and hCG from -3 to 31 h of GnRH or hCG administration. LH (GnRH) = LH concentrations in cows receiving GnRH treatment, LH (hCG) = LH concentrations in cows receiving hCG treatment, hCG (GnRH) = hCG concentrations in cows receiving GnRH treatment, this group presented non-detectable levels of hCG during whole sampling period. hCG (hCG) = hCG concentrations in cow receiving hCG treatment. Arrow indicates time of GnRH or hCG administration. Asterisks denote significant differences in least square means for LH between GnRH and hCG treatments (LH pooled SEM = 0.2, hCG pooled SEM = 1.1 *P* < 0.05). Concentrations of hCG between GnRH and hCG groups were significantly different (*P* < 0.05) from 1 h after hCG injection to the end of the sampling period.
**Table 4.** Time to LH or hCG maximum concentration (peak) after CIDR removal and GnRH/hCG administration.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Time to peak from CIDR-r (h)</th>
<th>Time to peak from injection (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LH</td>
<td>hCG</td>
</tr>
<tr>
<td>GnRH</td>
<td>11</td>
<td>50.8</td>
<td>ND</td>
</tr>
<tr>
<td>hCG</td>
<td>15</td>
<td>N/A</td>
<td>58.0</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>P-Value</td>
<td></td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>FSH</td>
<td>10</td>
<td>50.7</td>
<td>59.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAL</td>
<td>16</td>
<td>50.9</td>
<td>57.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>0.2</td>
<td>0.8</td>
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<tr>
<td>P-Value</td>
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<td>0.499</td>
<td>0.019</td>
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</tbody>
</table>

<sup>a,b</sup>Least square means differ among treatments within a column.

<sup>1</sup>Time to peak from CIDR-r and injection are reported as least square means.

<sup>2</sup>SEM = pooled standard error of the mean.

<sup>3</sup>ND = not detectable.

<sup>4</sup>N/A = not applicable.
removal ($P = 0.499$) or GnRH administration ($P = 0.499$) in FSH-GnRH or SAL-GnRH treatment groups (Table 4).

4.2.5. Human Chorionic Gonadotropin

Concentrations of hCG are presented for hCG-treated and GnRH-treated (non-detectable during whole sampling period) cows in Figure 9. Maximum hCG concentration in hCG treated cows was $27.1 \pm 2.8$ mIU/mL and did not differ between FSH-hCG and SAL-hCG treatment groups ($24.5 \pm 2.9$ and $29.7 \pm 2.7$ mIU/mL, respectively; $P = 0.226$). Maximum levels (peak) of hCG in plasma of all treated cows were reached $58.0 \pm 0.8$ h after CIDR removal or $9.0 \pm 0.8$ h post hCG injection (Table 4 and Fig. 9). A significant difference in time to hCG maximum concentrations (peak) after CIDR removal and GnRH/hCG administration was observed between Folltropin-V and Saline cows ($P = 0.019$; Table 4).

4.3. Follicle Development and Ovulation

Size of the ovulatory follicle at time of CIDR removal (Day 7) which is coincident with the application of Folltropin-V and Saline did not differ between cows assigned to either one of the treatments ($13.7 \pm 0.6$ and $14.7 \pm 0.5$ mm, for Folltropin-V and Saline respectively; $P = 0.099$). Further, there was not an effect of Folltropin-V or Saline treatment on the size of the ovulatory follicle at the time.
of either GnRH or hCG administration ($P = 0.521$). At this time point, size of the ovulatory follicle was similar for Folltropin-V and Saline treatment groups (17.1 ± 0.7 and 17.7 ± 0.6 mm, respectively).

No significant differences were observed in the size of the ovulatory follicle prior to ovulation between Folltropin-V and Saline treatment groups ($P = 0.585$), GnRH and hCG groups ($P = 0.287$) or the interaction between first (FSH vs. SAL) and second treatment (GnHR vs. hCG) factors ($P = 0.297$; Table 5).

Total growth of the ovulatory follicle from time of CIDR removal (Day 7) to time of GnRH or hCG administration (Day 9) did not differ between Folltropin-V and Saline treated cows (3.2 ± 0.6 and 3.3 ± 0.4 mm, respectively; $P = 0.891$). Daily growth of the ovulatory follicle during the same time period was not different between Folltropin-V and Saline treatment (1.6 ± 0.3 and 1.6 ± 0.2 mm; $P = 0.891$). Total and daily growth of the ovulatory follicle from CIDR removal to GnRH or hCG administration was affected by parity of the cows ($P = 0.001$), DIM ($P = 0.022$), milk yield ($P = 0.005$) and body weight ($P = 0.007$). Parity, DIM and milk yield presented a negative relationship with growth meaning that an increase in the values of any of these variables was related to a decrease in growth rate of the ovulatory follicle. On the other hand, body weight was positively related to the growth of the ovulatory follicle indicating that cows with higher body weight presented a higher growth rate of the ovulatory follicle.

Total growth of the ovulatory follicle from time of CIDR removal (Day 7) to ovulation (Day 10) did not differ between Folltropin-V and Saline treatment groups ($P = 0.102$), GnRH and hCG treatment groups ($P = 0.261$) or the
Table 5. Size of the ovulatory follicle prior to ovulation for first treatment factor, second treatment factor, and their interaction (combinations).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Size of ovulatory follicle prior to ovulation (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Trt. Factor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>10</td>
<td>18.1</td>
</tr>
<tr>
<td>SAL</td>
<td>16</td>
<td>17.5</td>
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<td>SEM(^2)</td>
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<tr>
<td><strong>Second Trt. Factor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnRH</td>
<td>11</td>
<td>18.4</td>
</tr>
<tr>
<td>hCG</td>
<td>15</td>
<td>17.2</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>P-Value</td>
<td></td>
<td>0.287</td>
</tr>
<tr>
<td><strong>Combinations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH-GnRH</td>
<td>3</td>
<td>19.3</td>
</tr>
<tr>
<td>FSH-hCG</td>
<td>7</td>
<td>17.0</td>
</tr>
<tr>
<td>SAL-GnRH</td>
<td>8</td>
<td>17.6</td>
</tr>
<tr>
<td>SAL-hCG</td>
<td>8</td>
<td>17.5</td>
</tr>
<tr>
<td>SEM</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>P-Value</td>
<td></td>
<td>0.297</td>
</tr>
</tbody>
</table>

1 Size of the ovulatory follicle is reported as least square means.
2 SEM = pooled standard error of the mean.
interaction of first (FSH vs. SAL) and second treatment (GnRH vs. hCG) factors 

\( (P = 0.293; \text{Table 6}) \).

There was a tendency \((P = 0.081)\) for Folltropin-V treated cows to have a higher daily growth rate from time of CIDR removal (Day 7) to ovulation (Day 10); whereas, there was not a significant effect of either GnRH or hCG treatment 

\( (P = 0.198) \) and the interaction of first and second treatment factors (combinations; \(P = 0.241; \text{Table 6}) \).

A significant interaction of first (FSH vs. SAL) and second (GnRH vs. hCG) treatment factors was noted when the time elapsed from CIDR removal (Day 7) to ovulation was evaluated \((P = 0.013)\). Cows receiving GnRH presented the earliest ovulation but only when combined with Folltropin-V treatment (Table 7). Indeed, when GnRH was combined with Saline treatment, cows ovulated a time not different from hCG treated cows. Therefore, administration of Folltropin-V followed by GnRH 49 h later showed to be the most beneficial treatment to decrease the time elapsed from CIDR removal to ovulation in the present study. On the other hand, administration of Folltropin-V followed by hCG at a similar timing than GnRH produced the longest time to ovulation after CIDR removal (Table 7).

When time to ovulation was evaluated from GnRH or hCG administration the same situation was noted as when evaluated from time of CIDR removal. There was a significant interaction between first (FSH vs. SAL) and second (GnRH vs. hCG) treatment factors \((P = 0.013)\). As with the previous analysis, cows receiving GnRH presented the earliest ovulation but only when combined
Table 6. Total and daily growth of the ovulatory follicle from time of CIDR removal (CIDR-r) to ovulation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Total from CIDR-r to ovulation (mm)</th>
<th>Daily from CIDR-r to ovulation (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Trt. Factor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>10</td>
<td>4.5</td>
<td>1.4</td>
</tr>
<tr>
<td>SAL</td>
<td>16</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td>SEM²</td>
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<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>P-Value</td>
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<td></td>
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</tr>
<tr>
<td><strong>Second Trt. Factor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnRH</td>
<td>11</td>
<td>4.3</td>
<td>1.4</td>
</tr>
<tr>
<td>hCG</td>
<td>15</td>
<td>3.2</td>
<td>1.0</td>
</tr>
<tr>
<td>SEM</td>
<td>0.7</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>P-Value</td>
<td>0.261</td>
<td></td>
<td>0.198</td>
</tr>
<tr>
<td><strong>Combinations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH-GnRH</td>
<td>3</td>
<td>5.6</td>
<td>1.8</td>
</tr>
<tr>
<td>FSH-hCG</td>
<td>7</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>SAL-GnRH</td>
<td>8</td>
<td>3.0</td>
<td>0.9</td>
</tr>
<tr>
<td>SAL-hCG</td>
<td>8</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td>SEM</td>
<td>0.9</td>
<td>0.9</td>
<td>0.3</td>
</tr>
<tr>
<td>P-Value</td>
<td>0.293</td>
<td></td>
<td>0.241</td>
</tr>
</tbody>
</table>

1 Growth of the ovulatory follicle is reported as least square means.
2 SEM = pooled standard error of the mean.
Table 7. Time to ovulation from CIDR removal (CIDR-r), GnRH/hCG administration, and LH or hCG maximum concentration (peak).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Time to ovulation from CIDR-r (h)</th>
<th>Time to ovulation from GnRH or hCG administ. (h)</th>
<th>Time to ovulation from LH or hCG max. conc. (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Trt. Factor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH</td>
<td>10</td>
<td>76.9</td>
<td>27.9</td>
<td>21.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAL</td>
<td>16</td>
<td>78.0</td>
<td>29.0</td>
<td>24.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td>0.6</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>P-Value</td>
<td></td>
<td>0.196</td>
<td>0.196</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Second Trt. Factor</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GnRH</td>
<td>11</td>
<td>75.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>hCG</td>
<td>15</td>
<td>79.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.6</td>
<td>0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>P-Value</td>
<td></td>
<td>0.001</td>
<td>0.001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**Combinations**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Time to ovulation from CIDR-r (h)</th>
<th>Time to ovulation from GnRH or hCG administ. (h)</th>
<th>Time to ovulation from LH or hCG max. conc. (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSH-GnRH</td>
<td>3</td>
<td>74.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FSH-hCG</td>
<td>7</td>
<td>79.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAL-GnRH</td>
<td>8</td>
<td>77.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>28.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>26.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SAL-hCG</td>
<td>8</td>
<td>78.5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>29.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
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<td>0.9</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>P-Value</td>
<td></td>
<td>0.013</td>
<td>0.013</td>
<td>0.066</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Least square means differ among treatments within a column.

<sup>1</sup>Time of ovulation is reported as least square means.

<sup>2</sup>SEM = pooled standard error of the mean.
with Folltropin-V treatment (Table 7). When GnRH was combined with Saline treatment, cows ovulated a time not different from hCG treated cows. Therefore, administration of Folltropin-V followed by GnRH 49 h later showed to be the most beneficial treatment to decrease the time elapsed from induction of ovulation to its occurrence in the present study. On the other hand, administration of Folltropin-V followed by hCG at a similar timing than GnRH produced the longest time to ovulation (Table 7).

When timing of ovulation was analyzed from time of LH or hCG maximum concentrations (peak), it was observed a significant effect of Folltropin-V treatment when compared to Saline ($P < 0.0001$), as well as a significant effect of hCG which resulted in the shortest time from peak to ovulation when compared to GnRH treated cows ($P < 0.001$; Table 7). In addition, there was an interaction between first (FSH vs. SAL) and second (GnRH vs. hCG) treatment factors to influence the time of ovulation after LH or hCG maximum concentration (peak; $P = 0.066$). According to this interaction, hCG treated cows presented a tendency to have the earliest ovulation but only when combined with Folltropin-V since SAL-hCG treated cows did not differ from those cows administered the FSH-GnRH combination. As the effect of Folltropin-V treatment alone was to reduce the time from peak to ovulation it is likely that it contributed to decrease the time elapsed from peak to ovulation in GnRH treated cows limiting the chances of finding differences with the SAL-hCG group. The fact that FSH-hCG treated cows ovulated earlier than SAL-hCG, is in agreement
with the finding that the effect of Folltropin-V was to decrease ovulation time when measured from the LH or hCG maximum concentration (Table 7).

The multiple regression model developed for ovulation time indicated the presence of a relationship between size of the ovulatory follicle at the time of GnRH/hCG administration, time from GnRH/hCG administration to LH/hCG maximum concentration (peak) and for time to maximum estradiol concentration with time of ovulation ($P < 0.0001$). The model explained 64% of the variation in the dependent variable ovulation time. Size of the ovulatory follicle at the time of GnRH/hCG showed a negative relationship with time to ovulation; whereas, time from injection of GnRH/hCG to GnRH/hCG maximum concentration peak and time to maximum estradiol concentration showed a positive relationship with time to ovulation. Time from GnRH/hCG administration to LH/hCG maximum concentration explained 41% of the variation in ovulation time when considering the other variables. Size of the ovulatory follicle at the time of GnRH/hCG administration and time to maximum estradiol concentration explained 18% and 14% of the variation in ovulation time respectively when considering the other variables.

**4.4. Hormonal Profiles and Follicle Features of Cows Presenting Premature Ovulation after Folltropin-V Administration (FSH-EARLY)**

As previously described in chapter 3, a subgroup of cows presented ovulation following treatment with Folltropin-V prior to the administration of
GnRH/hCG and was denominated as FSH-EARLY group. Animal and production traits for FSH-EARLY group were as follow: age 37 ± 6.5 months, body condition score 2.5 ± 0.2, body weight 632.4 ± 52.3 kg, days in milk 91.4 ± 5.8, milk yield 42.8 ± 3.0 kg, and parity 1.8 ± 0.5.

Mean progesterone concentrations during whole sampling period were 5.7 ± 0.5 ng/mL, and decreased at a rate of 1.7 ± 0.2 ng/mL/12 h after CIDR removal and PGF$_{2\alpha}$ administration (Day 7) reaching subluteal levels (< 1 ng/mL) by 24 to 36 h after CIDR removal and PGF$_{2\alpha}$. Mean estradiol-17β concentrations from approximate time of initiation of a new follicular wave (Day 2) to time of CIDR removal (Day 7) were 1.5 ± 0.2 pg/mL. Following CIDR removal mean concentrations of estradiol-17β were 1.7 ± 0.5 pg/mL. Cows in the FSH-EARLY group did not present an elevation of estradiol-17β consistent with a preovulatory peak. Mean concentrations of FSH from 48 h prior to CIDR removal and Folltropin-V administration (Day 5) to the end of sampling period for FSH (Day 9) were 1.0 ± 0.3 ng/mL. Mean concentrations of LH from 48 h prior to CIDR removal and Folltropin-V administration (Day 5) to the time of GnRH/hCG administration (Day 9) were 0.6 ± 0.1 ng/mL. Neither LH nor hCG were measured in FSH-EARLY cows following GnRH/hCG administration (Day 9) since they presented ovulation and were removed from the study prior to this time point.

Size of the ovulatory follicle at the time of CIDR removal (Day 7) was 13.9 ± 0.8 mm. Prior to ovulation, size of the ovulatory follicle was 14.9 ± 1.1 mm. Total growth of the ovulatory follicle from time of CIDR removal to
ovulation was $0.7 \pm 1.0$ mm. Daily growth of the ovulatory follicle from time of CIDR removal to ovulation was $0.6 \pm 0.4$ mm. Time elapsed from CIDR removal to ovulation in the FSH-EARLY group was $38.4 \pm 1.2$ h.
The major goal of the present study was to evaluate possible therapeutic strategies to hasten exportation of the oocyte to the oviduct following induction of ovulation with gonadotropins in lactating dairy cows. Development of therapeutic strategies aimed to reduce the time from onset of oocyte maturation (induced by gonadotropin surge in vivo) to ovulation may be a first step necessary for release of a fertile mature oocyte in cows undergoing environmental HS (Edwards et al., 2005). In this study it was tested the hypothesis that a possible strategy to reduce the time to ovulation may be to induce ovulation of larger follicles. Therefore, it was evaluated if the use of Folltropin-V (FSH/LH) given at the time of induction of luteolysis may stimulate supplemental growth of the DF during its final growth phase. Then, a possible larger ovulatory follicle may be more sensitive to the gonadotropin surge and respond with early ovulation. Besides inducing larger follicles more sensitive to ovulation treatment with gonadotropins, the use of Folltropin-V may have another advantage when used in lactating dairy cows under HS conditions by offsetting the effect of elevated temperatures to decrease follicle size (Badinga et al., 1993; Wilson et al., 1998a,b; Cunha et al., 2007). In addition to the evaluation of growth response of the DF to Folltropin-V (FSH/LH) administration, this experiment was also intended to examine if the administration hCG would have an impact in the timing of ovulation. Unlike GnRH, the use of hCG to induce ovulation in heat stressed cows may have some advantages since
its mechanism of action is independent of the pituitary gland which function might be altered by the effects of elevated temperatures in lactating dairy cows (Gilad et al., 1993). Furthermore, hCG presents a prolonged plasma half-life (Schmitt et al., 1996), greater affinity for LH receptors (Henderson et al., 1984), and the capability to remain bound to LH receptors for longer periods of time than LH (Mock and Niswender 1983; Mock et al., 1983; Henderson et al., 1984) which may be possibly advantageous for induction of early ovulation.

In the current study, the therapeutic model to induce ovulation was examined using non-heat stressed lactating dairy cows. The results of this study demonstrate that administration of hormones, utilizing current estrus synchronization protocols aimed at manipulating follicular growth and ovulation, was successful in decreasing time from the gonadotropin surge to ovulation. However, most of the difference observed among treatment groups can be attributed to the specific dynamic of the gonadotropin responsible for induction of ovulation. In particular, the average time to maximum concentrations of hCG (peak) in hCG-treated cows were reached ~ 7 h later than maximum LH concentrations (peak) in GnRH-treated cows. Therefore, if the onset of oocyte maturation is triggered by hCG maximum concentrations (peak) and not by the rise, administration of hCG to lactating dairy cows under HS would be a possible strategy to delay onset of oocyte maturation in relation to rupture of the ovulatory follicle. Furthermore, treatment with Folltropin-V (combination of both FSH and LH) failed to boost final growth of the ovulatory follicle and increase size at the time of GnRH/hCG administration. However, if data are considered from the
respective maximum concentrations (i.e. LH or hCG), Folltropin-V treated cows had a shorter time to ovulation when compared to Saline-treated cows, suggesting a possible beneficial effect of Folltropin-V in timing of ovulation. Despite the observed differences, it is unclear which mechanisms may be involved in the induction of early ovulation in Folltropin-V treated cows. As previously discussed, this group of animals did not have larger follicles at the time of GnRH/hCG impeding any possibility of testing the hypothesis that larger follicles would be more responsive to either LH or hCG due to increased number of LH receptors. Even with sufficient evidence that larger follicles present higher numbers of LH receptors (Ireland and Roche 1982; Stewart et al., 1996), whether these follicles ovulate earlier than smaller ones can not be concluded from the present data as ovulatory follicles were of similar sizes. In fact, FSH-GnRH treated cows (and to a lesser extent FSH-hCG treated cows) presented a numerical difference in growth from CIDR removal to the time of ovulation when compared to Saline-treated cows and there was also a weak tendency for higher daily growth from CIDR removal to ovulation for Folltropin-V than for Saline treated cows; however, it is likely that the low number of cows in FSH-GnRH group limited the probability of observing statistical differences.

Considering that the dose administered to Folltropin-V treated group induced earlier ovulation in a subgroup of cows (FSH-EARLY), it is unclear why this treatment did not simulate complementary growth of the ovulatory follicle. Therefore, administration of Folltropin-V (FSH/LH) at the dose and timing utilized in this study is not a promising alternative to increase DF size in lactating
dairy cows undergoing HS. In addition, it is somewhat surprising that animals treated with Folltropin-V did not present a significantly different rise in FSH concentrations after treatment when compared to Saline-treated cows. Only a numerical difference was observed with concentrations increasing after Folltropin-V administration in Folltropin-V treated cows. A possible explanation for the lack of difference is that the frequency of blood sampling was not sufficient to detect the rise in FSH concentrations or the high levels of FSH observed in the Saline group. In our study FSH concentrations in both groups are within the range of values previously reported for dairy cows after treatment with PGF$_{2a}$ to induce luteolysis (Parfet et al., 1989).

Based on the observations of this study it is logical to suggest that the use of hCG to induce ovulation in lactating dairy cows undergoing HS may be successful only if hCG triggers the mechanisms necessary for follicle wall breakdown and onset of oocyte maturation at different times. It would be required for hCG to trigger the process of follicular wall breakdown soon after administration when blood concentrations are rising. In this way, rupture of the ovulatory follicle may occur within a reasonable time after hCG administration (28-30 h). Whereas, the onset of oocyte maturation is delayed during a period of time sufficient to allow completion of the process while being released before aging begins. In regard to the process of follicle wall breakdown, Liu and Sirois (1998) indicated that prostaglandin production by the preovulatory follicle in hCG-treated cattle paralleled that of GnRH-treated cattle. In this study, PGE$_2$ and PGF$_{2a}$ increased by 24 h after hCG administration. The fact that PG’s production
has been associated with the process of follicle wall breakdown, and PG’s levels rise at similar times after GnRH or hCG treatment, suggests that both hormones may induce changes at the follicular wall level at a similar time and that hCG triggers the process soon after administration. Unfortunately, oocyte maturation was not assessed in the study of Liu and Sirois (1998), limiting comparison with studies which evaluated maturation of the oocyte after an induced LH surge.

The classical description of the series of events leading to ovulation and oocyte maturation, under naturally occurring and induced estrous cycles in cattle, considers the preovulatory gonadotropin surge as the trigger of the process (Kruip et al., 1983; de Loos et al., 1991). However, information in the literature on the specific portion of the LH/hCG surge responsible for triggering ovulation and oocyte maturation is lacking. In particular, it is not clear if the initial rise in LH/hCG concentration is responsible for initiating the process of follicle wall breakdown and oocyte maturation or, a threshold concentration close to maximum values must be attained before these two processes are initiated. Analysis of the ultra structural nuclear and cytoplasmic changes in bovine oocytes obtained from single and multiple ovulating cattle at different times after the LH surge indicates that the first observable changes occurs ~ 4 to 7 h after the surge and the process is completed 20 to 25 h later (Kruip et al., 1983; de Loos et al., 1991). Unfortunately, there is no mention of the duration and timing of the LH surge in any of these studies where the authors describe changes in oocyte structure from the LH surge. On the other hand, it is uncertain whether or not hCG triggers the process of oocyte maturation in a “timely” manner similar to that of LH in cattle.
since the morphological changes associated with oocyte maturation have not been described for cows induced to ovulate with hCG.

A shift in steroid production from estradiol-17β to progesterone is clearly observed in preovulatory follicles following the gonadotropin surge (Dieleman and Bevers 1993; Sirois 1994; Li et al., 2007). In the present study, the decline in serum estradiol-17β concentrations started at a time coincident with LH maximum concentrations in cows treated with GnRH (~ 50 h); whereas, in hCG-treated cows, the decline in estradiol-17β concentrations started earlier (~ 51.5 h) than the occurrence of hCG maximum concentrations (~ 58 h). Thus, if the decrease in concentrations of estradiol-17β is an indicator of the preovulatory follicle response to gonadotropins, the fact that estradiol-17β concentrations in hCG cows started to decrease prior to maximum hCG plasma concentrations suggests that follicles responded to hCG during the “rise” portion of the surge. Conversely, FSH-hCG, and to a lesser extent SAL-hCG treated cows (not significant difference) presented a slower rate of decrease of estradiol-17β concentrations since the slope of estradiol-17β decay in these cows was different. In agreement with this observation, when comparing intrafollicular concentrations of estradiol after either hCG administration in the study of Sirois (1994) or GnRH administration in Li et al. (2007), a difference of ~ 100 ng/mL in estradiol-17β concentrations can be observed 12 h post either hCG or GnRH administration. Although concentrations are compared from two different studies, this large difference (~ 100 ng/mL) in estradiol-17β suggests that the shift in steroid synthesis after gonadotropin treatment is dissimilar in hCG and GnRH treated
cows. It is uncertain how follicles may respond differently to LH or hCG given that both gonadotropins bind the same receptor in follicular cells (Lee and Ryan 1973; Minegishi et al., 1997). However, a slower decrease in estradiol-17β concentrations may have an impact on the onset of oocyte maturation although this is very controversial. Exposure of bovine oocytes matured in vitro to estradiol-17β during the first 8 h of maturation induced a block at metaphase I (germinal vesicle stage) while it did not compromise the ability of oocytes to complete the maturation process once estradiol-17β was removed (Beker-van Woudenberg et al., 2004). It is then logical to speculate that under in vivo conditions, estradiol-17β may exert a similar effect on the oocyte. Thus, in hCG-treated cows, estradiol could possibly delay onset of oocyte maturation for a longer period of time until concentrations decrease to basal levels. Even when the effects of steroids prior to, and during oocyte maturation are evident, it seems plausible that the dynamics of steroid secretion in preovulatory follicles (decrease in estradiol and increase in progesterone), instead of the effect of a specific steroid, may be responsible for triggering resumption of oocyte maturation. This is supported by the in vitro studies of Wang et al., (2006) who reported that bovine oocyte maturation can be prevented by addition of a steroid synthesis inhibitor to COC culture media; whereas, addition of either progesterone or estradiol without resembling the dynamics of steroids in vivo after the gonadotropin surge failed to initiate oocyte maturation. Whether or not changes in serum estradiol after hCG administration affect oocyte maturation can not be concluded from the current study since oocyte maturation was not evaluated.
The onset of oocyte maturation in relation to the gonadotropin surge is of particular interest for interpretation of the results in the present study given the clear differences observed in timing to reach serum LH and hCG maximum concentrations. Whereas LH peaked in average 1.8 h after GnRH injection and returned to baseline levels by 4 to 5 h, in agreement with other reports using Holstein cows (Kadokawa et al., 1998; Martinez et al., 2003), hCG maximum concentrations were not reached until after 9.0 h of administration. It is uncertain how this clear difference in hormone dynamics may have affected the time course of oocyte maturation since it was not analyzed in the present study. Unfortunately, there is limited available information on hCG dynamics and the relationship between hCG concentrations and onset of oocyte maturation. Schmitt et al. (1996) reported hCG maximum concentrations 6 h after administration of 3000 IU of hCG (1000 IU i.v. and 2000 IU i.m.) with a decay in concentrations by 12 h post injection; however, neither ovulation time nor oocyte maturation was assessed. In the current study, the average time to reach maximum concentrations after hCG administration for hCG-treated cows was 9.0 h which differed in 3 h from the previous report of Schmitt et al. (1996). Differences in route of hCG administration and sampling frequency between experiments (every 2 h in the present study and every 6 h in Schmitt et al., 1996) are the most likely reasons for disparity in time to maximum concentrations. However, both studies agree that hCG remains above baseline levels for a prolonged period of time, 30 h in the present study and 66 h in Schmitt et al. (1996). Results of the present study also indicate that cows administered hCG to induce ovulation (49 h after CIDR
removal) do not present an endogenous LH surge. Concentrations of LH in cows receiving hCG stayed at baseline levels during the entire sampling period after hCG administration. The most likely explanation is that hCG triggered the shift in steroids secretion decreasing the production of estradiol-17β hence, there was not sufficient estradiol-17β in the system to trigger an endogenous LH surge. As a consequence, it is logical to consider that in hCG treated cows ovulation was the result of hCG stimulation of the follicle without any involvement of LH.

When compared from CIDR removal or GnRH/hCG administration, differences in ovulation time among treatments were opposite to those observed from time of gonadotropin maximum concentrations (peak). Cows administered GnRH presented ovulation ~ 3.3 h earlier than hCG-treated cows whereas, when measured from the peak, ovulation occurred ~ 4.3 h earlier in hCG-treated cows. Unlike the discrepancies observed for time to ovulation in cows treated with either GnRH or hCG, cows receiving Folltropin-V ovulated earlier independently of analyzing ovulation time from GnRH administration or the respective peaks of LH/hCG. Cows receiving the FSH-GnRH combination presented the earliest ovulation which was ~ 3.5 h earlier after GnRH injection than in SAL-GnRH. Taken together these observations indicate that if ovulation time is to be measured from the time of induction of ovulation, GnRH may induce earlier ovulation than hCG specially when combined with Folltropin-V given 49 h earlier.

Despite clear variation in timing to ovulation across treatments, a high synchrony in time of ovulation was observed among treatments with no more than 5 h difference in the group presenting the widest range of ovulation times
measured from GnRH/hCG. Other studies using synchronization of the ovulation protocols in dairy cattle have reported greater differences in timing of ovulation (Pursley et al., 1995; Peters and Benboulaid 1998; Tenhagen et al., 2003). The good synchrony of ovulation in our study may be attributed to the pre-synchronization of estrus which resulted in the initiation of a new follicular wave in most of the cows after treatment with GnRH and CIDR insertion at Day 0 of the synchronization of ovulation protocol. Most cows included in the experiment presented a dominant follicle responsive to LH (evidenced by ovulation and formation of a new CL after treatment) at the time of CIDR insertion and GnRH administration. Consequently, cows responded with ovulation and beginning of a new follicular wave 48 to 72 h after the day of CIDR insertion and GnRH administration. Our results are consistent with previous reports of high incidence of ovulation in response to GnRH administration between days 5 to 9 of the estrous cycle in lactating dairy cows (Vasconcelos et al., 1999).

The regression analysis performed to determine the relative importance of parameters measured during the experimental protocol indicated that the time elapsed from the administration of GnRH or hCG to the respective LH or hCG maximum concentrations was the most relevant factor in determining the time of ovulation. Specifically, the model showed that the longer the time to reach maximum concentrations, the longer the time from injection to ovulation which is in agreement with the observation that hCG treated cows presented the latest ovulation. In addition, size of the ovulatory follicle at the time of GnRH/hCG administration as well as the time to estradiol maximum concentration was related
to time of ovulation. According to the regression analysis, ovulation time was shorter when follicles were larger and the estradiol maximum concentration was reached earlier. These results suggest that differences may have occurred in ovulation time if follicles would have been of different sizes at the time of GnRH/hCG. However, as the size of follicles in all groups were similar in the present study is not possible to draw any conclusion on this regard.

Animal and production traits were similar among cows assigned to Folltropin-V and Saline, or GnRH and hCG treatments indicating that a homogenous group of cows was utilized for the experiment. The differences observed in DIM for the treatment combinations are not concerning since the largest difference between groups was ~ 17 days which is not expected to have a significant effect on the values of the variables of interest. Moreover, prior to application of Folltropin-V or Saline, cows were similar in endocrine status (progesterone and estradiol-17β). Concentrations of progesterone were consistent across treatment groups during the period of CIDR insertion as well as the slopes of progesterone decrease after CIDR removal and PGF$_{2α}$ administration. Concentrations of progesterone decreased to subluteal levels (< 1 ng/mL) between 24 to 36 h after PGF$_{2α}$ administration in agreement with other studies applying the same PGF$_{2α}$ analogue to dairy cattle (Hittinger et al., 2004; Starbuck et al., 2006) and in cows receiving a gonadotropin preparation (FSH and LH) at the time of PGF$_{2α}$ administration (Stevens et al., 1993). Therefore, it is unlikely that any of the differences observed in follicular dynamics and timing of ovulation are
associated with concentrations of progesterone during the whole experiment or estradiol from CIDR insertion to removal.

Contrary to what would be expected in animals receiving supplemental gonadotropins (Folltropin-V), results of estradiol-17β analysis suggest that Folltropin-V treatment was responsible for lower concentrations of this hormone. Concentrations of estradiol-17β from time of follicular wave turnover to time of CIDR removal (prior to Folltropin-V administration) were similar in both treatment groups; however, estradiol-17β was reduced during the 48 h period following Folltropin-V treatment compared to controls (Saline). Under physiological conditions, FSH and LH stimulate estradiol secretion by the dominant follicle (Hunter 2003; reviewed by Lucy 2007); therefore, it is not clear why cows receiving these two gonadotropins in the present study had lower estradiol concentrations. A possible explanation includes premature luteinization of cells in the dominant follicle (proposed ovulatory follicle) in response to LH present in the gonadotropin preparation (Folltropin-V), as previously described for superovulated cattle (Boland et al., 1991). If this hold true, the amount of LH delivered to Folltropin-V treated cows was not sufficient to induce premature ovulation in the majority of animals treated; whereas, the quantity of LH was sufficient to trigger partial luteinization, consequently decreasing maximum serum concentrations of estradiol-17β.

Another particular aspect of the current study was the earlier ovulation of a subgroup of cows (FSH-EARLY) after Folltropin-V (FSH/LH) treatment and prior to GnRH/hCG administration. If considered from time of CIDR removal,
these cows presented the shortest time to ovulation (38.4 ± 1.2 h). A similar response to an FSH preparation was observed when given along with PGF$_{2\alpha}$ during early or mid diestrus (Day 8 and 10 of the estrous cycle; Stevens et al., 1996). In this study a subgroup of cows treated with a gonadotropin preparation (FSH/LH) presented ovulation within 48 h of treatment unlike the rest of the animals which ovulated between 2 to 5 days after treatment. However, in the study of Stevens et al., (1993) the gonadotropin preparation utilized was different to the preparation utilized in the current study with no mention to the LH content. In agreement with these observations (current study and Stevens et al., 1993), a 13% incidence of premature ovulations has been reported for cattle receiving gonadotropin treatment for superstimulation purposes (Callesen et al., 1987). Variability of LH content in different batches of gonadotropin preparations was previously reported (Murphy et al., 1984; Lindsell et al., 1986) and may account for these early ovulations (Callensen et al., 1987). In our study, cows in the Folltropin-V group received a single application of 4 ml Folltropin-V, equivalent to 80 mg of NIH-FSH-PI and approximately 12.8 mg of LH (16% LH content in Folltropin-V; Mapletonf et al., 2002). This amount is three times higher than the dose previously demonstrated to induce ovulation of follicles $\geq$ 12 mm in Holstein cattle (Sartori et al., 2001). Therefore, it was not surprising to observe premature ovulation in this subgroup of cows, since the average size of the ovulatory follicle was 13.9 mm at the time of CIDR removal. Unexpectedly, estradiol-17\(\beta\) concentrations in FSH-EARLY cows were lower than cows that ovulated during the expected time.
Because we were unable to find significant literature to draw conclusions about the specific portion (initial rise, maximum plasma level or decrease) of the gonadotropin surge responsible for triggering oocyte maturation, we have discussed our results from the timing of the LH/hCG maximum concentration (peak), GnRH/hCG administration and CIDR removal. Therefore, if oocyte maturation begins after hCG reaches maximum levels, this hormone must be considered as a possible therapeutic strategy to decrease the time from onset of maturation to release from the follicle in HS lactating cows. Indeed, synchronization of the ovulation protocols utilizing hCG to induce ovulation in dairy cattle during the summer months have been shown to improve cumulative pregnancy rates at 90 and 135 d when compared to AI at detected estrus (de Rensis et al., 2002). However, it can not be concluded from this study if hCG-treated cows had better pregnancy rates because of higher conception rates or just by increasing AI rate (hCG group was timed AI). Conception rates reported were averages for winter and summer, limiting possible comparison between treatments. In the same study, no differences were observed between a protocol using GnRH instead of hCG for synchronization of ovulation (de Rensis et al., 2002).

One of the working hypotheses of this study was that the presence of larger follicles at the time of an exogenously induced gonadotropin ovulatory surge may decrease time to ovulation and release of the oocyte. Furthermore, ovulation of larger follicles in lactating dairy cows may present other advantages. Previous studies have shown a positive relationship between larger size of the
preovulatory follicle and pregnancy success in dairy (Vasconcelos et al., 2001; Peters and Pursley 2003; Lopez et al., 2004) and beef cattle (Perry et al., 2007) induced to ovulate with hormonal treatments. Cows ovulating larger follicles have larger CL’s (Vasconcelos et al., 2001) and higher pregnancy rates than cows ovulating smaller follicles (Stevenson et al., 2006; Perry et al., 2007). Conversely, other studies have shown a negative relationship between greater preovulatory follicle size and pregnancy rates as well as maintenance of pregnancy in dairy cattle (Vasconcelos et al., 1999).

The overall size of follicles at the time of GnRH/hCG in our study (17.4 mm) was slightly larger than the size of preovulatory follicles in cows ovulating spontaneously after estrus (16.6 mm) and even larger than preovulatory follicles of cows included in different synchronization of ovulation protocols (~ 15.4 mm) in the same experiment (Cartmill et al., 2001). However, size of follicles in Folltropin-V and Saline-treated groups did not differ at time of GnRH/hCG indicating no differences in growth after Folltropin-V treatment. Only those cows presenting premature ovulation (FSH-EARLY) seemed to ovulate smaller follicles (already disappeared at time of GnRH/hCG) when compared to the other groups. The ovulatory follicle in this group had lower total growth when compared to the other treatments as a result of the shorter growth period. In addition, the FSH-EARLY group presented a clearly shorter time from CIDR removal to ovulation. However, timing of ovulation should not be compared between FSH-EARLY and the rest of treatment combinations because it is likely that these cows ovulated in response to the LH present in Folltropin-V unlike the
remainder of the cows which ovulated in response to the ovulatory treatment (GnRH/hCG) administered 49 h later.
CHAPTER 6
SUMMARY AND CONCLUSIONS

These data imply that the time elapsed from the preovulatory gonadotropin maximum concentration to ovulation can be modified through the application of a therapeutic model capable of manipulating growth of follicles and induce ovulation in lactating dairy cows. Combination of Folltropin-V (FSH/LH) at the time of CIDR removal and PGF$_{2\alpha}$ administration with hCG to induce ovulation, produced the shortest period of time from gonadotropin maximum concentration (peak) to ovulation. This treatment resulted in $\sim$ 6.5 h difference in time to ovulation measured from hCG maximum concentration when compared to cows induced to ovulate with GnRH (SAL-GnRH). Comparisons of timing of ovulation are made with cows induced to ovulate with GnRH (after synchronization of follicle growth with CIDR and GnRH), since this treatment is widely accepted and utilized in dairy cattle. However, most of the difference in ovulation time can be attributed to the different blood dynamics of hCG, since it reached maximum blood levels $\sim$ 9 h after intramuscular administration. The potential success of a therapeutic combination such as Folltropin-V and hCG to improve conception rates of lactating dairy cows suffering HS is uncertain and depends on a divergence between the mechanism responsible for the onset of follicle wall breakdown and the onset of oocyte maturation. Therefore, future research is warranted to determine if onset of oocyte maturation in cattle treated with hCG is delayed until hCG reaches blood maximum levels or if maturation is initiated as a
result of the rise in blood hCG concentrations. Since our experimental design was only intended to evaluate timing of ovulation, not oocyte maturation, further inference is limited.

When time to ovulation was measured from administration of GnRH/hCG, Folltropin-V and GnRH treated cows presented the shortest time to ovulation stressing the fact that Folltropin-V treatment had an effect in time to ovulation. In addition, earlier ovulation in FSH-GnRH group when assessed from GnRH/hCG indicates that the dynamics of hCG was responsible for the shorter time to ovulation when measured from the maximum concentration of gonadotropins.

Another particular aspect of this study was the premature ovulation of a subgroup of cows following Folltropin-V administration and the lower production of estradiol-17β in FSH-treated cows. Therefore, if Folltropin-V is to be used in estrus synchronization protocols, the dose may need adjusting to avoid these premature ovulations and decreased estradiol-17β secretion.

In conclusion, we demonstrated that the time elapsed from gonadotropin maximum concentration to ovulation can be modified in lactating dairy cows; however, further research is necessary to determine the exact timing of oocyte maturation in hCG-treated cows under non-heat stress and heat stress conditions.
LIST OF REFERENCES


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VITA

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