

MANIPULATING THE OVULATORY FOLLICLE: IMPACTS ON REPRODUCTIVE
TISSUE GENE EXPRESSION AND FERTILITY OF DAIRY COWS

By

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To my parents for their perseverance and endless support

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LIST OF ABBREVIATIONS

3 β -HSD	3-beta-hydroxysteroid dehydrogenase / Δ^5, Δ^4 isomerase
AI	artificial insemination
ANOVA	analysis of variance
AOR	adjusted odds ratio
Bax	B-cell lymphoma 2 associated X protein
Bcl-2	B-cell lymphoma 2
BCS	body condition score
bFGF	basic fibroblast growth factor
bTP-1	bovine trophoblast protein-1
BMP	bone morphogenic protein
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CI	confidence interval
CIDR	controlled internal drug release
CL	corpora lutea / corpus luteum
COX	cyclooxygenase
Ct	comparative threshold
CV	coefficient of variation
DAG	1,2-diacylglycerol
DIM	days in milk
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid

EGA	embryonic genome activation
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
FW	first follicular wave of the estrous cycle
GDF	growth differentiating factor
GH	growth hormone
GlyCAM-1	glycosylated cell adhesion molecule 1
GnRH	gonadotropin-releasing hormone
GnRH1	the first injection of gonadotropin-releasing hormone of a protocol for synchronization of the estrous cycle and timed artificial insemination
hCG	human chorionic gonadotropin
HDL	high-density lipoprotein
HGF	hepatocyte growth factor
ICM	inner cell mass
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding proteins
INF- τ	interferon-tau
IP ₃	inositol 1,4,5-trisphosphate
IU	international units
LDL	low-density lipoprotein
LH	luteinizing hormone
LU	laboratory units of antiviral activity
mRNA	messenger ribonucleic acid
MSY2	multifunctional Y-box protein 2

MDBK	Madin-Darby Bovine Kidney
mTOR	mammalian target of rapamycin
OCT	optimal cutting temperature
oTP-1	ovine trophoblast protein-1
P450arom	cytochrome P450 aromatase
P450c17	cytochrome P450 17-alpha-hydroxylase
P450scc	cytochrome P450 side-chain cleavage
P/AI	pregnancy per artificial insemination
PAPP-A	plasma-associated pregnancy protein-A
PGF _{2α}	prostaglandin F2-alpha
PGFM	15-keto-13,14-dihydro-prostaglandin F2-alpha
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
qRT-PCR	quantitative real-time reverse transcription polymerase chain reaction
RIA	radioimmunoassay
RNA	ribonucleic acid
ROC	receiver operating characteristic
RT	reverse transcriptase
SPP1	secreted phosphoprotein 1 or oestopontin
StAR	steroidogenic acute regulatory protein
SW	second follicular wave of the estrous cycle
TBP	TATA box-binding protein
TMR	total mixed ration
VEGF	vascular endothelial growth factor

VSV	vesicular stomatitis virus
VWP	voluntary waiting period

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By

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Experiments were conducted to further elucidate the effects of the environment on which the ovulatory follicle develops and its relationship with the reduction of fertility observed in anovular dairy cows, and to evaluate alternative management programs that incorporate this knowledge to improve reproductive performance of dairy cows.

In the first study, data from 5,607 lactating cows enrolled in synchronization programs for artificial insemination (**AI**) were evaluated. Cows had blood analyzed for progesterone 7 to 14 days apart, with the second sample collected on the day of the first injection of gonadotropin-releasing hormone (**GnRH1**). Cows were classified as cyclic if progesterone ≥ 1 ng/mL in at least one of the two samples, and anovular if both samples were < 1.0 ng/mL. Cyclic cows were categorized as low (**CLOW**; < 1.0 ng/mL) or high (**CHIGH**; ≥ 1.0 ng/mL) based on progesterone concentration at GnRH1, which would result in ovulation of the dominant follicle of the first (**FW**) and second (**SW**) follicular wave, respectively, at AI. Pregnancy per AI (**P/AI**) on day 30 after AI was greater for CHIGH than anovular and CLOW cows (43.0 vs. 29.7 vs. 31.3%, respectively). Short inter-AI intervals differed among groups and were 7.1, 11.9, and 15.7% for CHIGH, anovular and CLOW, respectively. Pregnancy loss differed only

between anovular and CLOW (10.0 vs. 15.0%), and it was intermediate for CHIGH (13.5%). In a second experiment, 220 cyclic Holstein cows received 2 injections of prostaglandin (**PG**) $F_{2\alpha}$ administered 14 days apart. The Ovsynch protocol (day 0 GnRH, day 7 $PGF_{2\alpha}$, day 9 GnRH, day 9.5 timed AI) was initiated either 3 or 10 days after the second $PGF_{2\alpha}$ to result in AI to the FW or SW dominant follicles. Blood was analyzed for progesterone and ovaries were scanned to determine ovulatory responses and follicle diameter. The proportion of cows with progesterone > 1.0 ng/mL at GnRH1 was 9.8 and 97.2% for FW and SW, respectively. Concentrations of progesterone at GnRH1 and $PGF_{2\alpha}$ injections of the Ovsynch protocol were greater for SW than FW. Pregnancy per AI was greater for SW than FW (41.7 vs. 30.4%) despite less ovulation to GnRH1 in SW than FW (78.7 vs. 88.3%). Results indicate that follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows.

Objectives of the second study were to investigate 2 intervals from induction of ovulation to AI in synchronization programs, and the effect of supplemental progesterone for resynchronization on fertility of lactating dairy cows subjected to a 5-day timed AI program. In experiment 1, 1,227 Holstein cows had their estrous cycle presynchronized with 2 injections of $PGF_{2\alpha}$ at 46 and 60 days in milk (**DIM**). Timed AI protocols were initiated with GnRH at 72 DIM, followed by 2 injections of $PGF_{2\alpha}$ at 77 and 78 DIM and a second injection of GnRH at either 56 (**OVS56**) or 72 hours (**COS72**) after the first $PGF_{2\alpha}$ of the timed AI protocols. All cows received timed AI at 72 hours after the first $PGF_{2\alpha}$. The proportion of cows detected in estrus at AI was greater for COS72 than OVS56 (40.6 vs. 32.4%); however, P/AI on day 32 after AI did not differ between OVS56 (46.4%) and COS72 (45.5%). In experiment 2, 675 nonpregnant

Holstein cows had their estrous cycle resynchronized starting at 34 days after the first AI. Cows received the OVS56 with (**RCIDR**) or without (**RCON**) supplemental progesterone from GnRH1 to the first PGF_{2α}. Subsets of cows had their ovaries scanned by ultrasonography at GnRH1, the first PGF_{2α}, and second GnRH injections. Blood was analyzed for progesterone on the day of AI and 7 days later. Cows were considered to have a synchronized ovulation if progesterone was < 1 ng/mL and > 2.26 ng/mL on the day of AI and 7 days later, respectively, and if no ovulation was detected between the first PGF_{2α} and second GnRH injections. Cows supplemented with progesterone had greater P/AI compared with unsupplemented cows (51.3 vs. 43.1%). Premature ovulation tended to be greater for RCON than RCIDR cows (7.5 vs. 3.6%), although synchronization of the estrous cycle after timed AI was similar between treatments. Results indicate that timing of induction of ovulation with GnRH relative to insemination did not affect P/AI of dairy cows enrolled in a 5-day timed AI program, and that supplementation with progesterone improved P/AI in cows subjected to the 5-d timed AI protocol during resynchronization starting on day 34 after the first AI.

In the third study, the effects of wave of the ovulatory follicle and concentrations of progesterone during follicular growth on reproductive responses of dairy cows were evaluated. Non-pregnant and non-lactating Holstein cows had their estrous cycle presynchronized with an injection of GnRH and a controlled internal drug-release (**CIDR**) insert containing progesterone, followed 7 days later by CIDR removal and 2 injections of PGF_{2α} 24 hours apart. All cows received an injection of GnRH 1 day after the second PGF_{2α}, which was the first GnRH of the synchronization protocol (day -9 GnRH1, day -2 and day -1 PGF_{2α}, day 0 GnRH) for cows induced to ovulate a FW

follicle (**FW**, n = 13) or a FW follicle supplemented with progesterone (**FWP4**, n = 8). Cows induced to ovulate a SW follicle (**SW**, n = 12) received the GnRH1 on day 6 of the new estrous cycle. Cows in FWP4 received 3 CIDR inserts at 12, 24 and 48 hours after the GnRH1 (day -9). Inserts were removed at the injection of PGF_{2α} (day -2). Cows were inseminated at the final GnRH (day 0) and again 16 hours later. Cows were killed on day 17 after the final GnRH. Progesterone concentration during follicular growth was greater for SW, intermediate for FWP4 and lesser for FW. Cows in FW ovulated larger follicles, which resulted in larger corpora lutea (**CL**) and greater concentrations of progesterone from days 4 to 16 of the estrous cycle after AI. The relative mRNA abundance for steroidogenic acute regulatory protein (**StAR**) was greater for FW than for other treatments. Nonetheless, the ratio between circulating progesterone and CL volume was not affected by treatment. A greater proportion of FW cows had a peak concentration of estradiol on the day before the final GnRH in comparison to other treatments. Maximum concentration of estradiol was greater for FW than for FWP4 and SW. The proportion of pregnant cows on day 17 tended to be less for FW than for FWP4 and SW. The length of conceptuses did not differ among treatments. There was a trend for SW conceptuses to produce less INF- τ ; however, no differences in the relative abundance of INF- τ mRNA were detected. Ovulation of FW follicles reduced fertility in dairy cows and this negative effect was mediated by suboptimal concentrations of progesterone during ovulatory follicle growth. Furthermore, luteal function during early gestation and capability of elongated conceptuses to produce IFN- τ were not involved with reduction of fertility in cows that ovulated FW follicles.

CHAPTER 1 INTRODUCTION

Resumption of ovulation postpartum in dairy cows is affected by a multitude of factors, such as health disorders and nutritional status during early lactation (Walsh et al., 2001; Santos et al., 2009). Studies in the US have shown that 24.1% of the lactating dairy cows have not resumed ovulation by 65 days postpartum, with within herd prevalence of anovular cows of 18.6 up to 41.2% (Santos et al., 2009). This is particularly relevant because this period coincides with the end of the voluntary waiting period (**VWP**) in most of commercial dairy herds. The negative impact of anovulation on fertility responses is well documented. Anovular cows are less likely to be detected in estrus, which clearly reduces their chance to be inseminated in herds that adopted traditional reproductive management systems. Protocols for synchronization of ovulation and timed artificial insemination (**AI**) allow for the insemination of anovular cows (Chebel et al., 2006); however, independent of the method of insemination, anovular cows have reduced pregnancy per AI (**P/AI**) and are at a greater risk of losing their pregnancies compared with cyclic cows (Gümen et al., 2003; Santos et al., 2004c; Santos et al., 2009).

It has been suggested that the mechanisms involved with the reduced fertility of anovular cows is the lack of exposure to progesterone in the postpartum period up to the first insemination (Crowe, 2008). Nonetheless, recent studies have suggested that even a short term exposure to reduced concentration of progesterone during the development of the ovulatory follicle is capable of modulating fertility of dairy cows. Induction of low concentrations of progesterone during follicular growth altered the morphology of uterine glands on the subsequent cycle (Shaham-Albalancy et al., 1997),

enhanced oxytocin-induced release of prostaglandin (**PG**) $F_{2\alpha}$ (Shaham-Albalancy et al., 2001; Cerri et al., 2008a), and increased the proportion of cows with short luteal phase (Cerri et al., 2008a). Similarly, the first wave (**FW**) dominant follicle grows under lower systemic concentrations of progesterone than the second wave (**SW**) follicle. Therefore, it is expected that cows induced to ovulate the FW dominant follicle would have reduced fertility compared with cows that ovulate SW dominant follicles. The ovulatory response to the first gonadotropin-releasing hormone (**GnRH1**) is high in anovular cows (Gümen et al., 2003), which synchronizes the emergence of the FW. Therefore, when subjected to GnRH-PGF $_{2\alpha}$ -based synchronization programs, anovular cows ovulate the FW at AI. Collectively, these observations indicate that the ovulation of a FW follicle at AI might reduce fertility responses of dairy cows and, it is likely that, follicular wave of the ovulatory follicle and the inherit low concentration of progesterone during the development of the FW dominant follicle are part of the of the mechanisms that lead to poor fertility in anovular cows.

Systematic breeding programs based on protocols for synchronization of ovulation and timed AI have been shown to maximize submission of eligible cows to AI without decreasing P/AI or altering the risk of pregnancy loss (Pursley et al., 1997b; Chebel et al., 2006). It has been demonstrated that prolonged periods of follicular dominance reduced embryo quality (Cerri et al., 2009). Accordingly, Santos et al. (2010) found that reducing the interval between the GnRH1 and the injection of PGF $_{2\alpha}$ from 7 to 5 days in a timed AI protocol, thus shortening the period of dominance by 2 days, improved P/AI in lactating dairy cows. Nonetheless, it also reduced ovulatory diameter, estradiol concentrations in plasma and the proportion of cows detected in estrus on the day of AI,

which have been shown to be associated with fertility in previous studies (Vasconcelos et al., 2001; Lopez et al., 2007). In beef cows, restricting follicular dominance was beneficial only when the period of proestrus was extended from 60 to 72 hours (Bridge et al., 2008). Taken together, these results suggest that, in a 5-day timed AI protocol, the ovulatory follicle might need additional time to mature. The administration of the final GnRH 16 hours before AI resulted in the greatest P/AI in a 7-day protocol (Pursley et al., 1998; Brusveen et al., 2008); however, a delay of the ovulatory stimulus might be beneficial in the 5-day protocol.

The stage of the estrous cycle on which the synchronization protocol is initiated affects the response to hormonal treatments, overall synchrony and subsequent fertility (Vasconcelos et al., 1999). Programs of presynchronization conveniently allow for the initiation of the timed AI protocol during early diestrus and enhanced P/AI of dairy cows (Moreira et al., 2001; El-Zarkouny et al., 2004). Nevertheless, presynchronization is only suitable for the first AI postpartum because it would delay re-insemination if used in subsequent services. Supplementation with exogenous progesterone reduces the pulse frequency of luteinizing hormone (**LH**) and prevents ovulation (Rathbone et al., 2001). It also serves to boost concentrations of progesterone in blood of cows with low endogenous concentrations. Therefore, it becomes an attractive alternative to improve synchrony of ovulation and to increase progesterone concentrations during development of the ovulatory follicle during resynchronized inseminations.

Reproductive physiology of dairy cows, aspects of early embryonic development, and current status of programs for synchronization of ovulation and timed AI were reviewed in Chapter 2. Chapters 3 through 5 describe original research on reproductive

biology of dairy cows. The study presented in Chapter 3 describes the effects of cyclic status and wave of the ovulatory follicle on fertility responses of lactating dairy cows. Chapter 4 provides further understanding on the mechanisms associated with luteal function and embryonic development that underlies the changes in fertility observed following the ovulation of FW and SW dominant follicles in dairy cows. Finally, Chapter 5 describes the effects of timing of AI in relation to induction of ovulation using GnRH on P/AI and risk of pregnancy loss in dairy cows. It also describes the effects of supplemental progesterone during resynchronization on subsequent synchrony of the estrous cycle and fertility responses of lactating dairy cows.

CHAPTER 2 LITERATURE REVIEW

The Estrous Cycle of Dairy Cows

Early studies on the duration of the estrous cycle in dairy cows reported a modal length of 22 days and that 82.8% of the inter-estrus intervals ranged from 17 to 26 days (Olds and Seath, 1951). The use of ultrasonography to access ovarian dynamic has somewhat confirmed previous results. Evaluation of non-inseminated lactating dairy cows resulted in an average length of the estrous cycle, defined as the interval between two consecutive ovulations, of 23 days (Savio, et al., 1990; Sartori et al., 2004).

The estrous cycle can be divided into four periods, namely proestrus, estrus, metestrus, and diestrus (Senger, 2003a; Peter et al., 2009). The proestrus initiates with structural and functional regression of the corpora lutea (**CL**) and is characterized by increasing synthesis of estradiol by the pre-ovulatory follicle (Wettermann et al., 1972). Estradiol concentrations in plasma of lactating dairy cows rise from approximately 2.5 pg/mL on the day of luteolysis to 7.9 pg/mL during estrus (Sartori et al., 2004). The maximal concentration of estradiol preceding ovulation reported for lactating dairy cows was less than that observed in dairy heifers (9.7 to 11.3 pg/mL; Wettermann et al., 1972; Sartori et al., 2004) or non-lactating cows (16.7 pg/mL; De La Sota et al., 1993). Elevated concentrations of estradiol in the absence of progesterone act on the mediobasal hypothalamus to induce sexual behavior (Blache et al., 1991; Allrich, 1994), which determines the end of proestrus and beginning of estrus (Senger, 2003a; Peter et al., 2009).

The period of estrus extends from the onset of sexual receptivity to the ovulation of the dominant follicle (Senger, 2003a; Peter et al., 2009). Gonadotropin-releasing

hormone (**GnRH**) is produced by neurons in the arcuate nucleus region of the hypothalamus and by the pre-optic chiasmatic area. The neurons located in the arcuate nucleus are responsible for the tonic release of GnRH, whereas those in the pre-optic chiasmatic area (pre-optic nuclei, anterior hypothalamic area, and supra-chiasmatic nucleus) are responsible for the large surges in GnRH release. During estrus, the elevation in ovarian estrogens induces the pre-ovulatory surge of GnRH from the surge center in the hypothalamus. The release of GnRH, in turn, stimulates the pre-ovulatory release of luteinizing hormone (**LH**) by the pituitary gland (Hafez et al., 2008). The pre-ovulatory surge of LH is detected approximately 2.8 hours after the onset of estrus and is followed by ovulation roughly 22 hours later (Chenault et al., 1974). An abrupt increase in the circulating concentrations of estradiol (from 6 to 7.4 pg/mL) was observed concurrently with the pre-ovulatory surge of LH. After the peak, estradiol declined 50% within the first 5 hours and returned to basal concentrations by 14 hours (Chenault et al., 1974). The characteristics of estrus in dairy cows have been evaluated using HeatWatch[®], a radiotelemetric system for estrous detection (Walker et al., 1996; Xu et al., 1998; Lopez et al., 2002; Dransfield et al., 2008). The average duration of the estrus was 7.2 hours and it ranged from 3.6 to 9.5 hours, which is slightly shorter than previously reported (10 hours; Chenault et al., 1974). Cows were mounted 8.5 times per period of estrus (4.3 to 11.2 mounts/estrus) and the average duration per mount was 2.8 seconds (2.4 to 3.4 seconds/mount). It has been demonstrated that duration of estrus is negatively associated with milk yield (Lopez et al., 2004). The average length of estrus of high producing Holstein cows housed in a free-stall barn was 8.7 hours and

it was shorter for cows with milk production above the average (≥ 39.5 kg/day, 6.2 hours) than for cows with production below the average (< 39.5 kg/day, 10.9 hours).

Proestrus and estrus compose the follicular phase of the estrous cycle, which generally ranges from 2 to 5 days (Savio et al., 1990; Sartori et al., 2004) and represent 20% of the duration of the entire estrous cycle (Peter et al., 2009). Longer follicular phases have been reported for cows on which the dominant follicle present on the day of luteolysis fails to ovulate (Sartori et al., 2004). For those cows, the ovulatory wave emerged after the regression of the CL and the follicular phase length averaged 14 days. Interestingly, cows were detected in estrus in the presence of the anovulatory follicle, in other words, they displayed estrus but did not ovulate.

In contrast to the follicular phase, the luteal phase of the estrous cycle is characterized by the presence of a functional CL. The pre-ovulatory surge of LH induces structural and functional changes of the granulosa cells of the pre-ovulatory follicle called luteinization, which support further development after ovulation and formation of the CL. The period from ovulation to the presence of a mature CL capable of producing large amounts of progesterone is called metestrus (Peter et al., 2009). The first significant increase in circulating concentrations of progesterone is detected on day 4 after ovulation (Stabenfeldt et al., 1969), an event that terminates the metestrus. During diestrus, the concentration of progesterone increases rapidly from days 4 (1.5 ng/mL) to 8 of the estrous cycle (5.5 ng/mL; Stabenfeldt et al., 1969). After this point, progesterone concentration increases at a slower rate and reaches its maximum value on day 16 of the estrous cycle at approximately 7 ng/mL (ranging from 6.1 to 10.2 ng/mL; Stabenfeldt et al., 1969). These absolute values change considerably with

lactational status and level of milk production because of the increased catabolic rate of ovarian steroids in the liver as dry matter intake increases (Sartori et al., 2004). Non-pregnant cows undergo spontaneous luteolysis between days 17 and 19 of the estrous cycle in response to the pulsatile release of prostaglandin (**PG**) $F_{2\alpha}$ by the endometrium (Kindahl et al., 1976a; Savio et al., 1990; Sartori et al., 2004).

Follicular Growth and Oocyte Development

The development of ovarian follicles in ruminants begins during fetal life and is a multifaceted process controlled at the endocrine, paracrine and autocrine levels (McNatty et al., 2007). Bovine follicles have been classified according to the stage of development into 6 types, namely primordial (type 1), transitory (type 1+), primary (type 2), small pre-antral (type 3), large pre-antral (type 4), and small antral follicles (type 5; Braw-Tal and Yossefi, 1997). Primordial follicles (< 40 μm) are composed by the oocyte (29.7 μm) surrounded by a single layer of flattened granulosa cells (< 10 cells at the largest cross-section). In the transitory stage, multiplying granulosa cells begin to become cuboidal. Primary follicles (40 to 80 μm) are composed of an oocyte (31.1 μm) surrounded by 1 or 1.5 layers of cuboidal granulosa cells (10 to 40 cells at the largest cross-section). Small pre-antral follicles (81 to 130 μm) are composed of an oocyte (49.5 μm) surrounded by 2 to 3 layers of cuboidal granulosa cells (41 to 100 cells at the largest cross-section). Large pre-antral follicles (131 to 250 μm) are composed of an oocyte (68.6 μm) surrounded by 4 to 6 layers of cuboidal granulosa cells (101 to 250 cells at the largest cross-section). Finally, small antral follicles (250 to 500 μm) are composed of an oocyte (92.9 μm) surrounded by more than 6 layers of cuboidal granulosa cells (> 250 cells at the largest cross-section) and presence of an antrum. The zona pellucida first appears in small pre-antral follicles in a sparse fashion,

becoming a complete ring around the oocyte only in large pre-antral follicles. A distinguishable theca interna layer was present in some of the large pre-antral and in all small antral follicles. The first significant change in oocyte diameter was observed in small pre-antral type 3 follicles with 41 to 60 granulosa cells in the largest cross-section.

Expression of several growth and differentiating factors, such as bone morphogenic protein 6 (**BMP-6**) and growth differentiating factor 9 (**GDF-9**), can be detected at the primordial stage (McNatty et al., 2007). The underlying mechanism that activates primordial follicles to start developing is not fully understood. Culture of bovine ovarian cortex explants suggested that a paracrine communication with the ovarian stroma is required to prevent activation of primordial follicles and control the pace of follicular development (Fortune et al., 1998). Nevertheless, results from *in vitro* studies demonstrated that primordial follicles entered the growing phase without gonadotropic stimulus (Braw-Tal and Yossefi, 1997; Fortune et al., 1998), which is in agreement with the absence of receptors for follicle stimulating hormone (**FSH**) and LH in primordial follicles (McNatty et al., 2007). With the advance to the primary stage, the ovine follicle becomes responsive to progestins, estrogens, androgens, and gonadotropins; however, an acute control by FSH/LH such that observed in antral follicles is still absent.

Furthermore, copious factors involved in the control of follicular growth begin to be synthesized by the oocyte (BMP-6, BMP-15, GDF-9), granulosa (inhibin, follistatin), and theca cells (transforming growth factor β 1 and 2, progestins, androgens; McNatty et al., 2007). Interaction between GDF-9 and BMP-15 produced by the oocyte induces cell proliferation and controls responsiveness to gonadotropins in a spatial-dependent manner because cells that diverge to become the cumulus and mural granulosa are

affected differently (McNatty et al., 2007). This interaction is critical for the development of ovarian follicles in ruminants as immunization of ewes against GDF-9 and BMP-15 resulted in impaired ovulation and subsequent luteal function (Juengel et al., 2002).

The transition from pre-antral to antral stages is characterized by a decline in somatic cell proliferation and enhanced differentiation into cells capable of responding to gonadotropins to produce increasing amounts of steroids (Webb and Campbell, 2007). Beginning of antrum formation was observed in follicles with 200 to 500 μm of diameter that had at least 250 granulosa cells in the largest cross-section, a clearly defined theca interna, and an oocyte surrounded by a thick zona pellucida (Turnbull et al., 1977; Braw-Tal and Yossefi, 1997). Development of antral follicles in cows occurs in a wave fashion, with the development of 2 to 3 follicular waves per estrous cycle (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989). The emergence of follicular waves was detected on days -0.2 and 9.6 relative to previous ovulation in heifers that had 2 follicular waves in an estrous cycle, compared with days -0.5, 9.0 and 16.0 in heifers that had 3 follicular waves (Ginther et al., 1989). The emergence of follicular waves is preceded by a surge of FSH (Adams et al., 1982). Circulating concentrations of FSH began to increase 2 to 2.3 days before follicular wave emergence, whereas maximum concentrations were observed between 0.4 and 0.9 days before emergence.

The vast majority of recruited follicles will cease growing and become atretic, which allows for the specie-specific control of the final number of ovulatory follicles. In cows, generally only one follicle out of the developing cohort is selected to become dominant over its counterparts. The time of follicular deviation has been defined as the beginning of the difference in growth rates between the future dominant follicle and its

largest subordinate (Ginther et al., 1996a). In Holstein heifers, deviation occurs, on average, 2.8 days after the emergence of follicular wave, when the future dominant follicle reaches a diameter of 8.5 mm and is 1.3 mm larger than the largest subordinate follicle. The underlying mechanisms that lead to follicular deviation in cows involve a shift in primary gonadotropin dependency from FSH to LH by the dominant follicle, whereas the FSH-dependent subordinates are deprived of FSH. In fact, circulating concentrations of FSH declines as the cohort of selected follicles develops and become basal at the time of deviation (Ginther et al., 1996a). The suppression of FSH is mainly determined by the presence of dominant follicles. Castration of the dominant follicle induced a transient increase in circulating concentrations of FSH (Adams et al., 1992). Conversely, treatment of heifers with proteinaceous fraction of follicular fluid, presumably a source of inhibin, suppressed FSH concentrations during the treatment period (Adams et al. 1992). The inhibition of FSH by the dominant follicle appears to be selective and does not affect LH concentrations (Quirk and Fortune et al., 1986). In spite of a transient increase on the expression of FSH receptors in theca cells on day 4 after emergence, the expression of FSH receptors in granulosa and theca cells of growing follicles was somewhat constant and independent of changes in follicular diameter (Xu et al., 1995). Nevertheless, messenger ribonucleic acid (**mRNA**) encoding for LH receptors in theca cells increased with follicular size. Expression of LH receptors in granulosa cells was detected only in healthy follicles past the diameter of deviation (Xu et al., 1995a). Furthermore, mRNA expression of LH receptors in granulosa cells in the largest follicle occurred at a smaller diameter than differences in size or

intrafollicular estradiol concentrations between the largest compared to the second largest follicles (Beg et al., 2001).

Changes in the intrafollicular insulin-like growth factor (**IGF**) system have been suggested to trigger the variations in responsiveness to gonadotropins that lead to morphological and functional deviation. Supplementation with IGF-1 increased proliferation and production of estradiol by granulosa cell *in vitro* (Spicer et al., 1993; Gong et al., 1994). The same authors reported that IGF-1 acted in synergy with FSH to enhance steroidogenesis in granulosa cells. The bioavailability of IGF-1 and -2 *in vivo* is modulated by the presence of insulin-like growth factor binding proteins (**IGFBP**). Accordingly, follicular diameter and steroidogenic activity have been described to be negatively correlated with intrafollicular amounts of IGFBP-2, -4 and -5 *in vivo* (Echternkamp et al., 1994). Moreover, increased amounts of free IGF-1 associated with reduced concentrations of IGFBP were detected in the largest compared to the second largest follicles (Beg et al., 2001). Mihm et al. (2000) used an interesting approach to evaluate changes in intrafollicular factors associated with deviation in heifers. Follicular fluid was collected from the 3 largest follicles approximately 1 day after the emergence of the wave, therefore before deviation. Collection was performed surgically using a very thin needle (27-gauge) inserted approximately 2 mm below the interface of the surface of the follicle with the ovary to minimize leakage of intrafollicular fluid after needle withdrawal. The technique associated with the small amount of fluid collected (15 to 20 μ L) allowed follicular development and deviation following aspiration. The authors reported that, before morphological deviation, the cohort follicle which later became the dominant follicle already had reduced amounts of IGFBP-4 in the follicular

fluid. The control of intrafollicular IGFBP-4 in humans has been associated with the proteolytic activity of plasma-associated pregnancy protein-A (**PAPP-A**) secreted by granulosa cells (Conover et al., 2001).

On the day of deviation, the concentration of estradiol in the follicular fluid of the future dominant follicle was slightly, but not significantly greater than those of its largest subordinate (Ginther et al., 1997). Nevertheless, one day after deviation the estradiol concentration in follicular fluid of the dominant follicle was approximately 300-fold greater than that of its largest subordinate. The results from Ginther et al. (1997) agree with the pattern of expression of cytochrome P450 side-chain cleavage (**P450scc**), cytochrome P450 17-alpha-hydroxylase (**P450c17**), and cytochrome P450 aromatase (**P450arom**) in bovine follicles around the time of deviation (Xu et al., 1995b). From the emergence of the cohort to just before deviation, no changes in mRNA expression of P450scc and P450c17 were detected, whereas expression of P450arom was higher on day 2 after emergence than on day 0. Nonetheless, maximal expression for all 3 enzymes was observed following deviation. During diestrus, the presence of a functional CL and elevated circulating concentrations of progesterone prevent the increase in frequency of LH pulses released by the pituitary and the pre-ovulatory surge of gonadotropins. Because of inadequate LH support, the dominant follicle of non-ovulatory waves stops growing and undergoes atresia. On day 6 after emergence, expression of P450scc and P450c17 mRNA begins to decline (Xu et al., 1995b). Following the emergence of the second wave, dominant follicles from the first wave were at an advanced stage of atresia. By that time, P450scc and P450arom mRNAs were undetectable in granulosa cells, and very low P450scc and P450c17 mRNAs

expression could be detected in theca cells (Xu et al., 1995b). Conversely, the dominant follicle present at the time of luteolysis continues to grow and secretes increasing amounts of estradiol. In absence of elevated progesterone, follicular estradiol acts in the hypothalamus to induce a pre-ovulatory surge of GnRH, followed by a surge of gonadotropins by the pituitary that culminates with ovulation.

Formation of the CL, Luteal Function and Luteolysis

The CL is a transient gland that remains functional for 17 to 19 days for non-pregnant cows with regular estrous cycle (Kindahl et al., 1976a; Savio et al., 1990; Sartori et al., 2004). Its primary function is to synthesize progesterone, which is required for adequate control of the estrous cycle, development of the embryo/conceptus and maintenance of gestation.

The CL is formed following morphological and functional changes on the remaining cells of the ovulated follicle induced by the pre-ovulatory surge of LH, in a process entitled luteinization. The first event associated with CL formation is the shift from estradiol to progesterone production by luteal cells. Studies on granulosa and theca cells gene expression during the peri-ovulatory period and cell culture systems indicate that steroidogenesis is initially inhibited in a coordinated manner. The transport of cholesterol across the outer mitochondrial membrane remains unaffected during the late stages of development of the ovulatory follicle based on steroidogenic acute regulatory protein (**StAR**) mRNA abundance (Nimz et al., 2009). The expression of P450scc, 3-beta-hydroxysteroid dehydrogenase / Δ^5, Δ^4 isomerase (**3 β -HSD**), P450arom in the granulosa cells, and P450c17 and 3 β -HSD in the theca cells were extensively reduced within 18 hours of the pre-ovulatory surge of LH (Voss and Fortune, 1993a; Voss and Fortune, 1993b). As a result, concentrations of androstenedione,

testosterone and estradiol in the follicular fluid were reduced between 4 and 10 hours after the pre-ovulatory surge of LH (Komar et al., 2001). Accordingly, circulating concentrations of estradiol declined 50% within the first 5 hours of the pre-ovulatory surge of LH, and returned to basal values within the next 9 hours (Chenault et al., 1974). After 72 hours of culture, the expression of P450_{scc} and 3 β -HSD, but not P450_{c17} and P450_{arom}, was restored in follicular cells (Voss and Fortune, 1993a; Voss and Fortune, 1993b). These results are consistent with increasing production of progesterone *in vitro* by granulosa and theca cells (i.e.: luteinization) between 24 and 72 hours of culture not associated with an increase in androgen production (Voss and Fortune, 1993a; Voss and Fortune, 1993b).

The secretory function of the CL does not rely exclusively on the steroidogenic capacity of luteal cells, but also on the amount of steroidogenic tissue and the blood flow through the gland. It has been shown that ovulation of larger follicles results in subsequent formation of a larger CL which resulted in greater concentration of progesterone in serum of dairy cows (Vasconcelos et al., 2001), possibly because of increased number of secretory luteal cells. The CL is composed by small and large luteal cells, vascular tissue, immune cells and extracellular matrix (Farin et al., 1986). Large and small luteal cells originate from granulosa and theca cells, respectively (Alila and Hansel, 1984) and are responsible for the production of progesterone. In the sheep, large luteal cells range from 20 to 31 μ m in diameter and encompass only 4% of the total number of cells in a CL; however, they represent approximately 25% of the total volume of the gland and are responsible for 80 to 90% of the progesterone production (Fitz et al., 1982; Farin et al., 1986). Small luteal cells (16 to 18 μ m) represent 19% of

the total number of cells and 18% of the luteal volume. In spite of a greater basal steroidogenic capacity, large luteal cells are not stimulated by LH, whereas the production of progesterone by small luteal cells increased from 2.3 to 12.4-fold when cells were cultured in the presence of LH (Fitz et al., 1982). The absence of LH-induced progesterone production by large luteal cells is not caused by lack of LH receptors; however, large luteal cells from bovine CL had less binding sites per unit of area than small luteal cells (Chegini et al., 1991).

After ovulation, the remaining follicular tissue is extensively reorganized during CL formation in non-primates mammals. Electronic microscopy analysis of the rat CL revealed that membranes and interstitium are restructured so as to minimize transport distances (Dharmarajan et al., 1985). Consequently, approximately 60% of luteal cell surface directly faces a capillary, 37% faces interstitial spaces which probably extended to a capillary surface, and only 3% is in direct contact with a neighboring luteal cell. Another important feature of the developing CL is the fast rate of tissue growth and cellular proliferation that results in a 17.5-fold increase in luteal mass within a few days (Farin et al., 1986; Jablonka-Shariff et al., 1993). The number of large luteal cells remains somewhat constant throughout the development of the ovine CL; whereas their volume increases 3-fold between days 4 and 16 of the estrous cycle (Farin et al., 1986). During the same period, the total number of fibroblasts, small luteal cells, and endothelial cells increases 2.5, 3, and 6.5 times, respectively (Farin et al., 1986). Exposure to LH after ovulation is critical for CL development, cellular differentiation, and production of increasing amounts of progesterone in ruminants. Hypophysectomy of ewes on day 5 of the estrous cycle reduced the number of small luteal cells, fibroblasts,

capillary endothelial cells and pericytes per CL, as well as the diameter of steroidogenic and non-steroidogenic cells (Farin et al., 1990a). Consequently, hypophysectomized ewes had a 4-fold decrease in CL weight and content of progesterone per milligram of CL on day 12 of the estrous cycle. These changes are associated with reduced abundance of mRNA encoding for StAR, P450_{scc}, and 3 β -HSD (Juengel et al., 1995a; Juengel et al., 1995b). The same authors reported that the parameters mentioned above were either reestablished or improved in hypophysectomized ewes following supplementation with exogenous LH. The treatment of heifers with a GnRH antagonist from days 2 to 7 of the estrous cycle diminished LH pulsatility, reduced luteal function and compromised CL formation (Peters et al., 1994). This is in agreement with data from *in vitro* studies on which adding LH to the culture media increased the expression of P450_{scc} and 3 β -HSD in luteinized cells and the production of progesterone (Voss and Fortune, 1993).

The control of angiogenesis and angiolysis is crucial for CL development and maintenance. Nearly 45% of the ovine CL is composed of non-steroidogenic tissue (Farin et al., 1986). The establishment of a new vascular network during early CL development involves interaction of the vascular endothelial growth factor (**VEGF**) and basic fibroblast growth factor (**bFGF**) systems. Intraluteal injection of specific antibodies against VEGF and bFGF during metestrus clearly reduced CL volume and concentrations of progesterone in plasma of Holstein cows (Yamashita et al., 2008). Suppression of bFGF and VEGF were associated with reduction of mRNA expression of angiotensin-1 and -2, respectively. Angiotensin-1 is necessary to maintain and stabilize blood vessels (Yancopoulos et al., 2000). Nevertheless, stable vessels are unsuitable to

angiogenic sprouting. An increase in the angiotensin-2:angiotensin-1 ratio destabilizes the vascular structure and, in the presence of angiogenic factor such as VEGF, reverts endothelial tissue into a more plastic state and allows for vascular network formation (Yancopoulos et al., 2000). Therefore, the balance in the angiotensin system is likely to play an important role in the development of the CL. Surprisingly, $\text{PGF}_{2\alpha}$ produced by the newly formed CL acts as a regulator to enhance luteal growth and progesterone secretion. Infusion of bFGF and VEGF into a microdialysis system increased the production of progesterone and $\text{PGF}_{2\alpha}$ by the early cycle bovine CL (Kobayashi et al., 2001). Furthermore, infusion of $\text{PGF}_{2\alpha}$ induced a transient increase in the release of progesterone by the early cycle bovine CL, whereas infusion of $\text{PGF}_{2\alpha}$ together with angiotensin-2 induced a sustained increase in progesterone release (Kobayashi et al., 2001).

The substrate for luteal synthesis of progesterone is cholesterol, generally obtained from the blood circulation as low-density lipoprotein (**LDL**) and high-density lipoprotein (**HDL**) or from hydrolysis of cholesterol esters stored in lipid droplets within the cytoplasm by cholesterol esterase (Caffrey et al., 1979; Niswender et al., 2002). In the bovine, the uptake of cholesterol from the bloodstream is expected to be mainly in the form of HDL. This is because approximately 90% of the pre-formed cholesterol circulates into HDL in this species. Once in the cytoplasm, free cholesterol is transported to the mitochondria via sterol binding proteins and cytoskeleton interaction. Cholesterol is then transported from the outer to the inner mitochondrial membrane by StAR. In the mitochondrial matrix, cholesterol's side chain is cleaved by P450_{scc} to form pregnenolone, which is converted into progesterone by 3β -HSD associated with

the smooth endoplasmic reticulum (Niswender et al., 2002). Continuous support of LH and growth hormone (**GH**) is required for CL maintenance and expression of StAR, P450scc and 3 β -HSD in ruminants; however, pulsatile release of LH by the pituitary gland was shown to be unnecessary to preserve progesterone production by fully mature CL in heifers (Peters et al., 1994). Gonadotropins also control the synthesis of progesterone in an acute manner without affecting gene expression patterns (Niswender et al., 2000). The binding of LH to its receptor in small luteal cells activates adenylate cyclase, leading to increased concentrations of cyclic adenosine monophosphate (**cAMP**) and ultimately activation of protein kinase A (**PKA**). Activation of PKA increases cholesterol esterase activity and the amount of StAR in the phosphorylated form, stimulating the transport of cholesterol to the inner mitochondrial membrane. Progesterone has been shown to stimulate its own synthesis by bovine luteal cells. Treatment of luteal cells with onapristone, a highly specific progesterone inhibitor, reduced *in vitro* production of progesterone by 35% (Okuda et al., 2004). This reduction might be partially explained by a genomic effect of progesterone. Incubation of bovine luteal cells with progesterone increased the expression of StAR, P450scc and 3 β -HSD in comparison to the control group, suggesting that the ablation of this effect could compromise synthesis of progesterone (Rekawiecki et al., 2005).

If the cow is not pregnant, the CL must be regressed at the end of the estrous cycle to provide another opportunity for ovulation and conception. Because the circulating PGF_{2 α} is extensively degraded after its first passage through the lungs in Guinea-pigs, and presumably cows (Piper et al., 1970; Davis et al., 1985), PGF_{2 α} dynamics is assessed by measuring circulating concentrations of its main metabolite

15-keto-13,14-dihydro-PGF_{2α} (**PGFM**; Kindahl et al., 1976b). The process of luteolysis is triggered by the endometrial release of 4 to 5 pulses of PGF_{2α} within a 2-day interval (Kindahl et al., 1976a). The duration of each pulse, measured as the interval between two consecutive nadirs, ranges from 6 to 8 hours (Ginther et al., 2010). Low-amplitude pulses of PGFM (120 pg/mL) have been shown to occur before a noticeable reduction in circulating concentrations of progesterone (Ginther et al., 2010). During the period of luteal regression, the amplitude of the PGFM pulses decreases from the first (578 pg/mL) to the final pulse (131 pg/mL). Consistent with previous data (Kindahl, et al., 1976a), progesterone concentrations declined after the first pulse of PGF_{2α} (Ginther et al., 2010). A transient increase on concentrations of progesterone was observed between 1 and 2 hours after the first luteolytic pulse of PGF_{2α}, followed by a constant decrease until basal values (Ginther et al., 2010).

It has been suggested that the coordinated actions of progesterone, estradiol and oxytocin are responsible for the endometrial synthesis of PGF_{2α} in ruminants (McCracken et al., 1999). The induction of endometrial synthesis of PGF_{2α} by oxytocin in the sheep is mediated by an increase in the activity of phospholipase A₂ (Lee and Silvia, 1994). Nevertheless, *in vitro* treatment of bovine endometrial cells with oxytocin failed to enhance phospholipase A₂ mRNA expression; however, it induced synthesis of PGF_{2α} associated with greater expression of cyclooxygenase 2 (**COX-2**; Asselin et al., 1997). Treatment of heifers with oxytocin between days 17 and 19 of the estrous cycle induced an acute peak of PGFM 5 to 15 minutes after the treatment (LaFrance and Goff, 1985). However, a similar response was not observed when treatment was administrated between days 6 and 13 of the estrous cycle (LaFrance and Goff, 1985).

This temporal change in uterine responsiveness to oxytocin is likely to set the proper timing of luteolysis. Endometrial expression of oxytocin receptors in the luminal epithelium and superficial glands is low during diestrus (Robinson et al., 2001). Greater abundance of mRNA and protein encoding for oxytocin receptors are detected only after day 16 of the estrous cycle, after downregulation of progesterone receptors on day 12 (Robinson et al., 2001). In spite of this temporary inhibition of oxytocin receptors, exposure to progesterone was shown to be needed for oxytocin-induced release of $\text{PGF}_{2\alpha}$. Treatment with oxytocin failed to increase PGFM concentration in bilaterally ovariectomized heifers if no progesterone priming was provided (LaFrance and Goff, 1988). Interestingly, receptors for oxytocin were present in the uterus of ovariectomized cows that did not respond to an injection of oxytocin (Lamming and Mann, 1995). Progesterone has been associated with accumulation of triglycerides into cytoplasmic lipid droplets in endometrial cells of rats (Boshier et al., 1981). In ewes, most of the lipids in the endometrium are present as phospholipid and linoleic and arachidonic acids increase during mid to late luteal phase before luteolysis (Meier et al., 1997). Therefore, exposure to progesterone during diestrus enriches the phospholipid pool of endometrial cells with precursors for prostaglandin synthesis possibly to stimulate the luteolytic process. In addition, administration of exogenous progesterone enhanced expression of mRNA encoding for COX-2 in uterine luminal epithelium and superficial glands of sheep (Eggleston et al., 1990; Charpigny et al., 1997).

The permissive effect of progesterone on oxytocin-induced release of $\text{PGF}_{2\alpha}$ in ovariectomized heifers was enhanced following treatment with estradiol (LaFrance and Goff, 1988). Estradiol enhanced expression of mRNA encoding for COX-2 in the uterus

of ovariectomized sheep, but only after priming with progesterone (Charpigny et al., 1997). Moreover, estradiol stimulated phospholipase A₂ activity in human endometrium (Bonney and Franks, 1987). In fact, a single injection of estradiol on day 18 of the estrous cycle induced an acute release of PGF_{2α} beginning from 2 to 3 hours after the treatment (Knickerbocker et al., 1986a). Similar responses were observed in heifers treated with estradiol on days 13 of the estrous cycle, which was also associated with anticipated luteolysis compared with non-treated heifers (Thatcher et al., 1986). Conversely, suppression of follicular growth and, consequently, reduction of plasmatic concentrations of estradiol have been shown to delay luteolysis. Treatment of heifers with follicular fluid free of steroids, a source of inhibin, restrained follicular development and extended luteal lifespan (Salfen et al., 1999). Electrocauterization of all visible follicles and subsequent X-irradiation of ovaries either on days 9, 12 or 15 (Villa-Godoy et al., 1985) or on day 10 of the estrous cycle (Fogwell et al., 1985) prevented spontaneous regression of the CL, which was associated with inhibition of pulsatile release of PGF_{2α} (Fogwell et al., 1985). Nevertheless, regression of the CL was inhibited only transiently by ablation of ovarian follicles. In Holstein heifers from which follicles ≥ 4 mm were aspirated between days 9 and 21 of the estrous cycle, luteolysis occurred during the period of aspiration and preceded the first significant rise in estradiol concentration (Araujo et al., 2009).

In ruminants, uterine PGF_{2α} is transported to the ovaries through a direct exchange between the uterine vein and the ovarian artery. This countercurrent mechanism was described initially in sheep (McCracken et al., 1971). Later studies used the cow as an animal model and confirmed that intrauterine infusion of PGF_{2α}

induced an increase in the luteolysin concentration in the ovarian artery that was not associated with an increase in the carotid artery (Hixon and Hansel, 1974). Accordingly, unilateral hysterectomy ipsi-lateral to the CL inhibits luteolysis in response to oxytocin in heifers (Ginther et al., 1967). This mechanism is crucial for efficient regression of the CL because of the extensive degradation of $\text{PGF}_{2\alpha}$ in the lungs (Piper et al., 1970).

The increase in luteal peripheral blood flow is one of the earliest physiological events observed during the luteolytic cascade in the cow. Using color Doppler ultrasonography, an acute increase in luteal blood flow was observed within 30 minutes to 2 hours of the administration of $\text{PGF}_{2\alpha}$ to cows between days 10 and 12 of the estrous cycle (Acosta et al., 2002). This response was followed by a sustained reduction in luteal blood flow, CL volume and circulating concentrations of progesterone. Endothelin-1 has been implicated as a mediator of the early effects of the luteolytic cascade. Bovine endothelial cells have receptors for $\text{PGF}_{2\alpha}$ (Mamluck et al., 1998) and $\text{PGF}_{2\alpha}$ -induced synthesis of endothelin-1 has been reported from *in vitro* and *in vivo* systems (Girsh et al., 1996a; Ohtani et al., 1998). Furthermore, these changes in CL blood flow result from $\text{PGF}_{2\alpha}$ action in vascular tissue, since endothelin-1 can be produced by endothelial cells of the bovine CL, but not by luteal cells (Girsh et al., 1996b). Endothelin-1 is a potent vasoconstrictor and may initiate luteolysis by depriving the gland of nutrients, substrates for steroidogenesis, and luteotropic support (Niswender et al., 2002). Moreover, high affinity binding sites for endothelin-1 are present in large and small luteal cells. Endothelin-1 inhibited basal and LH-stimulated production of progesterone in small luteal cells, whereas incubation with a selective inhibitor for endothelin-1 prevented the anti-steroidogenic effect of $\text{PGF}_{2\alpha}$ on luteal cells

(Girsh et al., 1996b). Elevated concentrations of endothelin-1 and expression of prepro-endothelin-1 were observed in the bovine CL around the time of spontaneous luteolysis and as soon as 2 hours after exogenous treatment with $\text{PGF}_{2\alpha}$ (Girsh et al., 1996b). A positive feedback loop between endothelin-1/angiotensin-II and $\text{PGF}_{2\alpha}$ synthesis in the CL has been proposed as one of the mechanisms involved in regression of the CL in cows (Miyamoto et al., 2009). Angiotensin-II also suppressed progesterone secretion stimulated by LH in bovine luteal cells (Stirling et al., 1990).

Either mediated by vasoactive factors or via direct action in luteal cells, the administration of $\text{PGF}_{2\alpha}$ was shown to reduce mRNA expression for StAR by 50% within 12 hours and to undetectable concentrations within 24 hours of treatment (Pescador et al., 1996). The same authors reported that the downregulation of P450_{scc} was observed after that of StAR. Prostaglandin- $\text{F}_{2\alpha}$ acts in large luteal cells by binding to a high affinity specific G protein-coupled receptor and inducing activation of membrane-bound phospholipase C (**PLC**; Niswender et al., 2002). Phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (**IP₃**) and 1,2-diacylglycerol (**DAG**), stimulating the release of free Ca^{2+} from the smooth endoplasmic reticulum into the cytoplasm. Free Ca^{2+} and DAG stimulate the activity of Ca^{2+} -dependent protein kinase C (**PKC**) and Ca^{2+} -dependent endonucleases, which are expected to reduce StAR activity and stimulate apoptosis (Niswender et al., 2002). In addition, progesterone exerts a protective effect in luteal cells, which might impact CL development and steroidogenesis. Treatment of bovine luteal cells with progesterone stimulated the expression of B-cell lymphoma 2 (**Bcl-2**) mRNA and reduced the ratio of B-cell lymphoma 2 associated X protein (**Bax**):Bcl-2 in comparison to untreated cells

(Liszewska et al., 2005), which was shown to reduce cell death in the human CL (Sugino et al., 2000). Conversely, incubation of bovine luteal cells with onapristone enhanced apoptosis through stimulation of Fas antigen and caspase-3 mRNA expression and caspase-3 activation (Okuda et al., 2004). The upregulation of the caspase apoptotic pathway reduced cell viability to 70% of the control group. Therefore, the acute reduction of luteal progesterone in the mid-cycle CL might activate apoptotic pathways (Okuda et al., 2004; Liszewska et al., 2005) that in association with reduced blood supply, leads to complete regression of the CL.

The involvement of vascular responses in the initiation of CL regression might be a component of the inability to induce luteolysis of newly formed CL (Acosta et al., 2002; Santos et al., 2010). In fact, the absence of receptors for $\text{PGF}_{2\alpha}$ or the affinity of those receptors for its ligand does not seem to explain the unresponsiveness of the young CL (Wiltbank et al., 1995). The early CL has a much smaller mass of vascular tissue to respond to a luteolytic stimulus (Farin et al., 1986). Accordingly, increase in the mRNA expression for endothelin-1 was observed following treatment of exogenous $\text{PGF}_{2\alpha}$ on day 10, but not on day 4 of the estrous cycle (Levy et al., 2000). Furthermore, no changes in blood flow were observed following administration of $\text{PGF}_{2\alpha}$ when cows were treated on day 4 of the estrous cycle (Acosta et al., 2002). It was reported that exogenous $\text{PGF}_{2\alpha}$ stimulated COX-2 luteal mRNA expression on day 11 of the estrous cycle, whereas it inhibited this response in a day 4 CL (Tsai and Wiltbank, 1998). The lesser production of $\text{PGF}_{2\alpha}$ and endothelin-1 by the newly formed CL could compromise the positive feedback that stimulates luteolysis.

Early Embryogenesis: From the Zygote to Shedding of the Zona Pellucida

After the zygote stage, embryos undergo sequential mitotic divisions. The interval between cellular divisions has been determined for bovine embryos using *in vitro* culture systems and it varies according to the stage of the embryo. The first cleavage occurs from 27 to 32 hours after fertilization (Holm et al., 2002; Lequarre et al., 2003) and it is followed by two short cell cycles leading to the 4-cell (8.1 hours) and the 8-cell stages (7.1 hours; Holm et al., 2002). Early embryogenesis is mainly controlled by maternal gene expression. In fact, the competent ooplasm was shown to contain the mRNA needed to support embryonic development up to the fourth cell cycle (Plante et al., 1994; Memili and First, 1998). Moreover, it has been suggested that oocytes with a greater mRNA content in the cytoplasm generate embryos that are less likely to express cell death transcripts later in development and more likely to develop to the blastocyst stage (Meirelles et al., 2004).

The process by which the embryo becomes able to synthesize its own mRNA has been named transition from maternal to embryonic control of development or embryonic genome activation (**EGA**). The initial reports on embryonic gene expression stated that bovine embryos are transcriptionally inactive before the fourth cell cycle (i.e.: during the 8-cell stage) and rely exclusively on maternal mRNA and proteins synthesized during oogenesis (Kopency, 1989). Recent studies have contested the classic view that EGA occurs only after the 8-cell stage. Incorporation of uridine triphosphate labeled with radioactive sulfur (i.e.: ^{35}S) was shown to occur in 2-cell embryos and to remain constant until the 8-cell stage, demonstrating active RNA translation during early embryogenesis (Memili et al., 1998). Furthermore, the expression of ribonucleic acid (**RNA**) polymerase II was detected in 2-cell embryos and inhibition of RNA polymerase

II-dependent transcription during any of the first four cell cycles blocked embryonic development beyond the 16-cell stage (Memili and First, 1998). The classic view that EGA takes place at the 8-cell stage (Kopency, 1989) is somewhat supported by recent data, which shows a sharp increase on embryonic transcription during the 8-cell stage (Memili et al., 1998). Therefore, genome activation in bovine embryos has been described as a gradual process that encompasses a minor EGA between the 1- and 4-cell stages and a major EGA starting at the 8-cell stage (Memili and First, 2000).

The mechanisms by which the embryonic genome is activated in mammals include depletion of maternal mRNA and acquisition of essential transcription factors (Schier, 2007). Although the maternal mRNA stored in the oocyte is protected from degradation through the binding of MSY2, a germ cell-specific Y-box protein (Yu et al., 2004), this protection begins to be removed because of MSY2 phosphorylation following germinal vesicle breakdown and MSY2 is entirely absent as early as the 2-cell stage, which correspond to the EGA in the mouse embryo (Yu et al., 2001). Similar responses were observed in bovine embryos, on which the amount of MSY2 protein decreased beginning at the germinal vesicle breakdown and was reduced significantly at the 8-cell stage (Vigneault et al., 2009). The unstable maternal mRNA is then largely degraded during this period (Paynton et al., 1988, Wang et al., 2004), including gene products that might interfere with later development (Schier, 2007). Concomitantly, the amount of TATA box-binding protein (**TBP**) begins to increase from the 8-cell through to the blastocyst stages in bovine embryos (Vigneault et al., 2009). The TBP is the first component of the pre-initiation complex that binds to the TATA box motif of a typical RNA polymerase II promoter in order to initiate gene transcription (Nelson and Cox,

2008). Another mechanism considered to be responsible for the embryonic genome inactivation before the major EGA is the occurrence of rapid cell cycles lacking G1 and G2 phases (Schier, 2007). In fact, the fourth cell cycle that leads the bovine embryo to the 8-cell stage and to the major EGA is longer (45.1 hours) than the second and third cycles (8.1 and 7.1 hours, respectively; Holm et al., 2002).

Bovine embryos reach the stage of morula between days 4 and 5 of gestation (Holm et al., 2002), which is characterized by lack of polarization and free movement of molecules across the embryo. Compaction of the morula is the first morphogenetic event of pre-implantation development (Watson et al., 2004). Cell-to-cell contact increases until the individual blastomere outlines disappear. Initial attachment is promoted by heteromeric cadherin-based structures, called adherens junctions. E-cadherin is a single-pass transmembrane protein which extra-cellular domain interacts with other E-cadherins molecules via a Ca^{2+} -dependent homophilic association and approaches adjacent blastomeres. E-cadherin's cytoplasmic domains interact with either β - or γ -catenin, which links cadherins to the actin microfilament network via α -catenin and reinforces cell-to-cell adhesion. Furthermore, activation of embryo compaction coincides with cell apicobasal polarization of blastomeres (Pratt et al., 2002), which is an important step on cell differentiation. The process of compaction involving E-cadherin has been shown to be crucial for further embryonic development (Kan et al., 2007).

Bovine embryos leave the oviduct and reach the uterine horn between days 4 and 7 of gestation. Blastocyst formation occurs between days 6 and 7 of gestation (Holm et al., 2002; Senger, 2003b). It marks the segregation of the first two cell lineages during

embryogenesis: the inner cell mass (**ICM**) and the trophectoderm (Senger, 2003b). Proper cell differentiation relies on downregulation of signals for pluripotency on trophectoderm cells, but not in the ICM. The initial differentiation of the trophectoderm depends on the asymmetric positional signals generated following compaction that induces cell polarization and epithelialization (Yamanaka et al., 2006). Using the mouse embryo as a model, the expression of the transcription factor *Cdx2* was detected at the 8-cell stage and its protein was identified in the outer cells of early morula (Strumpf et al., 2005). Expression of *Cdx2* is critical for trophectoderm cell fate specification. In the absence of this transcription factor, such as in *Cdx2*^{-/-} mutant embryos, *Oct4*, a repressor of the trophectoderm cell fate (Nichols et al., 1998; Niwa et al., 2000) was expressed by the trophectoderm (Strumpf et al., 2005). Consequently, *Cdx2*^{-/-} blastocysts failed to maintain their blastocel, did not express specific markers of differentiated trophoblast giant cells such as *Hand1* and *Pl1*, and had greater incidence of programmed cell death at the expanded blastocyst stage (Strumpf et al., 2005). Conversely, the expression of *Cdx2* has to be restricted to the trophectoderm in order to prevent embryonic stem cells to differentiate into trophectoderm cells (Nichols et al., 1998). In fact, by the expanded blastocyst stage *Cdx2* protein is restricted to trophectoderm nuclei (Strumpf et al., 2005). The ICM further differentiates to give rise to epiblast pluripotent cells and cells committed with the primitive endoderm fate (Plusa et al., 2008). Initially, lineage-specific transcription factors for epiblast (*Nanog*; Chambers et al., 2003; Mitsui et al., 2003) and primitive endoderm (*Gata6*; Fujikura et al., 2002) cell lineages are stochastically expressed among the ICM, often by the same cell (Plusa et al., 2008). These pathways become mutually inhibitory, which generates exclusive

epiblast and primitive endoderm precursors. This is followed by cell sorting and reallocation involving cell movement, adhesion and apoptosis (Plusa et al., 2008).

These changes in gene expression are accompanied by morphological alterations of the embryo. Early after embryo compaction, tight junctions begin to be assembled (Eckert and Fleming, 2008). Tight junction formation is affected by cell contact pattern and is restricted to the outer trophectoderm cells of the blastocyst. Differently from the non-compact embryo, the movement of molecules across the embryo is no longer unrestrained. The dependency on Na⁺/K⁺-ATPase pumps in the basolateral membrane of trophectoderm cells (α 2 and α 3 isoforms for bovine embryos) to induce the formation of the blastocel has been challenged (Watson et al., 2004). The osmotic gradient across the rabbit trophectoderm averages 8 mOsm, demonstrating that the blastocel is near iso-osmolar compared with the culture media (Borland et al., 1977). This defies the main mechanism attributed to ion pumps. In fact, Na⁺/K⁺-ATPase null mice are able to develop to the blastocyst stage (James et al., 1999). Aquaporins are capable of moving water in presence of low osmotic gradients and have been shown to be present in the trophectoderm and to participate in diffusion processes during blastocel filling (Barcroft et al., 2003).

Until the stage of expanded blastocyst, embryonic growth is restricted by the zona pellucida. Bovine embryos hatch from the zona pellucida between days 8 and 9 of gestation (Holm et al. 2002; Senger, 2003b) primarily because of the physical distention induced by the expanding blastocoel (Coates and Menino, 1994). Results from cinematographic and morphometric analyses of bovine blastocysts cultured *in vitro* have shown that the expansion preceding hatching can be either continuous or

discontinuous, but in a pulsatile fashion (Massip et al., 1982). Furthermore, low to moderate pulsatile activity were compatible with normal hatching, whereas high pulse frequency was associated with failure to hatch (Massip et al., 1982). The involvement of proteases in the process of hatching of the zona pellucida has been discarded based on results of *in vitro* production of bovine embryos. Bovine embryos produce plasminogen activator, a serine protease (Menino and Williams, 1987). Moreover, embryos that are able to hatch *in vitro* produce more plasminogen activator than those that fail to hatch (Kaaekuahiwi and Menino, 1990). Nevertheless, culture of embryos in the presence of a human plasminogen activator inhibitor did not reduce the proportion of hatched blastocysts, whereas incorporation of ouabain, a Na⁺/K⁺-ATPase that inhibits blastocelic expansion, impaired hatching in a dose dependent manner (Coates and Menino, 1994). The lack of proteolytic activity involved in hatching of bovine embryos agree with the observation that empty zonae can be recovered from uterine flush, meaning that they were not degraded during hatching (Besenfelder et al., 2001).

Elongation of the Conceptus

Early after hatching, the bovine embryo remains detached from the uterus and relies on a synchronous cross-talk with the maternal unit during the pre-implantation period for further development and maintenance of pregnancy (Spencer et al., 2007). Initially, the blastocyst remains spherical and grows from 160-180 µm at hatching to 375 µm of diameter on day 11 of gestation (Betteridge and Fléchon, 1988). Embryonic shape changes to ovoid during a transitory phase preceding elongation of the conceptus (i.e.: embryo and extraembryonic membranes). The conceptus begins to enlongate between days 12 and 14 of gestation and rapidly develops into a filamentous structure that can reach 25 cm within 3 to 5 days (Lucy et al., 1995). The fast elongation

of the conceptus is critical for maintenance of gestation, since its capability to produce interferon-tau (**IFN- τ**) and prevent the regression of the CL relies on its length between days 17 and 19 of gestation (Robinson et al., 2006).

Proper establishment of gestation after hatching from the zona pellucida encompasses embryonic contact with uterine luminal and glandular epithelium, orientation of the blastocyst, apposition between the trophoctoderm and endometrium, extensive elongation, inhibition of CL regression, and adhesion of trophoctoderm to uterus (Bazer et al., 2008). The stimuli and nutritional support for conceptus elongation in ruminants are provided by copious molecules secreted by the endometrial glandular epithelium, collectively referred as to histotroph. The importance of endometrium secretory function has been demonstrated in sheep using the uterine gland knockout model (Gray et al., 2002). The authors reported that the blastocysts from ewes which uterine glands were absent hatched normally; however they failed to elongate. Seven out of 8 control ewes had filamentous conceptuses on day 14 of gestation, whereas uterine gland knockout ewes had either no conceptus present (5 of 12), tubular conceptuses (6 of 12), or a fragmented filamentous conceptus (1 of 12). Histotroph secretion has been shown to be stimulated by progesterone. Nevertheless, the expression of progesterone receptors is downregulated in luminal epithelium and superficial glandular epithelium between days 10 and 12 of the estrous cycle in cows (Robinson et al., 2001), which seems contradictory. It has been hypothesized that after day 12 of the estrous cycle, progesterone acts on receptors located in the uterine stroma and the effects on endometrium and conceptus are then mediated by progestamedins such as fibroblastic growth factors (**FGF**) and hepatocyte growth factors

(**HGF**; Bazer et al., 2008). In fact, progesterone receptors are present in the sub-epithelial stroma throughout the entire estrous cycle in cows (Robinson et al., 2001). The uterine expression of FGF-2 and FGF-10 in cows and the expression of its receptors in the ovine luminal endometrium and pre-attachment bovine embryos have been described (Michael et al., 2006; Satterfield et al., 2008; Cooke et al., 2009). Fibroblastic growth factor-2 has been associated with increase in deoxyribonucleic acid (**DNA**) synthesis, and IFN- τ mRNA and protein abundance in bovine trophectoderm cell lines (Michael et al., 2006). Conversely, the levels of FGF-2 mRNA were not affected by pregnancy status (Michael et al., 2006), suggesting that a maternal factor other than conceptus-derived factors controls uterine FGF expression. The treatment of ewes with progesterone from days 1.5 to 9 of the estrous cycle increased the levels of FGF-10 mRNA in the subepithelial stroma compared with untreated control group. Furthermore, the administration of progesterone antagonist reduced FGF-10 mRNA abundance on day 12 of the estrous cycle compared with animals treated with progesterone only (Satterfield et al., 2008).

Mucin is a large glycosylated protein constituent of the endometrial glycocalyx which main function is to prevent molecules from attaching to the endometrium. Mucin is locally reduced at implantation sites by the activity of cell-surface proteases, which indicates that this process is triggered by the conceptus (Spencer et al., 2004). The exact mechanism responsible for initial adhesion of the trophectoderm to endometrial epithelium in ruminants is not fully understood; however it is likely to be a multifaceted process (Spencer et al., 2004; Bazer et al., 2010). Glycosylated cell adhesion molecule 1 (**GlyCAM-1**) functions as the carbohydrate ligand for L-selectin, which has been

shown to mediate adhesion of humans embryos (Genbacev et al., 2003). In non-pregnant ewes, GlyCAM-1 expression increases in the endometrial luminal and superficial glandular epithelium between days 1 and 5 of the estrous cycle and declines from day 11 to 15 (Spencer et al., 1999). Conversely, GlyCAM-1 expression increases between days 15 and 19 in pregnant ewes. Furthermore, the reduced amount of immunoreactive GlyCAM-1 measured in uterine flushing samples collected between days 11 and 13 of gestation is largely increased from day 15 to 17 of gestation.

Galectins are proteins with a conserved carbohydrate recognition domain that bind b-galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on cell surface. Galectin-15 mRNA was not detected in the endometrium of ewes before day 10 of the estrous cycle. Nonetheless, galectin-15 expression increased 13-fold between days 10 and 14. This increase was only transient for non-pregnant females, but not for pregnant ewes (Gray et al., 2004). It is interestingly to notice that expression of galectin-15 in the endometrium was induced by progesterone and further stimulated by IFN- τ , a response suitable for the specific control of conceptus elongation (Gray et al., 2004).

Integrins are heterodimeric transmembrane glycoprotein receptors that mediate cellular adhesion and motility. Integrins have been suggested to bind extracellular matrix ligands, such as secreted phosphoprotein 1 or oostopontin (**SPP1**), IGFBP-1, and Galectin-15, to induce reorganization of the cytoskeleton and enhance cell migration during initial adhesion of the conceptus (Spencer et al., 2004; Bazer et al., 2010). In fact, the expression of integrin subunits α_v , α_4 , α_5 , β_1 , β_3 and β_5 were detected on the apical surfaces of trophoctoderm and endometrial luminal epithelium during the peri-implantation period in the sheep (Johnson et al., 2001). Furthermore, changes in the

expression of integrins have been linked with infertility in women (Lessey, 1998). Finally, the interaction between integrins, extracellular matrix ligands, and contractile actin microfilaments from the cytoskeleton allows for the trophectoderm to respond to internal and external mechanical forces, which might enhance cell migration (Bazer et al., 2010).

In face of increasing energetic demands, embryonic energetic metabolism switches from oxidation of pyruvate to a more complex process that involves anaerobic glycolysis during morula compaction (Thompson et al., 1996). Because elongating conceptuses are incapable of gluconeogenesis, the glucose used by the conceptus is dependent on the availability of this molecule within the uterine lumen (Kalhan and Parimi, 2000; Riley and Moley, 2006). Bovine embryos express both facilitative (GLUT1, GLUT3, GLUT8) and sodium-dependent glucose transporters (SGLT-1) as early as the 2-cell stage and additional transporters begin to be expressed at the 16-cells stage (GLUT5), blastocel formation (GLUT4) and during elongation (GLUT2; Augustin et al., 2001). Moreover, the expression of additional glucose transporters coincides with the timing of rises in glucose uptake by the embryo (Rieger et al., 1992; Thompson et al., 1996). Hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate and allows the influx of hexoses into the glycolytic pathway. Phosphofructokinase catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate and is considered the primary site of control of glycolysis. There are evidences that the expression of these glycolitic enzymes is enhanced as the embryo develops and might be associated with the elongation process (Lequarre et al., 1997). There are indicatives that the cross-talk between the conceptus of ruminants and the maternal unit is

responsible to enhance the transport of glucose into the uterine lumen. Total recoverable glucose in the uterine fluid increased as the pregnancy develops and was greater for pregnant compared with cyclic ewes during the period of conceptus elongation (Gao et al., 2009a). Endometrial expression of glucose transporters is stimulated by progesterone and IFN- τ , and this effect was abolished after the administration of a progesterone receptor antagonist (Gao et al., 2009b). Accordingly, the increase in uterine levels of glucose was coordinated with the expression of both facilitative (GLUT1 and GLUT4) and sodium-dependent (SGLT-1 and SGLT-6) glucose transporters, which was also greater in pregnant compared with cyclic ewes (Gao et al., 2009b). Conceptually, this remarkable mechanism supports conceptus development in pregnant ewes, but alternatively allows for the non-pregnant females to spare glucose.

Similarly to the transport of glucose into the uterine lumen, the mechanisms that ensure adequate supply of amino acids to support embryonic development are also modulated by progesterone and IFN- τ during early pregnancy in ruminants. The uterine concentrations of the vast majority of basic, acidic and neutral amino acids between days 13 and 16 of the estrous cycle were highly affected by pregnancy (Gao et al., 2009a). Amino acids are required for tissue production and can be used as a source of energy by the elongating conceptus. Fluctuations in arginine availability during early embryogenesis have been evaluated because the effects of this amino acid in cell signaling pathways. No changes in uterine concentration of arginine were observed in non-pregnant ewes throughout the estrous cycle (Gao et al., 2009a). Conversely, concentrations for pregnant animals increased 138% from day 3 to 14 of gestation. Intrauterine concentrations of arginine were controlled at the transporter level (Gao et

al., 2009c). Accordingly, Na⁺-independent arginine transporter CAT1 (SLC7A1) increased slightly on days 14 of gestation and markedly increased on day 20, whereas CAT2 (SLC7A2) increased linearly after day 16 of gestation (Gao et al., 2009c). Abundance of mRNA encoding CAT1 in luminal and glandular endometrium was slightly upregulated following long term exposure to progesterone (1.5- and 1.8-fold increase, respectively). Furthermore, endometrial CAT2 mRNA expression was 4.1-fold greater in animals primed with progesterone and an additional 1.7-fold increase was observed in animals that also received IFN- τ . Arginine has been linked with activation of the mammalian target of rapamycin (**mTOR**) pathway in ovine trophectoderm cells (Bazer et al., 2010). The mTOR pathway is composed by 2 complexes, the mTORC1 involved in cell proliferation and mRNA translation, and the mTORC2 involved in cytoskeletal alterations, cell migration and survival.

Maternal Recognition of Pregnancy

Spontaneous regression of the CL between days 17 and 19 of the estrous cycle must be inhibited for successful maintenance of the gestation in cows. It was shown early that the elongating conceptus plays a key in the maternal recognition of pregnancy. Removal of embryos from the uterus on day 13 of gestation had no effect on the length of the estrous cycle compared with non-inseminated heifers, whereas heifers that kept their embryos until days 17 or 19 of gestation had the inter-estrus interval extended from 21 to 25 and 26 days, respectively (Northey and French, 1980). Furthermore, the same authors reported an extension of luteal lifespan in non-inseminated heifers following intrauterine infusions with a homogenate prepared from 17 to 18 days old conceptuses. Infusions were administered between days 14 and 18 of the estrous cycle. Knickerbocker et al (1986b) demonstrated that the anti-luteolytic

factor secreted by elongating conceptus is a protein and suggested that it acts to prevent the pulsatile release of $\text{PGF}_{2\alpha}$ by endometrial cells. Helmer et al. (1987) demonstrated that the protein complex secreted by bovine conceptuses cross-reacted with antiserum to ovine trophoblast protein-1 (**oTP-1**) and labeled it as bovine trophoblast protein-1 (**bTP-1**). Analysis of complementary DNA (**cDNA**) revealed that bTP-1 entire sequence was 85% identical to that of oTP-1 and 79% identical to that of bovine interferon-alpha II (Imakawa et al., 1989). Bovine and ovine trophoblast protein-1 were then labeled as IFN- τ .

Interferon-tau prevents CL regression by inhibiting the endometrial release of $\text{PGF}_{2\alpha}$ stimulated by estradiol and oxytocin. Intrauterine infusion of enriched bTP-1 twice daily between days 15 and 21 of the estrous cycle abolished pulses of $\text{PGF}_{2\alpha}$ measured in the vena cava and resulted in extension of CL function in Jersey cows (Helmer et al., 1989). After 5 intrauterine infusions of oTP-1 between days 12 and 14 of the estrous cycle, ewes had no increase in PGFM concentrations following an injection of estradiol on day 14 of the estrous cycle, whereas concentrations increased from 6 to 10 hours after the injection in controls (Vallet et al., 1988). Similarly, ewes treated with oTP-1 had a smaller increase in PGFM after an injection of oxytocin on day 15 of the estrous cycle than did untreated ewes (Vallet et al., 1988). The presence of IFN- τ in the uterine lumen of non-pregnant ewes reduced mRNA expression and, consequently, the abundance of receptors for estrogen and oxytocin (Spencer and Bazer, 1996; Spencer et al., 1996).

The expression and, consequently, production of IFN- τ has been shown to be developmentally regulated in the bovine embryo. Low mRNA expression of IFN- τ was

detected as early as the blastocyst stage (Bertolini et al., 2002). The production of IFN- τ by bovine conceptuses increases after day 16 of gestation, reaches a maximum level on day 22 and is no longer present after day 24 (Bartol et al., 1985). Studies that evaluated mRNA expression of bTP-1 demonstrated an earlier upregulation (day 13 of gestation) and showed that the expression was restricted to the trophectoderm (Farin et al., 1990b). Nonetheless, the massive increase in IFN- τ production around the period of maternal recognition of pregnancy is considered to be a function of extensive elongation of the conceptus and increase in cell mass capable of producing the protein. Similar gene expression was reported for conceptuses between d 14 and 18 of gestation in spite of increasing amounts of IFN- τ in the uterus (Robinson et al., 2006). Likewise, when conceptuses were categorized according to their length into smaller than 5, from 5 to 10, and greater than 10 cm, no difference in mRNA abundance was reported in face of a 500-fold increase in the amount of IFN- τ (Robinson et al., 2006).

Synchronization of Estrus and Ovulation and Timed AI

Reproductive efficiency has a major impact on profitability of high-producing dairy herds, with the average value of a pregnancy estimated in \$278.00 for US farms (De Viries, 2006). The advent of large-scale artificial insemination (**AI**) allowed for a faster selection and spreading of superior genes for selected traits, which largely improved the potential for milk production since the application of AI over 6 decades. Nevertheless, implementation of AI generated the need for detection of estrus. Visual detection of standing estrus is challenging and might be poor and inaccurate. Dairy cows remain in estrus for 7.2 hours on average and stand 8.5 when mounted during this period (Dransfield et al, 1998). Previous studies reported that the efficiency of estrous detection is usually below 50% in dairy herds (Senger, 1994). Furthermore, evaluation

of individual herds revealed that the proportion of cows subjected to AI without being in estrus ranged from 0 to 60%, and was above 10% in at least 30% of the herds (Reimers et al., 1985; Nebel et al., 1987). This scenario can be worsened by health disorders and poor nutritional status that lead to delayed cyclicity postpartum (Walsh et al., 2007; Santos et al., 2009). Therefore, programs for synchronization of estrus and ovulation have been developed to maximize submission to AI after the end of the voluntary waiting period (**VWP**) and improve reproductive performance of dairy herds.

Initial attempts to synchronize the estrus of dairy cows were based on administration of $\text{PGF}_{2\alpha}$ to induce regression of the CL and anticipate the return to estrus (Lauderdale et al., 1974). Nonetheless, the use of $\text{PGF}_{2\alpha}$ to synchronize the return to estrus has little or no effect when administered during metestrus or to anovular cows (Rowson et al., 1972; Moreira et al., 2001). When 2 injections of $\text{PGF}_{2\alpha}$ are given 10 to 14 days apart, more than 90% of the cycling cows are expected to respond to the second injection; however, the proportion of cows detected in estrus following the second injection ranged between 50 and 60% (Bruno et al., 2005; Chebel et al., 2006). Furthermore, the interval from $\text{PGF}_{2\alpha}$ administration to the onset of estrus varies according to the phase of the estrous cycle and stage of follicular development. Treatment of dairy heifers with $\text{PGF}_{2\alpha}$ on days 7, 11 and 15 of the estrous cycle resulted in similar proportions of animals displaying estrus (Tanabe and Hann, 1984). Nevertheless, the interval between injection and onset of estrus was shorter (43.9 ± 8.2 hours) for heifers treated on day 7, intermediate (53.0 ± 12.2 hours) for heifers treated on day 15, and longer (71.5 ± 14.3 hours) for heifers treated on day 11. Approximately 60% of cows that were synchronized after 2 injections of $\text{PGF}_{2\alpha}$ administered 14 days

apart were detected in estrus between 3 and 6 days after the second injection; however, the interval that encompasses all detected estruses ranged from 0 to 13 days after the second injection (Chebel et al., 2006). The variability between days of estrous cycle when animals were treated, as well as the variation within the same day, makes the use of timed AI following administration of $\text{PGF}_{2\alpha}$ highly inefficient.

Improved synchrony of the estrous cycle was obtained when both luteal and follicular development were manipulated. Turnover of follicles can be achieved by treatment with GnRH, which induces an endogenous surge of LH and FSH culminating in the ovulation of a dominant follicle and subsequent recruitment of a new cohort of follicles (Fernandes et al., 1978; Macmillan and Thatcher, 1991). The efficacy of GnRH to recruit a new follicular wave depends upon the presence of a dominant follicle in the ovary that is responsive to LH. Follicular deviation, and subsequent acquisition of receptors for LH in granulosa cells, was observed at a diameter of 8.5 mm in Holstein heifers (Ginther et al., 1996a). Nonetheless, consistent ovulatory response to LH was observed only when dominant follicles reached 10 mm of diameter in Holstein cows (Sartori et al., 2001). It was shown that the treatment of cows with GnRH followed 7 days later by an injection of $\text{PGF}_{2\alpha}$ increased the number of animals synchronized within a 5-day period and also enhanced the precision of synchrony during days 2 and 3 after $\text{PGF}_{2\alpha}$ injection as compared to cows treated with $\text{PGF}_{2\alpha}$ only (Thatcher et al., 1989). The incorporation of a final injection of GnRH to synchronize the ovulation of a competent follicle gave rise to the Ovsynch protocol and allowed for AI at fixed time (Pursley et al., 1995). The protocol consisted of an injection of GnRH followed 7 days later by an injection of $\text{PGF}_{2\alpha}$. A second injection of GnRH was administered 48 hours

after the PGF_{2α} and females were inseminated 20 to 24 hours later. Ovulation was detected from 26 to 32 hours after the final GnRH in 38 of 44 animals, and the protocol resulted in acceptable pregnancy per AI (**P/AI**).

From the report of the Ovsynch protocol, the impact of timed AI programs on reproductive efficiency of dairy herds has been compared with conventional breeding systems based on estrous detection. Injection of the final GnRH at 24 h after the PGF_{2α} and timed AI 15 hours later reduced P/AI in heifers compared with AI following estrous detection (25.8 vs. 48.7%; Schmitz et al., 1996). Conversely, P/AI in heifers that received the final GnRH 48 h after the PGF_{2α} and timed AI 15 hours later was similar to that observed in heifers inseminated in estrus (45.5 vs. 48.0%; Schmitz et al., 1996). Pursley et al. (1997a) evaluated P/AI in lactating dairy cows and heifers subjected to the Ovsynch protocol or to a synchronization program based on PGF_{2α}. Cows in the PGF_{2α} treatment received as many as 3 injections 14 days apart if signs of estrus had not been observed. All control cows not detected in estrus after the third injection of PGF_{2α} received timed AI 72 to 80 hours later. Pregnancies per AI were similar between the two programs. For the lactating cows, the proportion of cows detected in estrus after the first 2 injections of PGF_{2α} averaged 54.0 % following each injection, with an overall 81.8% for the 28-day period. Because of the low estrous detection rate in the PGF_{2α} group, cows enrolled in the Ovsynch protocol experienced greater pregnancy rate. In a subsequent study, lactating dairy cows were subjected to either the Ovsynch protocol or AI based on estrous detection with periodic use of PGF_{2α} (Pursley et al., 1997b). After a non-pregnancy diagnosis, cows were re-inseminated using the original treatment. Median days postpartum to first AI (54 vs. 83) and days open (99 vs. 118) were reduced

in cows subjected to the Ovsynch protocol compared with cows inseminated following detection of estrus. A meta-analysis of the results from timed AI in contrast to estrous detection programs, however, showed that the pregnancy rate for Ovsynch programs did not differ from that observed for systems based on estrous detection (Rabiee et al., 2005). Nonetheless, positive effects of timed AI compared with reproductive programs based on detection of estrus were only observed when P/AI was not reduced by timed AI and detection of estrus was deficient (Tenhagen et al., 2004).

Synchronization programs have been modified to improve fertility of lactating dairy cows in response to timed AI and to minimize the labor associated with administration of treatments. The timing of induction of ovulation after luteolysis and subsequent interval to AI has been shown to modulate P/AI. Pregnancy per AI was significantly reduced only when insemination was performed 32 hours after the final GnRH of the Ovsynch protocol (Pursley et al., 1998). Nonetheless, the same authors reported a quadratic effect of the interval between the final GnRH injection and timed AI, and maximal P/AI was observed when cows were inseminated at approximately 16 hours after the injection. In order to avoid the handling of cows twice within the same day, the Cosynch protocol was proposed and consisted of the administration of the final GnRH concurrently with the timed AI. Portaluppi and Stevenson (2005) reported increased P/AI and reduced pregnancy loss between day 40 of gestation and term for cows that received the final GnRH and timed AI at 72 hours after $\text{PGF}_{2\alpha}$ compared with cows that received the final GnRH 48 hours after the $\text{PGF}_{2\alpha}$ and were inseminated 48 or 72 hours later. However, others have reported either similar responses between Ovsynch and

Cosynch protocols (DeJarnette and Marshall, 2003) or a decrease in fertility for cows subjected to Cosynch programs (Brusveen et al., 2008).

Reduction in the period of follicular dominance was shown to improve embryo quality and subsequent fertility of dairy cows (Bleach et al., 2004; Cerri et al., 2009). An approach to incorporate this knowledge into synchronization protocols is to shorten the interval from follicle recruitment to luteal regression. Traditional GnRH/PGF_{2α}-based timed AI programs use 7 days between the first injection of GnRH and the injection of PGF_{2α}, which results in the ovulation of a follicle of approximately 10 days. Shortening the interval between the initial GnRH and the injection of PGF_{2α} from 7 to 5 days increased P/AI in lactating dairy cows (Santos et al., 2010). This 5-day protocol requires an additional injection of PGF_{2α} to ensure that a newly formed CL in response to the initial GnRH is fully regressed (Santos et al., 2010). Corpus luteum regression (96.3 vs. 91.5 %) and P/AI (37.9 vs. 30.9 %) were greater for cows subjected to the 5-day Cosynch with 2 injections of PGF_{2α} compared with cows that received the 7-day Cosynch.

The stage of the estrous cycle at which the synchronization protocol is initiated affects ovarian responses to hormonal treatments, synchrony of ovulation and, consequently, P/AI (Vasconcelos et al., 1999). Initiation of the Ovsynch protocol in various stages of the estrous cycle showed that the interval between days 5 to 9 resulted in greatest fertility. When the protocol was initiated after day 10 of the estrous cycle, a large proportion of cows have spontaneously regressed their CL before the injection of PGF_{2α}, which has been associated with a decline in fertility (Chebel et al., 2006). Furthermore, the ability of the initial GnRH to induce ovulation of the dominant

follicle and synchronize the emergence of a new follicular wave was largely reduced when the injection was administered between days 1 and 4 of the estrous cycle (Vasconcelos et al., 2009; Cerri et al., 2009). Failure to ovulate in response to the initial GnRH was associated with the presence of a larger follicle at the end of the protocol and with reduced fertility (Vasconcelos et al., 1999; Cerri et al., 2009). When given at random stages of the estrous cycle, it is expected that only 50 to 60% of the treated cows will ovulate in response to GnRH, although only 41.1% ovulated when this concept was tested in postpartum dairy cows (Navanukraw et al., 2004). Therefore, presynchronization protocols have been developed to optimize fertility in response to timed AI programs to assure that the first GnRH is administered at the stages of the estrous cycle with greatest ovulatory response. The use of 2 injections of PGF_{2α} 14 days apart with 12 days between the second PGF_{2α} and the initial GnRH of the timed AI protocol enhanced P/AI on day 32 after insemination from 36.6% for the control to 48.5% for the treated group (Moreira et al., 2001). This improvement in fertility was associated with an increase in the proportion of cyclic cows that had a functional CL at the initial GnRH and PGF_{2α} injections of the timed AI protocol. Consequently, presynchronization reduced the proportion of cyclic cows that had sub-luteal concentrations of progesterone at either the initial GnRH or PGF_{2α} injections, two conditions associated with reduced fertility (Vasconcelos et al., 1999; Chebel et al., 2006). Similarly, El-Zarkouny et al. (2004) demonstrated an increased proportion of cows with high progesterone concentrations at initiation of Ovsynch (59 vs. 72%) and greater P/AI (37.5 vs. 46.8%) for cows presynchronized with PGF_{2α}. Because the interval between the second injection of PGF_{2α} and the timed AI protocol affects the

stage of follicular development at the initial GnRH, different intervals have been evaluated. Although presynchronizing cows 14 days before initiating the Ovsynch improved P/AI compared with no presynchronization (Navanukraw et al., 2004), this interval is not optimal and results in poor ovulation rate to the initial GnRH of the Ovsynch (Chebel et al., 2006; Galvão et al., 2007). Recently, it was demonstrated that reducing the interval between presynchronization and timed AI from 14 to 11 days increased ovulatory response to the initial GnRH of the timed AI protocol and improved P/AI (Galvão et al., 2007).

The second injection of PGF_{2α} of presynchronization programs frequently coincides with the end of the VWP, in a way that cows that return to estrus are eligible for breeding. Inseminated cows do not receive any further treatment, whereas those not detected in estrus are enrolled in the timed AI program. Studies have shown that P/AI improves as the lactation progresses up to 70 to 90 days postpartum (Pursley et al., 1997a; Tenhagen et al., 2003). Accordingly, cows inseminated on estrus following the presynchronization had less P/AI than those subjected to timed AI in some (Bruno et al., 2005) but not all studies (Chebel et al., 2006). On the other hand, insemination of cows in estrus during presynchronization reduces the interval to first AI and costs associated with hormones and labor. Following a presynchronization protocol composed by PGF_{2α} injections on days 35 and 49 postpartum and a controlled internal drug release (**CIDR**) insert containing progesterone from days 42 to 49 postpartum, cows were assigned to either a short or a long VWP (Chebel and Santos, 2010). Cows in the short VWP were inseminated if observed in estrus from 49 to 62 days postpartum and those not inseminated were submitted to the Ovsynch timed AI protocol beginning at 62 days

postpartum, whereas cows assigned to the long VWP were all inseminated following timed AI at 72 days postpartum. The proportion of short VWP cows inseminated in estrus was 58.9% and the interval from parturition to first AI was shorter for short VWP cows than for long VWP cows (64.7 ± 0.4 vs. 74.2 ± 0.5 days). The P/AI after first insemination postpartum did not differ between short and long VWP on day 32 (33.0 vs. 39.6%, respectively) and 60 after AI (25.3 vs. 31.1%, respectively). Nevertheless, the median days to pregnancy for cows in the short VWP was 125 compared with 134.5 days for cows in the long VWP. Collectively, these results suggest that insemination of cows after the second $\text{PGF}_{2\alpha}$ of the presynchronization anticipates pregnancy and might be an alternative to the enrollment of all eligible cows in timed AI programs depending upon the costs of hormonal treatments and efficiency of estrous detection.

Excluding beneficial effects in uterine health, presynchronization with $\text{PGF}_{2\alpha}$ is only effective in cyclic cows (Moreira et al., 2001); therefore, it is possible that methods that expose cows to GnRH and/or progesterone prior to the first AI postpartum enhance synchrony of the estrous cycle, induce cyclicity in a portion of anovular cows, and benefits fertility. The comparison between conventional $\text{PGF}_{2\alpha}$ -based presynchronization protocols (2 injections of $\text{PGF}_{2\alpha}$ 14 days apart and initiation of the timed AI protocol 12 days later) and the insertion of a CIDR for 7 days associated with an injection of $\text{PGF}_{2\alpha}$ at insert removal 3 days before the initiation of the timed AI protocol showed no differences in P/AI and pregnancy loss (Rutigliano et al., 2008). A novel method of presynchronization has been developed on which an Ovsynch was performed before the Ovsynch that synchronizes ovulation for timed AI (Double-Ovsynch; Souza et al., 2008). The Double-Ovsynch (day 0 GnRH, day 7 $\text{PGF}_{2\alpha}$, day 10

GnRH, day 17 GnRH, day 24 PGF_{2α}, followed 56 hours later by an injection of GnRH and timed AI 16 hours later) enhanced P/AI in primiparous (65.2 vs. 45.2%), but not multiparous cows (37.5 vs. 39.3%) in comparison to a conventional Presynch protocol (2 PGF_{2α}, 14 days apart, 12 days before the Ovsynch protocol). In a recent study, similar strategies were evaluated for presynchronization of lactating dairy cows managed under a grazing system (Ribeiro et al., 2009). Cows were either subjected to a Presynch program (2 PGF_{2α}, 14 days apart, 11 days before the synchronization protocol) or to a G6G program (PGF_{2α} followed 3 days later by GnRH, 6 days before the synchronization protocol). The synchronization protocol consisted of an injection of GnRH followed 5 and 6 days later by 2 injections of PGF_{2α}. A second GnRH was administered concurrently with timed AI 3 days after the first PGF_{2α}. There were no overall differences in fertility between presynchronization programs.

Intravaginal inserts for controlled release of progesterone have been used to improve synchrony of ovulation and P/AI in response to timed AI protocols (Lima et al., 2009; Chebel et al., 2010). Progesterone released by the CIDR insert acts to reduce LH pulsatility and to block the pre-ovulatory LH surge (Rathbone et al., 2001); therefore, it potentially minimizes the negative effects of spontaneous luteolysis before the injection of PGF_{2α} and reduces the occurrence of premature ovulations during a timed AI protocol. When cows have their estrous cycle presynchronized before a timed AI program, incorporation of a CIDR insert to the synchronization protocol was not beneficial to fertility (El-Zarkouny et al., 2004; Galvão et al., 2004). Conversely, when cows were not presynchronized, the incorporation of a CIDR insert to the Ovsynch protocol improved P/AI from 40 to 50% on day 28 after AI, and from 33 to 38% on days

56 after AI (Stevenson et al., 2006). However, these results are not in agreement with others that reported no benefit for the incorporation of a CIDR insert to the Ovsynch protocol for non-presynchronized cows compared with those subjected to a Presynch program (El-Zarkouny et al., 2004).

After the first postpartum AI, 55 to 65% of the cows remain nonpregnant and should receive a new insemination soon after the nonpregnancy diagnosis. The initiation of the protocol for resynchronization with GnRH before the pregnancy diagnosis has been shown to anticipate the second AI postpartum without affecting P/AI and pregnancy loss to the first and second AI (Chebel et al., 2003). A single injection of GnRH administered from 28 to 38 days after previous AI induced ovulation in 57.1% of the treated cows, whereas the ovulatory response following administration of different doses of human chorionic gonadotropin (**hCG**) ranged from 35.7 to 85.7% (Buttrey et al., 2010). Induction of ovulation with hCG tended to increase the risk of pregnancy loss relative to previous AI in that particular study. Overall P/AI was low; however, did not differ between cows that received the resynchronization protocol before the nonpregnancy diagnosis. Chebel et al. (2003) reported that the initiation of the Ovsynch protocol on day 21 after previous AI resulted in acceptable P/AI following the second AI postpartum. Furthermore, P/AI for cows that received the resynchronization protocol beginning 7 days before the nonpregnancy diagnosis was similar to that observed for cows that received the resynchronization protocol beginning on the day of nonpregnancy diagnosis. The stage of the estrous cycle at which the resynchronization protocol is initiated affects ovarian responses to hormonal treatments, synchrony of ovulation and, consequently, P/AI (Vasconcelos et al., 1999; Fricke et al., 2003).

Pregnancy per AI was greater for cows that received the resynchronization protocol beginning on days 26 and 33 after previous AI than for cows that had this interval reduced to 19 days (Fricke et al., 2003). In the same study, no difference was found for P/AI between cows resynchronized on days 26 and 33 after previous AI. Conversely, Sterry et al. (2006) reported an increase in P/AI for cows resynchronized beginning on day 33 rather than 26 after previous AI. It was shown that the administration of PGF_{2α} 12 days before the initial GnRH of the resynchronization protocol reduced pregnancy losses between days 31 and 66 after AI, resulting in greater P/AI on day 66 after AI (Silva et al., 2007). Nonetheless, the stage of the estrous cycle at the beginning of resynchronization protocols is generally unknown, mainly because presynchronization would delay subsequent AI and increase overall days to pregnancy. As mentioned earlier, the use of CIDR inserts improved synchrony of ovulation (Lima et al., 2009; Chebel et al., 2010) and P/AI for cows that were not presynchronized before timed AI protocols (Stevenson et al., 2006).

Delayed Ovulation Postpartum in Dairy Cows

Elevated circulating concentrations of estradiol and progesterone during late gestation suppress the release of gonadotropins by the pituitary gland and development of ovarian follicles (Ginther et al., 1996b). Resumption of FSH release is observed within the first and second week postpartum (Beam and Butler, 1997); however, the pulsatile release of LH to support growth of dominant follicles and ovulation prevent early ovulation postpartum (Roche et al., 1992). The extent of anovulation postpartum is affected by several factors, such as health disorders and negative energy balance (Santos et al., 2009), and might range from 18 to 24 days up to 2 to 3 months. Wiltbank et al. (2002) characterized 3 primary physiological patterns of anovulatory conditions in

dairy cows. The terms inactive or static ovaries have been used to describe cows on which ovarian follicles do not grow larger than the diameter of deviation (e.g.: 8.5 mm). This condition is observed in cows that undergo extensive feed deprivation and severe emaciation. Nonetheless, delayed ovulation postpartum associated with inactive ovaries is rare in US dairy herds. Development of ovarian follicles past the diameter of deviation that fail to ovulate is the most prevalent anovulatory condition of dairy cows (Gümen et al., 2003). It has been associated with reduced production of estradiol by the dominant follicle and increased sensitivity of the hypothalamus to the negative feedback induced by follicular estradiol. Early lactation cows have high concentrations of GH and low concentrations of IGF-1 because of uncoupling of the somatotrophic axis in the liver. As feed intake increases and energy balance improves, the concentrations of insulin in plasma also increases because of the greater flux of propionate and synthesis of glucose by the liver. The increase in plasma concentrations of insulin as energy balance improves seems to be one of the signals to reestablish the GH receptor population in the liver of cows (Butler et al., 2003). This increase in GH receptor 1A in the hepatic tissue re-couples the GH/IGF-1 axis causing substantial increases in plasma concentrations of IGF-1 and enhancing the steroidogenic capacity of ovarian follicles (Butler et al., 2004). Furthermore, undernutrition has been linked to incapacity of the hypothalamus to sustain high frequency of LH pulses, which would compromise growth and steroidogenesis in the pre-ovulatory follicle (Schillo, 1992). Collectively, these conditions lead to reoccurring follicular waves that are anovulatory (Beam and Buttler, 1997). Finally, follicular cysts has been defined as structures larger than 18 mm in diameter in the absence of CL that are associated with variable sexual behavior, such

as lack of standing estrus, nymphomania, and masculinization. The underlying mechanism involves a lack of estrogen receptor activity in the hypothalamus, which blocks the estradiol positive feedback on GnRH/LH release and subsequent ovulation (Gümen and Wiltbank, 2002; 2005; Nanda et al., 1991).

The prevalence of anovular cows at 65 days postpartum averaged 24.1% in US dairy herds and ranged from 18.6 to 41.2% among herds (Santos et al. 2009). Cows that suffered from calving difficulty, twin births, retained placenta, displacement of abomasum, and lameness during the first 66 days of lactation were more prone to have delayed resumption of cyclicity (Walsh et al., 2007; Santos et al., 2009). Furthermore, the prevalence anovulation at the end of the VWP was greater for primiparous than multiparous cows (Santos et al., 2009). In accordance with the underlying mechanisms suggested by Wiltbank et al., (2002), cows with low body condition score (**BCS**) and cows that lost excessive BCS in the first weeks postpartum were at a higher risk of being diagnosed as anovular at the end of the VWP (Santos et al., 2009). It is important to indicate that, within herds, more productive cows are not at a higher risk of having delayed postpartum ovulation. In fact, Santos et al. (2009) observed that lower producing cows, those with average milk yield of 32.1 kg/d in the first 3 months postpartum, were 21% less likely to have resumed ovulation by 65 days postpartum compared with cows producing 50 kg/d in the same period postpartum.

Delayed return of cyclic activity in postpartum dairy cows typically results in impaired reproductive performance of the herd because of depressed estrous expression, reduced P/AI, and increased risk of pregnancy loss (Santos et al., 2009). Anovulation likely has a greater negative impact in herds that perform AI following

estrous detection because anovular cows would be less likely to be inseminated compared with cyclic cows. The ovulatory follicle of anovular cows subjected to GnRH/PGF_{2α}-based timed AI programs develops under low concentrations of progesterone as they are typically in metestrus and early diestrus when ovulation is induced and AI is performed. This is because anovular cows generally have high ovulatory response to an injection of GnRH (Gümen et al., 2003); therefore, a new estrous cycle is initiated following the first injection of GnRH of synchronization protocols. The development of the ovulatory follicle under low concentrations of progesterone influences the composition of the follicular fluid (Cerri et al., 2008a), alters subsequent morphology of uterine glands (Shaham-Albalancy et al., 1997), increases oxytocin-induced release PGF_{2α} (Shaham-Albalancy et al., 2001; Cerri et al., 2008a) and the incidence of shortened luteal phases (Cerri et al., 2008a), and compromise embryo quality (Cerri et al., 2008b; Rivera et al., 2009). Not only anovular cows have reduce P/AI after the first AI postpartum, but they also have increased risk of pregnancy loss (Santos et al., 2004; McDougall et al., 2005), and reduced overall pregnancy rate (Walsh et al., 2007).

Overall Hypotheses and Objectives

The understanding of the processes that orchestrate the biology of dairy cows is crucial to develop technology to improve the reproductive performance of dairy herds. The oocyte carries the “burden” needed to sustain embryonic development up to the 8-cell stage (Memili and First, 2000) and changes in the oocyte can be expressed later during embryonic development. The oocyte develops enclosed into the follicle and participates in a delicate concert that responds to endocrine, paracrine and autocrine signals (McNatty et al., 2007). Moreover, modulation of the endocrine milieu on which

the follicle develops has been shown to affect not only the oocyte, but also uterine morphology and luteal function in the first weeks after insemination (Shaham-Albalancy et al., 1997, 2001; Cerri et al., 2008a).

Minimizing the proportion of cows that have not resumed ovarian cyclicity by the end of the VWP is critical to fertility (Santos et al., 2004; Walsh et al., 2007; Santos et al., 2009). Meanwhile, the understanding of the mechanisms by which anovulation leads to impaired fertility would provide means to counteract these effects. Particularly, it might lead to development of new technologies that target the critical period in which postpartum anovulation depresses fertility. Previous results have suggested that the negative effects of anovulation on fertility of dairy cows subjected to timed AI protocols might be mediated by the ovulation of a first wave (**FW**) follicle that grows under reduced concentrations of progesterone (Shaham-Albalancy et al., 1997, 2001; Gümen et al., 2003; Cerri et al., 2008a, 2008b; Denicol et al., 2009; Rivera et al., 2009). Therefore, the hypotheses for the study presented in the Chapter 3 were that ovulation of FW dominant follicle at AI would result in fertility similar to that of anovular cows and lesser than that of cows that ovulate the second wave (**SW**) dominant follicle.

Furthermore, it was hypothesized in Chapter 4 that this reduction in fertility would be mediated by low concentrations of progesterone during follicular growth associated with impaired embryonic development, but not to poor luteal function during early gestation.

The length of follicular dominance affects oocyte competence, embryo viability, and subsequent fertility of dairy cows (Revah and Butler, 1996; Cerri et al., 2009; Bleach et al., 2004). This knowledge resulted in a 5-day timed AI protocol that enhanced P/AI in lactating dairy cows compared with the traditional 7-day program

(Santos et al., 2010). Nevertheless, the reduced ovulatory diameter, estradiol concentrations in plasma, and proportion of cows detected in estrus on the day of timed AI (Santos et al., 2010) suggest that the ovulatory follicle might benefit from a longer proestrus in this type of program (Bridges et al., 2008). It has been demonstrated that, within the 7-day protocol, induction of ovulation with GnRH 16 hours before AI resulted in the greatest P/AI (Pursley et al., 1998; Brusveen et al., 2008). It was hypothesized in the first experiment presented in Chapter 5 that, in a 5-day timed AI protocol, the delay of the final GnRH to induce ovulation from 16 hours before AI to the time of AI would not alter fertility. Finally, the stage of the estrous cycle on which cows have their ovulation resynchronized is generally unknown, which might reduce response to hormonal treatments and subsequent fertility (Vasconcelos et al., 1999). The hypothesis of the second experiment presented in Chapter 5 was that supplementation with progesterone during resynchronization would improve synchronization of ovulation by minimizing the effects of premature luteolysis, thereby enhancing fertility of lactating dairy cows.

CHAPTER 3
FOLLICULAR WAVE OF THE OVULATORY FOLLICLE AND NOT CYCLIC STATUS
INFLUENCES FERTILITY OF DAIRY COWS

Two experiments evaluated the influence of follicular wave at artificial insemination (**AI**) on fertility of dairy cows. In experiment 1, data from 5,607 lactating cows enrolled in estrous and ovulation synchronization programs for AI were evaluated. Cows' blood was analyzed for progesterone 7 to 14 d apart, with the second sample collected on the day of the first GnRH (**GnRH1**) of the synchronization protocol. Cows were classified as cyclic if progesterone was ≥ 1 ng/mL in at least 1 of the 2 samples and as anovular if both samples were < 1 ng/mL. Cyclic cows were categorized as low (**CLOW**; < 1 ng/mL) or high (**CHIGH**; ≥ 1 ng/mL) progesterone on the day of GnRH1, which would result in ovulation of the dominant follicle of the first (**FW**) and second (**SW**) follicular waves, respectively, at AI. Pregnancy per AI (**P/AI**) was determined 30 and 53 d after AI. In experiment 2, 220 cyclic Holstein cows received 2 injections of prostaglandin (**PG**) $F_{2\alpha}$ administered 14 d apart. The Ovsynch protocol (d 0 GnRH, d 7 PGF $_{2\alpha}$, d 9 GnRH, d 9.5 timed AI) was initiated either 3 or 10 d after the second PGF $_{2\alpha}$ of the presynchronization to result in insemination to the FW or SW dominant follicles. Blood was analyzed for progesterone and ovaries were scanned to determine ovulatory responses and follicle diameter. Pregnancy was determined on d 32 and 67 after timed AI. In experiment 1, P/AI on d 30 was greater for CHIGH cows than for CLOW and anovular cows (43.0, 31.3, and 29.7%, respectively), but because of pregnancy loss, P/AI on d 53 was lowest for anovular cows. Proportions of cows with short reinsemination intervals differed among groups and were 7.1, 15.7, and 11.9% for CHIGH, CLOW, and anovular cows, respectively. Pregnancy loss was greater for anovular cows than for CLOW cows (15.0 vs. 10.0%) and was intermediate for CHIGH cows (13.5%). In experiment 2, 9.8 and

97.2% of the FW and SW cows, respectively, had progesterone ≥ 1 ng/mL at GnRH1. Concentrations of progesterone at the GnRH1 and PGF_{2 α} injections of the Ovsynch protocol were greater for SW cows than FW cows. Pregnancy per AI was greater for SW cows than for FW cows (41.7 vs. 30.4%) despite less ovulation to GnRH1 in SW cows than in FW cows (78.7 vs. 88.4%). Collectively, these data indicate that follicular wave of the ovulatory follicle and not cyclic status caused the greatest reduction in P/AI in dairy cows. Whether the culprit is the follicle itself or the hormonal milieu characteristic of the first follicular wave and the early stage of the estrous cycle remains to be elucidated. Synchronization programs that induced ovulation of the FW follicle at AI reduced P/AI in lactating dairy cows, and ovulation of the FW follicle, or development of the ovulatory follicle under low progesterone concentrations, or both, might be mechanisms for reduced fertility in anovular cows.

Introduction

Protocols for synchronization of ovulation and timed AI based on GnRH and PGF_{2 α} maximize the proportion of cows inseminated early after the end of the voluntary waiting period and result in satisfactory pregnancy per AI when implemented in commercial dairy herds (Chebel et al., 2006). Optimal P/AI in response to the Ovsynch protocol in lactating dairy cows has been associated with successful ovulation to the first GnRH injection and the presence of a functional corpus luteum (**CL**) at the injection of PGF_{2 α} (Vasconcelos et al., 1999; Moreira et al., 2001; Chebel et al., 2006).

The proportion of lactating dairy cows that ovulate to the GnRH1 is greater when it is administered in the presence of a dominant follicle, usually between d 5 and 9 and d 17 and 21 of the estrous cycle (Vasconcelos et al., 1999; Bello et al., 2006; Cerri et al., 2009). However, the proportion of cows with concentrations of progesterone < 1 ng/mL

at the injection of PGF_{2α} and the incidence of ovulation before the final GnRH was higher when the Ovsynch protocol was initiated after d 10 of the estrous cycle (Vasconcelos et al., 1999). Inducing ovulation of the dominant follicle of the first follicular wave during early diestrus (i.e.: d 5 to 9 of the estrous cycle) with the GnRH1 results in recruitment of the second follicular wave which will generate the ovulatory follicle at timed AI. In contrast, when GnRH1 is administered before the acquisition of ovulatory capacity by the FW follicle (d 1 to 4 of the estrous cycle; Sartori et al., 2001) or when ovulation of the SW dominant follicle is induced during proestrus (d 17 to 21 of the estrous cycle), the FW dominant follicle is induced to ovulate at completion of the timed AI protocol before AI. The FW dominant follicle grows under smaller systemic concentrations of progesterone than the SW follicle, which has been associated with changes in the rate of follicle growth and follicular fluid composition (Cerri et al., 2008a), increased endometrial synthesis of PGF_{2α} (Shaham-Albalancy et al., 2001), and incidence of short estrous cycles (Cerri et al., 2008a). Consequently, cows that received the GnRH1 of the timed AI protocol with progesterone ≥ 1 ng/mL had greater P/AI than cows with progesterone < 1 ng/mL (Cerri et al., 2004; Stevenson et al., 2008).

Anovular cows are more likely to ovulate to GnRH1 (Gümen et al., 2003) and, among those that ovulate to GnRH1, the follicle induced to ovulate at the end of the synchronization protocol is the FW dominant follicle. Therefore, cyclic cows that initiate the timed AI protocol when progesterone is < 1 ng/mL and anovular cows are similar in that they both ovulate the FW dominant follicle at AI. It is known that anovular cows have reduced reproductive performance (Cerri et al., 2004; Santos et al., 2009). Therefore, if fertility of anovular cows and that of cyclic cows that ovulate the FW

dominant follicle are similar and they are both less than that of cyclic cows ovulating the SW dominant follicle, it is then reasonable to speculate that some of the underlying mechanisms responsible for the reduced P/AI are similar between them, particularly the fact that these cows are induced to ovulate follicles that develop under progesterone concentrations often <1ng/mL.

The hypothesis of the present study was that ovulation of the FW dominant follicle at AI reduces fertility in lactating dairy cows compared with ovulation of the SW dominant follicle. It was expected that cows ovulating the FW dominant follicle for AI would have reduced P/AI compared with cows induced to ovulate the SW dominant follicle for AI despite cyclic status at the beginning of the timed AI protocol. In experiment 1, the objectives were to determine the associations of cyclic status and follicular wave at AI with P/AI and risk of pregnancy loss in lactating dairy cows; in experiment 2, the objective was to evaluate the effect of follicular wave on ovulatory responses and P/AI of lactating dairy cows.

Materials and Methods

Experiment 1

Data from previously completed experiments (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004a,b; Chebel et al., 2006; Juchem, 2007; Hillegass et al., 2008; J. E. P. Santos; unpublished data) were collated into a single data set containing a total of 5,607 cows subjected to presynchronized timed AI or estrous synchronization protocols for first postpartum AI. Only cows that did not receive supplemental progesterone during the timed AI or estrous synchronization protocols were used. Detailed description of animals and management are presented in the published papers. In those studies, the proportion of cows calving in the fall, winter, spring, and

summer were 26.7, 52.2, 13.8, and 7.3%, respectively. Inseminations were performed between the months of September and May to avoid the negative effects of heat stress on fertility.

Reproductive management

Detailed description of reproductive management is presented in the published papers. Briefly, the estrous cycles of cows were presynchronized (Moreira et al., 2001) with 2 injections of 25 mg of PGF_{2α} (Lutalyse, 5 mg/mL of dinoprost tromethamine sterile solution, Pfizer Animal Health, New York, NY) administered 14 d apart, and either 12 or 14 d after the second injection of PGF_{2α} they were enrolled in an estrous or ovulation synchronization protocol. For both synchronizations, the initial portion of the protocols was similar and consisted of a 100-μg injection of GnRH (Cystorelin, 50 g/mL of gonadorelin diacetate tetrahydrate, Merial Ltd., Iselin, NJ) followed 7 d later by an injection of 25 mg of PGF_{2α}. Cows subjected to insemination upon detection of estrus did not receive any further treatment. These cows were observed for signs of estrus once daily, in the morning, by tail chalking using paint sticks (All-weather Paintstik, La-Co Industries, Chicago, IL), and those observed in estrus based on rubbed chalk were inseminated in the same morning in 3 studies (Cerri et al., 2004; Santos et al., 2004a,b). Cows assigned to timed AI either received an injection of 1 mg of estradiol cypionate (ECP, 2 mg/mL of estradiol cypionate, Pfizer Animal Health) and were inseminated 48 h later (Cerri et al., 2004; Galvão et al., 2004) or received an injection of GnRH 48 to 56 h after PGF_{2α} and were timed AI 12 to 24 h later (Santos et al., 2004a; Chebel et al., 2006; Juchem, 2007). In one study (Hillegass et al., 2008), all cows received GnRH concurrent with AI at either 48 or 72 h after PGF_{2α} to induce ovulation, and half of them were supplemented with estradiol cypionate 24 h after PGF_{2α}.

After the first AI, all cows observed to have the tail head paint removed were reinseminated. Cows with an inter-insemination interval between 5 and 17 d after the first AI were considered to have short inter-AI interval.

Blood sampling and progesterone analysis

Blood was sampled 7 to 14 d apart for analysis of progesterone. The second sample was collected on the day of the GnRH1. The first sample was collected either 7 (Hillegass et al., 2008), 12 (Santos et al., 2004a; J. E. P. Santos; unpublished data), 13 (Chebel et al., 2006), or 14 d earlier (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004b; Juchem, 2007). Approximately 7 mL of blood was collected by puncture of the median coccygeal vein or artery using evacuated tubes with K₂ ethylenediaminetetraacetic acid (**EDTA**; Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The samples were immediately placed in ice and were later centrifuged at 2,000 × g for 15 min for separation of plasma. Plasma samples were frozen at -25°C until later analysis by enzyme-linked immunosorbent assay (**ELISA**; Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004a; Chebel et al., 2006; Juchem, 2007; Hillegass et al., 2008; J. E. P. Santos; unpublished data) or radioimmunoassay (**RIA**; Santos et al., 2004b) for progesterone.

Classification of cows according to progesterone status

Cows were classified as having initiated estrous cycles if progesterone concentration was ≥1 ng/mL in at least 1 of the 2 samples, or as anovular when both samples were <1 ng/mL. Cyclic cows were further classified as having progesterone concentration <1 ng/mL on the day of GnRH1 (cyclic-low; **CLOW**) or as having ≥1 ng/mL on the day of GnRH1 (cyclic-high; **CHIGH**). Therefore, 3 groups of cows were created: anovular, CLOW, and CHIGH. The rationale for such categories was that

presynchronized cyclic cows that had low concentration of progesterone on the day of GnRH1 were in either proestrus, estrus, or metestrus and, therefore, when completing the synchronization protocol, were expected to have the FW dominant follicle induced to ovulate. On the other hand, presynchronized cows classified as CHIGH were expected to be in early or mid diestrus and likely ovulated the SW dominant follicle at the end of synchronization protocol. A small proportion of CHIGH cows could also ovulate the SW dominant follicle to GnRH1 if on d 13 of the estrous cycle, which would result in ovulation of the third wave dominant follicle at AI. Similar to FW cows, anovular cows subjected to the synchronization programs based on GnRH and PGF_{2α} were expected to be inseminated to an ovulation of the FW dominant follicle.

Pregnancy diagnosis

Cows were examined for pregnancy twice. The first diagnosis was performed by ultrasonography between 27 and 32 d after AI (Cerri et al., 2004; Galvão et al., 2004; Santos et al., 2004a,b; Chebel et al., 2006; Juchem, 2007; J. E. P. Santos; unpublished data) or by transrectal palpation at 40 d after AI (Hillegass et al., 2008). Pregnant cows in the first exam were reevaluated for pregnancy at 2 (Galvão et al., 2004; Santos et al., 2004a, b; J. E. P. Santos; unpublished data) or 4 wk later (Cerri et al., 2004; Chebel et al., 2006; Juchem, 2007; Hillegass et al., 2008). Throughout the paper, the first diagnosis is designated as d 30 and the second as d 53 after AI because those were the average intervals from AI to pregnancy diagnosis for all studies combined.

Pregnancy per AI was calculated as the proportion of inseminated cows pregnant at either 30 or 53 d after AI. Pregnancy loss was calculated by dividing the number of cows diagnosed not pregnant on d 53 by the number of cows pregnant on d 30.

Experiment 2

Animals, housing, and feeding

Holstein cows ($n = 220$) from a commercial dairy farm were enrolled in this experiment. Cows were housed in free-stall barns and primiparous and multiparous cows were housed separately throughout the experiment. Cows were fed diets formulated by CPM-Dairy software (Cornell-Penn-Miner version 3.0.8; Miner Institute, Chazy, NY) to meet the metabolizable energy and protein, mineral, and vitamin requirements for lactating Holstein cows weighing 650 kg and producing 45 kg of 3.5% FCM when consuming 26 kg/d of DM (NRC, 2001). All diets were mixed as complete rations and offered twice daily at 0600 and 1600 h to allow for 3 to 5% refusals. Ingredients were alfalfa hay, alfalfa silage, corn silage, steam-flaked corn, citrus pulp, whole cottonseed, solvent-extracted soybean meal, expeller-treated soybean meal, a protein blend of animal-marine byproducts, Ca salts of palm oil, and a mixture of vitamins and minerals.

Treatments

Weekly, cohorts of 8 to 22 lactating dairy cows at 37 ± 3 days in milk (**DIM**) were blocked by parity (lactation 1 or lactation >1) and milk yield in the first month postpartum and, within each block, were randomly assigned to 1 of the 2 treatments, insemination to the dominant follicle of the FW (42 primiparous and 70 multiparous) or SW (36 primiparous and 72 multiparous).

The estrous cycles of cows were presynchronized with 2 subcutaneous injections of 25 mg of $\text{PGF}_{2\alpha}$ as dinoprost tromethamine (5 mL of Lutalyse Sterile Solution, Pfizer Animal Health) given 14 d apart, with the injections given at 44 ± 3 and 58 ± 3 DIM for cows in treatment FW and at 37 ± 3 and 51 ± 3 DIM for cows in treatment SW (Figure 3-

1). These schemes for presynchronization were designed such that cows in FW and SW would initiate the timed AI protocol 3 and 10 d after the second PGF_{2α}, respectively, and they would be inseminated on the same day postpartum.

The Ovsynch protocol was initiated at 61 ± 3 DIM of and consisted of an intramuscular injection of 100 µg GnRH (Cystorelin, 50 µg/mL of gonadorelin diacetate tetrahydrate, Merial Ltd.), followed 7 d later by a subcutaneous injection of 25 mg of PGF_{2α} on d 68 ± 3 postpartum and 48 h later by an intramuscular injection of 100 µg of GnRH on d 70 ± 3 postpartum, with timed AI performed 12 h after the final GnRH injection of the Ovsynch protocol. The same technician inseminated all cows in the experiment with semen from 5 different sires equally allocated across treatments.

Ovarian ultrasonography

Transrectal ultrasonography of the ovaries was performed using an ultrasound equipped with a 7.5-MHz linear transducer (Sonovet 2000, Universal Medical System, Bedford Hills, NY) on the day of the second PGF_{2α} of the presynchronization protocol. Initially, 247 cows were evaluated, but only those with a visible CL > 18 mm in diameter remained in the experiment (n = 220 cows) to ensure that all cows were cyclic, which was later confirmed by a plasma sample with progesterone ≥ 1 ng/mL. The 220 cows that continued the experiment also had their ovaries scanned on the day of GnRH1 and again 7 d later, on the day of the PGF_{2α} injection of the Ovsynch protocol. Ovarian maps were drawn and diameter and position of the CL and follicles ≥ 8 mm in diameter were recorded. Cows with a follicle ≥ 8 mm on the day of GnRH1 injection of the timed AI protocol and with a newly formed CL 7 d later on the ipsilateral ovary were considered to have ovulated in response to GnRH1.

Blood sampling and progesterone analysis

Approximately 7 mL of blood was collected by puncture of the coccygeal artery or vein using evacuated tubes with K₂ EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Samples were collected on the day of the second PGF_{2α} of the presynchronization protocol, on the day of GnRH1, and again on the day of the PGF_{2α} of the Ovsynch protocol (Figure 3-1). Samples were placed in ice upon collection and were transported to the laboratory within 5 h. Blood samples were centrifuged and plasma was separated and frozen at -25°C and later analyzed for progesterone concentration by a validated ELISA (Cerri et al., 2004). Individual samples were analyzed in duplicate and samples with a coefficient of variation >15% were reanalyzed. Sensitivity of the assay was 0.10 ng/mL and the intra- and interassay coefficients of variation were 5.4 and 9.9%, respectively.

Body condition score (BSC) and milk yield

Cows were scored for body condition (1 = emaciated, 5 = obese) according to Ferguson et al. (1994) on the day of GnRH1. Cows were milked twice daily and milk yield from the first 3 mo postpartum was recorded for individual cows, and the average milk production in those months was used in the statistical analysis.

Pregnancy diagnosis and calculation of reproductive responses

Pregnancy was diagnosed by transrectal ultrasonography on d 32 after the timed AI. Presence of an amniotic vesicle with an embryo was used as indicators of pregnancy. Pregnant cows were reexamined for pregnancy 5 wk later, on d 67 of gestation.

Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at 32 or 67 d after AI by the number of cows receiving AI. The proportion of

cows having experienced pregnancy loss was calculated as the number of cows that lost pregnancy between 32 and 67 d after AI divided by the number of cows diagnosed pregnant on d 32 after AI.

Study Design and Statistical Analyses

Experiment 1

Information from individual studies was collated into a single data set for statistical analysis. Data were analyzed by a multivariate logistic regression using the LOGISTIC procedure of SAS (SAS/STAT version 9.2, SAS Institute Inc., Cary, NC). A backward stepwise regression model was used, and explanatory variables were sequentially removed from the model by the Wald statistic criterion if $P > 0.10$. Pregnancy per AI and incidence of pregnancy loss and short inter-AI interval were analyzed with a model that included group (anovular, CLOW, and CHIGH), study (1 to 8), season of calving (fall, winter, spring, and summer), parity (primiparous and multiparous), method of AI (timed AI or synchronized estrus), BCS, milk yield, and interaction between group and method of AI. Adjusted odds ratio (**AOR**) and 95% confidence interval were generated by the logistic regression. Comparisons were performed among individual groups (anovular vs. CLOW vs. CHIGH), and an orthogonal comparison was performed to determine the effect of follicular wave (anovular + CLOW vs. CHIGH) on reproductive responses of dairy cows.

Experiment 2

Experiment 2 was a completely randomized design with blocks. Based on results of experiment 1, it was expected that a 12 percentage units decrease (e.g., 31 vs. 43%) in P/AI would be observed in cows inseminated when the FW dominant follicle was induced to ovulate compared with cows inseminated following induction of ovulation of

the SW dominant follicle. One hundred eighty cows were initially planned (90/treatment) to provide enough replicates to detect statistical significance with a 12 percentage unit improvement in P/AI for SW cows ($\alpha = 0.05$, $\beta = 0.90$; one-tailed test). Because of potential attrition before completion of the study, 220 cows were enrolled.

Binary data were analyzed by logistic regression using the LOGISTIC procedure of SAS (SAS Institute Inc.). The models included the effects of treatment, parity, BCS, and milk yield. Additional covariates were included when relevant. Variables with $P > 0.10$ were sequentially removed from the model by a backward elimination based on the Wald's statistics. Treatment was forced in the final models.

Concentrations of progesterone on the day of GnRH1 and PGF_{2 α} of the Ovsynch protocol were analyzed by analysis of variance (**ANOVA**) using the GLM procedure of SAS. For concentration of progesterone on the day GnRH1, the model included the effects of treatment, parity, BCS, milk yield, and interaction of treatment and parity. For concentration of progesterone on the day of the PGF_{2 α} of the Ovsynch, the model also included the effects of ovulation to GnRH1 and interaction between treatment and ovulation to GnRH1.

Diameter of the largest follicle at PGF_{2 α} injection was analyzed by ANOVA using the GLM procedure of SAS, and the model included the effects of treatment, parity, BCS, milk yield, ovulation to GnRH1, and interactions between treatment and parity and treatment and ovulation to GnRH1. In both experiments, differences with $P \leq 0.05$ were considered significant, and those with $0.05 < P \leq 0.10$ were considered a tendency.

Results

Experiment 1

The proportion of cows classified as anovular at the beginning of the synchronization protocol was 22.6%. Among the cyclic cows, 14.8 and 85.2% had low and high progesterone at GnRH1, respectively.

Ovulation of the SW dominant follicle reduced ($P < 0.0001$) incidence of short inter-AI interval, and CLOW cows had increased ($P = 0.03$) incidence of short inter-AI interval compared with anovular cows (Table 3-1). Of the nonpregnant cows, a lesser ($P < 0.0001$) proportion had short inter-AI interval when inseminated in estrus than when receiving timed AI (4.0 vs. 11.1%; AOR = 0.31; 95% CI = 0.20 to 0.50); however, no interaction ($P = 0.16$) between method of insemination and cyclic status or follicular wave was observed for short inter-AI interval.

The overall P/AI on d 30 after first postpartum AI averaged 38.8% and differed according to the follicular wave at insemination (Table 3-1). Cows that ovulated the SW dominant follicle had greater ($P < 0.001$) P/AI compared with cows that ovulated the FW dominant follicle (43.0 vs. 30.2%; AOR = 4.18; 95% CI = 2.51 to 6.95). In addition, cows ovulating to the dominant follicle of the FW had similar ($P = 0.31$) P/AI whether they were previously cyclic or anovular. Pregnancy per AI at d 30 after AI was greater ($P < 0.0001$) for cows inseminated in estrus than those receiving timed AI, but no interaction ($P = 0.74$) between method of insemination and cyclic status or follicular wave was observed. For anovular cows, P/AI for those inseminated in estrus or receiving timed AI were 38.2 and 26.8%, respectively; for CLOW, they were 41.8 and 28.8%, respectively; and for CHIGH they were 50.0 and 39.8%, respectively.

Responses at d 53 after AI were similar to those observed on d 30 (Table 3-1). Cows ovulating the SW dominant follicle had greater ($P < 0.001$) P/AI compared with cows that ovulated the FW dominant follicle (36.9 vs. 26.0%; AOR = 2.45; 95% CI = 1.87 to 3.21). Of the cows ovulating the dominant follicle of the FW, those classified as CLOW had greater P/AI (AOR = 1.28; 95% CI = 1.02 to 1.62; $P = 0.04$) than anovular cows. Cows inseminated in estrus had greater ($P < 0.0001$) P/AI than those receiving timed AI (42.0 vs. 29.9%; AOR = 1.77; 95% CI = 1.52 to 2.06), but no interaction ($P = 0.44$) between method of AI and cyclic status or follicular wave was observed.

Pregnancy loss averaged 13.5% and there was no overall effect ($P = 0.30$) of cows ovulating the FW or SW on losses of pregnancy between 30 and 53 d of gestation (Table 3-1). Nevertheless, anovular cows had greater (AOR = 2.04; 95% CI = 1.12 to 3.72; $P = 0.02$) and CHIGH cows tended (AOR = 1.71; 95% CI = 1.00 to 2.93; $P = 0.06$) to have greater pregnancy loss than CLOW cows. Cows inseminated in estrus had less ($P = 0.05$) pregnancy loss than those receiving timed AI (10.4 vs. 15.2%), but no interaction ($P = 0.30$) between method of AI and follicular wave or cyclic status was observed.

Experiment 2

Milk yield during the first 3 mo postpartum was similar ($P = 0.76$) for both treatments and averaged 46.9 ± 0.7 kg/d for multiparous cows and 36.3 ± 0.9 kg/d for primiparous cows. Average BCS did not differ ($P = 0.61$) between treatments, but primiparous cows (2.96 ± 0.04) had greater ($P = 0.01$) BCS compared with multiparous cows (2.83 ± 0.03).

Ovarian responses and progesterone concentration during the Ovsynch

The proportion of cows with high concentration of progesterone on the day of GnRH1 was greater ($P < 0.001$) for SW than for FW cows (Table 3-2). Accordingly, the mean concentration of progesterone on the day of GnRH1 was greater ($P < 0.0001$) for SW than for FW cows (Table 3-2). The ovulatory response to GnRH1 was greater ($P = 0.05$) for FW cows. On the day of the PGF_{2α} of the Ovsynch protocol, the proportion of cows with a visible CL tended to be less ($P = 0.10$) for SW than for FW cows, although mean concentration of progesterone was greater ($P = 0.002$) for cows in the SW than for cows in the FW treatment (Table 3-1). The dominant follicle at the PGF_{2α} of the Ovsynch protocol was larger ($P < 0.001$) for FW cows compared to SW cows (Table 3-1).

Pregnancy per AI and pregnancy loss

Pregnancy per AI on d 32 and 67 after AI averaged 35.9 and 34.1%, respectively. Cows induced to ovulate to the SW had greater P/AI on d 32 and 67 after AI (Table 3-3). In addition, cows that ovulated to GnRH1 of the Ovsynch protocol across treatments were more likely ($P < 0.01$) to be diagnosed pregnant on d 32 (39.7 vs. 16.7%; AOR = 3.37; 95% CI = 1.30 to 8.74) and 67 (38.0 vs. 13.9%; AOR = 4.08; 95% CI = 1.47 to 11.28) after AI than cows that did not ovulate to GnRH1. No interaction between treatment and ovulation to GnRH1 was observed for pregnancy on d 32 ($P = 0.63$) and 67 ($P = 0.85$). Cows with high progesterone on the day of the PGF_{2α} of the Ovsynch protocol had greater ($P = 0.02$) P/AI on d 32 after insemination than cows with low progesterone (37.4 vs. 4.6%; AOR = 10.91; 95% CI = 1.40 to 84.91) across treatments.

Pregnancy loss between d 32 and 67 of gestation was not affected by treatment (Table 3-3). Similarly, ovulation to GnRH1 did not influence ($P = 0.22$) the risk of cows losing their pregnancy in the first 67 d of gestation.

Discussion

Studies have consistently shown that cows that have not resumed ovulation before the first postpartum AI have reduced P/AI and increased risk of pregnancy loss when inseminated following timed AI protocols or following estrus (Gümen et al., 2003; Santos et al., 2004c; Santos et al., 2009). Although the association between anovulation and impaired reproductive performance is clear, the specific mechanisms by which it reduces fertility remain undefined. Results from experiment 1 showed that P/AI did not differ between anovular cows and cyclic cows that were induced to ovulate the FW dominant follicle before AI. According to Wiltbank et al. (2002), anovular postpartum dairy cows generally have continuous development of follicles that fail to ovulate spontaneously and, in some cases, develop follicular cysts. Nevertheless, the majority of these cows respond to an injection of GnRH through either ovulation or luteinization (Gümen et al., 2003). In that sense, anovular cows that successfully respond to a GnRH-PGF_{2 α} based synchronization protocol (i.e., ovulate to GnRH1, regress the newly formed CL in response to the PGF_{2 α} , and ovulate after the final GnRH) are expected to ovulate the FW dominant follicle at AI. Because a large proportion of anovular cows are expected to respond to a GnRH-PGF_{2 α} based synchronization protocol (Gümen et al., 2003), results observed in experiment 1 indicate that the ovulation of a FW dominant follicle might be one of the components of the poor fertility observed in anovular dairy cows.

Cyclic status and follicular wave before AI were characterized in experiment 1 based on the concentration of progesterone in samples collected on the day of GnRH1 and 7 to 14 d earlier. For cyclic cows with progesterone ≥ 1 ng/mL on the day of GnRH1, it was assumed that these cows were in diestrus, which would likely result in the ovulation of the SW dominant follicle in lactating dairy cows after a GnRH-PGF_{2 α} based synchronization protocol. On the other hand, cyclic cows with progesterone < 1 ng/mL at GnRH1 would likely be in proestrus, estrus, or metestrus and they would likely ovulate the FW dominant follicle at AI. The exception would be cows in metestrus, around d 3 of the cycle, that might ovulate to GnRH1 and recruit a SW dominant follicle to ovulate at the end of the synchronization program. Nevertheless, this proportion is expected to be very low (Vasconcelos et al., 1999; Cerri et al., 2009) and unlikely to change the interpretation of the results of this study. In fact, when cows were on d 3 of the estrous cycle, ovulation to GnRH1 and recruitment of the SW was observed in only 7.1% of them (Cerri et al., 2009). In addition, the small proportion of cows in diestrus at GnRH1 in FW cows in experiment 2 reinforces the criteria used in the first experiment to stage the estrous cycle and determine the follicular wave at AI. Because cyclic cows initiating the synchronization protocol with progesterone < 1 ng/mL in experiment 1 had reduced P/AI, but similar to that of anovular cows, experiment 2 was designed to specifically test the hypothesis that ovulation of the FW dominant follicle impairs P/AI in lactating dairy cows. Results from experiment 2 confirm the findings of experiment 1: P/AI was greater for cows induced to ovulate the SW dominant follicle at the end of the protocol.

A major difference between FW and SW dominant follicles is the hormonal milieu on which they develop. The FW dominant follicle emerges early in cycle and grows

under low and increasing concentrations of progesterone, whereas the SW grows during diestrus concurrent with a mature CL. Progesterone exerts a negative feedback on the pulsatility of luteinizing hormone (**LH**), and the latter stimulates the growth and steroidogenesis of dominant follicles (Evans et al., 1997). In accordance, cows induced to ovulate the FW dominant follicle had lower concentration of progesterone from the GnRH1 to the PGF_{2α} of the Ovsynch protocol, resulting in a larger preovulatory follicle in experiment 2. Previous studies have reported that the ovulation of larger follicles with increased steroidogenic capacity resulted in larger CL, greater concentration of progesterone during the subsequent diestrus, and increased P/AI (Vasconcelos et al., 2001; Lopes et al., 2007). Despite evidence of a greater steroidogenic capacity in luteinized theca cells from FW dominant follicles, no difference in progesterone production was found in luteinized granulosa cells originated from FW and SW dominant follicles or in concentration of progesterone of cows ovulating FW or SW dominant follicles (Wolfenson et al., 1999). Therefore, although the FW ovulatory follicle was larger at AI than the SW follicle, it is unlikely that progesterone concentrations in the subsequent estrous cycle differed in experiment 2.

Low concentration of progesterone during the development of the ovulatory follicle reduced the concentration of total Insulin-like growth factor-1 (**IGF-1**) in the follicular fluid of the preovulatory follicle (Cerri et al., 2008a). Insulin-like growth factor-1 increases ovarian responsiveness to gonadotropins, steroidogenic capacity, and proliferation of granulosa cells and protects the oocyte and granulosa cells from apoptosis (Quirk et al., 2000; Wasielak and Bogacki, 2007; Velazquez et al., 2008). The FW dominant follicle develops under lower concentrations of progesterone than the SW

dominant follicle. Low concentrations of progesterone might result in increased LH pulsatility, which can disrupt oocyte quality (Revah and Butler, 1996), thereby influencing fertility.

Superstimulated lactating dairy cows used for embryo collection had improved embryo quality when follicle stimulating hormone (**FSH**) treatment was initiated at the emergence of the second follicular wave, or at the first follicular wave with supplemental progesterone compared with the first follicular wave without progesterone (Rivera et al., 2009). Denicol et al. (2009) observed a tendency for increased P/AI on d 38 after timed insemination in cows induced to ovulate the FW dominant follicle supplemented with progesterone during the development of the ovulatory follicle compared with unsupplemented cows. Progesterone concentrations during the development of the ovulatory follicle were approximately 1 and 3 ng/mL for unsupplemented and supplemented cows, respectively (Denicol et al., 2009). Collectively, these data suggest that induction of ovulation of the FW dominant follicle impairs fertility of dairy cows because of the low concentrations of progesterone during the development of the ovulatory follicle, which might compromise oocyte and embryo quality.

Anovulation and consequent long-term lack of exposure to progesterone has been associated with shortened luteal lifespan after the first postpartum ovulation and reduced P/AI in dairy and beef cattle (Crowe, 2008). Nevertheless, recent data suggest that insufficient concentration of progesterone during the development of the ovulatory follicle and not necessarily throughout the entire postpartum period is associated with poor fertility. In fact, supplementation with progesterone from 20 to 15 d before ovulation was unable to extend the interestrus interval (Kyle et al., 1992). Conversely, the

increase in the concentration of 13,14-dihydro-15-keto PGF_{2α} (**PGFM**) in response to an injection of oxytocin on d 15 or 16 of the estrous cycle was higher for cyclic cows with low compared with cows with high concentrations of progesterone during the development of the ovulatory follicle (Shaham-Albalancy et al., 2001; Cerri et al., 2008a). In fact, cows ovulating the SW dominant follicle that developed under low concentrations of progesterone had increased incidence of short luteal phase (25%) compared with cows developing the ovulatory follicle under high (0%) concentrations of progesterone (Cerri et al., 2008a). In experiment 1, CLOW cows had increased incidence of short inter-AI interval compared with CHIGH cows, suggesting either lack of synchronization or potentially premature luteolysis.

The endometrial expression of oxytocin receptors is prevented by the action of progesterone until mid diestrus, at which time progesterone receptors are downregulated in the luminal epithelium and superficial glands and the binding of oxytocin to its receptors triggers the luteolytic cascade (McCracken et al., 1999). It has been proposed that exposure to progesterone before ovulation programs the endometrial dynamics of progesterone and oxytocin receptors in the subsequent cycle and, consequently, has an effect on interestrus interval. Postpartum beef cows supplemented with a progestogen (i.e., norgestomet) during the development of the ovulatory follicle had increased endometrial expression of receptors for progesterone and reduced expression of receptors for oxytocin at d 5 of the subsequent cycle compared with untreated controls (Zollers et al., 1993). This is likely to be the cause of premature endometrial responsiveness to oxytocin in cows not exposed to progesterone in the preceding estrous cycle (Zollers et al., 1989). Sá Filho et al. (2009) reported

smaller abundance of mRNA for oxytocin receptors on d -2, 0, and 5 relative to ovulation in postpartum beef cows pretreated with progesterone, but no changes in expression of progesterone receptor mRNA were found during the same period. Therefore, it is plausible that inadequate exposure to progesterone during the development of the ovulatory follicle might enhance the responsiveness of the endometrium to oxytocin and result in premature luteolysis in more cows ovulating the FW compared with the SW dominant follicle.

The success of AI programs is related to cyclic status and the stage of the estrous cycle at which synchronization protocols are initiated (Vasconcelos et al., 1999; Santos et al., 2004c; Cerri et al., 2009). Increased ovulation to GnRH1 combined with the presence of a functional CL at the induced luteolysis and ovulation to the final GnRH were achieved when the protocol was initiated from d 5 to 10 of the estrous cycle (Vasconcelos et al., 1999; Bello et al., 2006) and have been linked with improved embryo quality (Cerri et al., 2009) and increased P/AI (Vasconcelos et al., 1999; Moreira et al., 2001; Chebel et al., 2006). Conversely, in experiment 2 of the present study the proportion of cows that ovulated to GnRH1 was greater and the proportion of cows with a functional CL at the PGF_{2α} of the Ovsynch protocol tended to be greater in FW cows than SW cows; however, these same cows had reduced P/AI on d 32 and 67 after AI. It is known that anovular cows and cows in proestrus are more likely to ovulate to GnRH1 (Gümen et al., 2003), but the growth of the FW dominant follicle under reduced concentrations of progesterone and the induced ovulation of the FW dominant follicle at AI likely negate the benefits of high ovulation to GnRH1 on P/AI. Because anovular cows subjected to GnRH-PGF_{2α} based synchronization protocols ovulated the

FW dominant follicle at AI, it is suggested that reduced P/AI in these cows is caused by the ovulation of the FW dominant follicle that develops under low concentrations of progesterone and not by complete lack of previous exposure to progesterone.

Conclusion

The follicular wave of the ovulatory follicle affected fertility of dairy cows submitted to synchronization protocols. Pregnancy per AI increased in cows that ovulated the SW dominant follicle compared with cows induced to ovulate the dominant follicle of the FW. Furthermore, the reproductive responses evaluated for cows that ovulated the FW dominant follicle and for anovular cows were similar. Collectively, these data indicate that follicular wave of the ovulatory follicle and not cyclic status had the greatest effect reducing P/AI of dairy cows. Nevertheless, in experiment 1, both follicle wave and, to a lesser extent, cyclic status influenced P/AI on d 53 after insemination. Whether the culprit for reduced fertility in cows ovulating the FW dominant follicle is the follicle itself or the hormonal milieu characteristic of the FW and the early stage of the estrous cycle remains to be elucidated. These data indicate that some of the underlying mechanisms by which delayed resumption of ovulation reduces fertility are associated with the ovulation of the FW dominant follicle and the related changes in the hormonal milieu during growth of the ovulatory follicle. Furthermore, they also indicate that synchronization programs for lactating dairy cows should be designed to result in ovulation of the SW dominant follicle at AI or a follicle that develops under high concentrations of progesterone to optimize fertility of lactating dairy cows.

Table 3-1. Effect of cyclic status and follicular wave at insemination on fertility responses of dairy cows – Experiment 1

	Group ¹			P
	Anovular	CLOW	CHIGH	
	% (n/n)			
Short AI interval ²	11.9 (106/891) ^b	15.7 (59/376) ^a	7.1 (154/2165) ^c	< 0.001
Pregnant				
Day 30	29.7 (377/1268) ^b	31.3 (171/546) ^b	43.0 (1630/3793) ^a	< 0.001
Day 53	25.1 (318/1265) ^c	28.1 (153/545) ^b	36.9 (1392/3773) ^a	< 0.001
Pregnancy loss	15.0 (56/374) ^a	10.0 (17/170) ^{b,e}	13.5 (218/1610) ^d	0.08

^{a-c} Means in the same row with different superscripts differ (P < 0.04).

^{d,e} Means in the same row with different superscripts tend to differ (P = 0.06).

¹ Anovular = cows with progesterone < 1 ng/mL in two sequential blood samples collected 7 to 14 d apart with the second sample collected on the day of the first GnRH of the synchronization protocol. These cows were expected to ovulate the dominant follicle of the first follicular wave; CLOW = cyclic cows but with progesterone < 1 ng/mL on the first GnRH of the synchronization protocol presumed to ovulate a first wave follicle at AI; CHIGH = cyclic cows with progesterone ≥ 1 ng/mL on the first GnRH of the synchronization protocol presumed to ovulate a second wave follicle at AI.

² Nonpregnant cows re-inseminated between 5 and 17 d after the first AI.

Table 3-2. Effect of follicular wave at insemination on ovarian responses of cyclic dairy cows during the Ovsynch protocol – Experiment 2

	Follicular wave ¹		AOR ²	95 % CI ³	P
	FW	SW			
 % (n/n)				
Ovulation to GnRH1 ⁴	88.4 (99/112)	78.7 (85/108)	0.47	0.22 - 1.00	0.05
CL at PGF _{2α}	96.4 (108/112)	90.7 (98/108)	0.36	0.11 - 1.20	0.10
Progesterone ≥ 1 ng/mL					
GnRH1	9.8 (11/112)	97.2 (105/108)	322	87 - 999	0.001
PGF _{2α}	92.0 (103/112)	88.0 (95/108)	0.63	0.26 - 1.55	0.31
Progesterone, ng/mL					
GnRH1	0.39 ± 0.08	2.55 ± 0.08	---	---	0.001
PGF _{2α}	2.10 ± 0.20	2.92 ± 0.17	---	---	0.002
Follicle at PGF _{2α} , mm	17.9 ± 0.3	15.7 ± 0.2	---	---	0.001

¹ Follicular wave of the ovulatory follicle at the timed AI. FW = first wave; SW = second wave.

² AOR = adjusted odds ratio (first wave set as reference for comparison).

³ CI = confidence interval.

⁴ First GnRH of the Ovsynch protocol.

Table 3-3. Effect of follicular wave at insemination following the Ovsynch protocol on fertility responses of cyclic dairy cows – Experiment 2

	Follicular wave ¹		AOR ²	95 % CI ³	P
	FW	SW			
Pregnant % (n/n)				
Day 32	30.4 (34/112)	41.7 (45/108)	1.85	1.04 - 3.30	0.04
Day 67	27.7 (31/112)	40.7 (44/108)	2.06	1.15 - 3.70	0.02
Pregnancy loss	8.8 (3/34)	2.2 (1/45)	0.20	0.02 - 2.18	0.19

¹ Follicular wave of the ovulatory follicle at the timed AI. FW = first wave; SW = second wave.

² AOR = adjusted odds ratio (first wave set as reference for comparison).

³ CI = confidence interval.

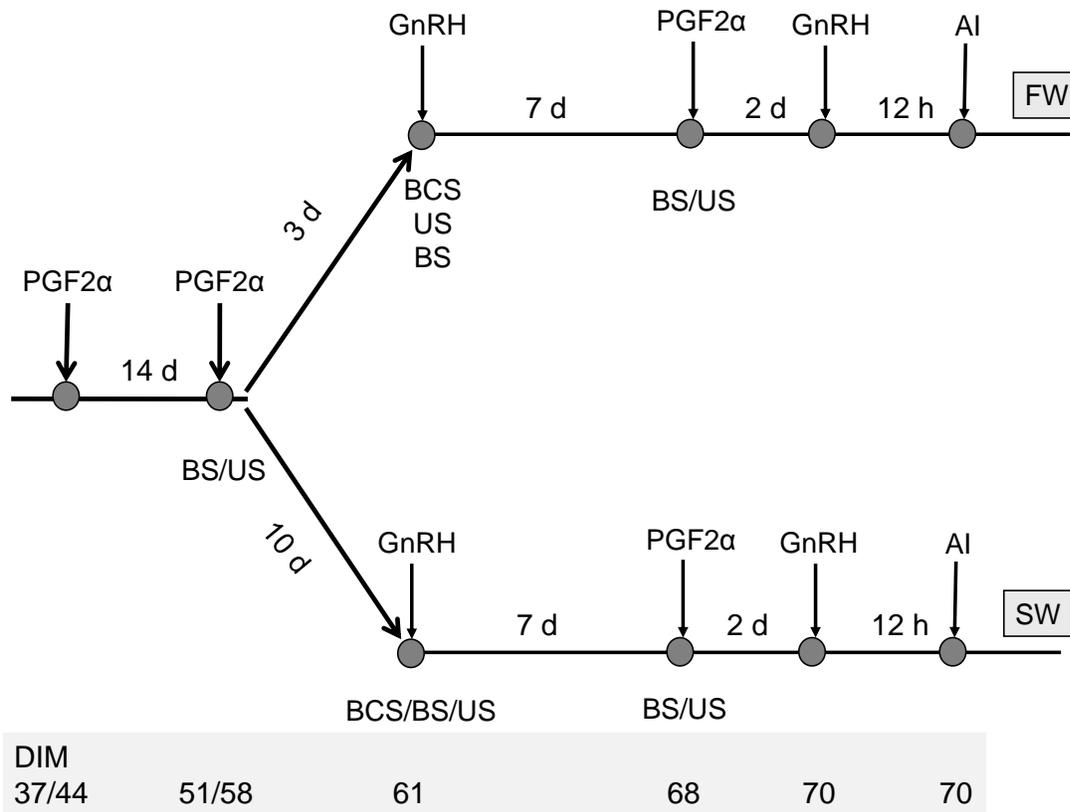


Figure 3-1. Diagram of activities during experiment 2. AI = artificial insemination; BCS = body condition score; BS = blood samples for analysis of progesterone concentration; DIM = days in milk; FW = ovulation of the first wave dominant follicle at AI; GnRH = injection of 100 µg of gonadorelin diacetate tetrahydrate; PGF_{2α} = injection of 25 mg of dinoprost as tromethamine salt; SW = ovulation of the first wave dominant follicle at AI; US = ultrasonography of the ovaries.

CHAPTER 4
EFFECT OF WAVE OF THE OVULATORY FOLLICLE AND CONCENTRATION OF
PROGESTERONE DURING FOLLICULAR GROWTH ON LUTEAL FUNCTION AND
CONCEPTUS DEVELOPMENT IN DAIRY COWS

Objectives were to determine the effects of wave of the ovulatory follicle and concentrations of progesterone during follicular growth on reproductive responses of dairy cows. Non-pregnant and non-lactating Holstein cows had their estrous cycle presynchronized with an injection of gonadotropin-releasing hormone (**GnRH**) and an intravaginal controlled internal drug release (**CIDR**) insert containing progesterone, followed 7 d later by CIDR removal and 2 injections of prostaglandin (**PG**) $F_{2\alpha}$ 24 h apart. All cows received an injection of GnRH at 1 d after the second $PGF_{2\alpha}$, which was the first GnRH of the synchronization protocol (d -9 GnRH, d -2 and d -1 $PGF_{2\alpha}$, d 0 GnRH) for cows induced to ovulate a first wave follicle (**FW**, n = 13) or a first wave follicle supplemented with progesterone (**FWP4**, n = 8). Cows induced to ovulate a second wave follicle (**SW**, n = 12) received the synchronization protocol beginning 6 d after the previous GnRH injection. Cows in FWP4 received 3 CIDR inserts at 12, 24 and 48 h after the injection of GnRH (d -9) that were removed at the injection of $PGF_{2\alpha}$ (d -2). Cows were inseminated at the final GnRH (d 0) and again 16 h later. Cows were killed on d 17 after the final GnRH. Progesterone concentration during follicular growth was greater for SW, intermediate for FWP4 and lesser for FW. Cows in FW ovulated larger follicles, which resulted in larger corpora lutea (**CL**) and greater concentrations of progesterone from d 4 to 16 after the final GnRH. The relative messenger ribonucleic acid (**mRNA**) abundance for steroidogenic acute regulatory protein (**StAR**) in the CL was greater for FW than for other treatments. Nonetheless, the ratio between circulating progesterone and luteal volume was not affected by treatment. A greater proportion of

FW cows had a peak of estradiol on the day before the final GnRH in comparison to other treatments. Maximum concentration of estradiol was greater for FW than for FWP4 and SW. Proportion of pregnant cows on d 17 tended to be less for FW than for FWP4 and SW. The length of conceptuses did not differ among treatments. There was a trend for SW conceptuses to produce less interferon-tau (**INF- τ**); however, no statistical difference in the relative abundance of INF- τ mRNA was detected among treatments. Ovulation of first wave dominant follicles reduced fertility in dairy cows, and this negative effect was mediated by suboptimal concentrations of progesterone during ovulatory follicle growth. Furthermore, luteal function during early gestation and capability of elongated conceptuses to produce INF- τ were not involved with reduction of fertility in cows that ovulated follicles from the first wave.

Introduction

Programs for synchronization of ovulation and timed artificial insemination (**AI**) have been widely used to improve reproductive efficiency in dairy herds (Chebel et al., 2006). The timing of events within a timed AI protocol affects not only the response to hormonal treatments (Vasconcelos et al., 1999), but also the environment on which the ovulatory follicle grows. Recent studies have shown that ovulation of a FW dominant follicle reduced the risk of pregnancy compared with the ovulation of a follicle from the SW and that this reduction in fertility is likely to be related with reduced concentrations of progesterone during follicular growth (Denicol et al., 2009; Bisinotto et al., 2010). Therefore, further understanding of the effects of wave of the ovulatory follicle and progesterone concentration during follicular growth is critical to improve timed AI programs.

During early gestation, the elongating conceptus must be able to produce large amounts of IFN- τ to inhibit endometrial release of PGF $_{2\alpha}$ and block luteolysis (Helmer et al., 1989). It has been suggested that a more rapid increase in concentrations of progesterone following AI enhances conceptus development and production of IFN- τ (Mann and Lamming, 2001; Robinson et al., 2006). The diameter of the ovulatory follicle is positively associated with subsequent CL volume and progesterone concentration in plasma (Vasconcelos et al., 2001). Because the dominant follicle of the FW develops concurrently with the newly formed CL under lesser concentrations of progesterone, it grows at a faster rate and reaches a larger diameter compared with a SW dominant follicle (Sirois and Fortune, 1988). In fact, concentrations of progesterone in plasma following the ovulation of FW follicles were either similar or greater than those observed following the ovulation of SW follicles (Wolfenson et al., 1999). Moreover, in vitro production of progesterone by luteinized theca cells was greater in samples derived from FW dominant follicles, suggesting that differences in size of the CL might not account for all the fluctuation in progesterone concentration observed in vivo (Wolfenson et al., 1999). Synthesis of progesterone encompasses the transport of cholesterol from outer to inner mitochondrial membrane by StAR, conversion into pregnenolone by cytochrome P450 side-chain cleavage (**P450scc**), and conversion into progesterone by 3 β -hydroxysteroid dehydrogenase / Δ^5, Δ^4 isomerase (**3 β -HSD**) associated with the smooth endoplasmic reticulum (Niswender, 2002). Modulation of these enzymatic pathways might be involved in the differential production of progesterone per cell described previously (Wolfenson et al., 1999). Hence, luteal

support during early embryogenesis is unlikely to be the cause of the poor fertility observed in cows that ovulate a FW follicle.

Exposure to progesterone during the development of the ovulatory follicle has been implicated in proper timing of the luteolytic cascade. The lack of previous exposure to progesterone advanced endometrial downregulation of progesterone receptors and responsiveness to oxytocin during the subsequent estrous cycle in beef cows (Zollers et al., 1989, 1993). Reduced concentrations of progesterone before ovulation enhanced subsequent uterine release of $\text{PGF}_{2\alpha}$ in response to oxytocin between d 15 and 16 of the estrous cycle in dairy cows (based on circulating concentrations of its major metabolite 13,14-dihydro-15-keto $\text{PGF}_{2\alpha}$: **PGFM**; Shaham-Albalancy et al., 2001; Cerri et al., 2008a). Accordingly, inadequate concentrations of progesterone before ovulation also increased the incidence of shortened luteal phases (Cerri et al., 2008a). Therefore, the ovulation of a FW dominant follicle might create an asynchrony between the production of $\text{IFN-}\tau$ by the conceptus and endometrial responsiveness to oxytocin, impairing maternal recognition of gestation. In addition to the effect of hastening luteolysis, the advancement in downregulation of progesterone receptors could disturb the secretory function of the endometrium. Progesterone stimulates histotroph secretion by uterine glands, which is critical for embryonic development and maintenance of gestation (Bazer et al., 2008). A reduction in the abundance of progesterone receptors could overcome the expected increase in luteal production of progesterone following the ovulation of a FW dominant follicle, diminish embryo development and its capacity to produce large amounts of $\text{IFN-}\tau$.

The hypotheses of the present study are that ovulation of a FW dominant follicle would result in a larger CL and greater concentrations of progesterone during the subsequent estrous cycle compared with the ovulation of a SW dominant follicle; however, this increase would be associated with reduced conceptus length and lesser capability to produce IFN- τ on d 17 after AI. Furthermore, we hypothesized that these effects would be mediated by the concentration of progesterone during the development of the ovulatory follicle.

Objectives were to evaluate ovarian dynamics, hormonal profiles, CL expression of steroidogenic and angiogenic factors, length of conceptuses and their capability to produce IFN- τ in non-lactating Holstein cows induced to ovulate either a FW or a SW dominant follicles. Moreover, to evaluate if the effects from the ovulation of a FW dominant follicle are induced by reduced concentrations of progesterone during follicular growth.

Materials and Methods

Animals

All experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and approved by the Animal Research Committee of the University of Florida (IFAS/ARC#: 018-09ANS). Non-lactating and non-pregnant Holstein cows were kept on pasture and had access to water ad libitum. Cows were fed a total mixed ration once a day, at 6:00 a.m., to meet or exceed the requirements of a 650 kg non-lactating Holstein cow (NRC, 2001). Diet was composed of corn silage, Bermuda grass silage, ground corn, citrus pulp, soybean meal, wet brewers grain, minerals, and vitamins.

Synchronization of Follicular Wave and Supplementation with Progesterone

Synchronization of follicular wave and supplementation with progesterone were performed as described in Figure 4-1. Cows were blocked by parity (nulliparous, primiparous, or multiparous) and body condition score (**BCS**; Ferguson et al., 1994) and, within each block, randomly assigned to one of three treatments: ovulation of a first wave dominant follicle (**FW**, n = 13); ovulation of a first wave dominant follicle and supplementation with progesterone during follicular development (**FWP4**, n = 8); or ovulation of a second wave dominant follicle (**SW**, n = 12). Cows had their estrous cycle presynchronized with an injection of GnRH (100 µg of gonadorelin diacetate tetrahydrate, i.m., Cystorelin, Merial Ltd., Duluth, GA) administered concurrently with the insertion of a intravaginal CIDR insert (1.38 g of progesterone, Eazi-Breed CIDR, Pfizer Animal Health, Kalamazoo, MI). Inserts were removed 7 d later and cows received two injections of PGF_{2α} (25 mg of dinoprost tromethamine, i.m., Lutalyse, Pfizer Animal Health, Kalamazoo, MI) administered at the moment of insert removal and 1 d later. All cows received an injection of GnRH at 1 d after the second PGF_{2α} of presynchronization, which was the first GnRH of the synchronization protocol (d -9 GnRH, d -2 and d -1 PGF_{2α}, d 0 GnRH) for FW and FWP4. Cows assigned to SW received the synchronization protocol beginning 6 d after the GnRH injection. Cows assigned to FWP4 received 3 CIDR inserts, placed sequentially at 12, 24 and 48 h after the first injection of GnRH and removed at the first injection of PGF_{2α} of the synchronization protocol. Cows were inseminated twice using the semen of a single bull of proven fertility concurrently with the final GnRH of the synchronization protocol and again 16 h later.

Ultrasonography of Ovaries and Eligibility Criteria

Cows had their ovaries assessed by ultrasonography (Aloka SSD-500 equipped with a 7.5 MHz linear transducer, Aloka Co., Tokyo, Japan) during presynchronization and synchronization protocols to ensure response to hormonal treatments (Figure 4-1). Position and diameter of all follicles > 4 mm and CL were recorded at each examination. Ovulation in response to GnRH was defined as the disappearance of one or more follicles ≥ 8.5 mm within 2 d followed by the formation of a CL in a compatible location. Only cows that ovulated in response to all injections of GnRH and had their CL regressed following injections of PGF_{2 α} (based on ultrasonographic evaluations) were kept in the study. Two FWP4 and three SW cows did not ovulate in response to the first GnRH of the synchronization protocol. Nonetheless, they were kept in the study because the emergence of a new follicular wave was detected within 48 h of the injection. Cows had their ovaries scanned on d 7 and 13 and diameters of the CL and its cavity (if present) were determined by averaging the largest cross-sectional width and height. Area and volume the CL were calculated as follows:

$$Area = (\pi \times R_{CL}^2) - (\pi \times R_{cavity}^2)$$

$$Volume = (4/3 \times \pi \times R_{CL}^3) - (4/3 \times \pi \times R_{cavity}^3)$$

where: W_{CL} = largest cross-sectional width and H_{CL} = largest cross-sectional height of the CL; R_{CL} = average radius of the CL $(W_{CL} + H_{CL})/2$; W_{cavity} = largest cross-sectional width and H_{cavity} = largest cross-sectional height of the cavity (if CL does not bear a cavity, $W_{cavity} = H_{cavity} = 0$); R_{cavity} = average radius of the cavity $(W_{cavity} + H_{cavity})/2$; and $\pi = 3.14159$.

Blood Collection and Analyses of Hormonal Concentrations in Plasma

Blood was collected daily from d -9 to 17 by puncture of the coccygeal artery or vein using evacuated tubes with K₂ ethylenediaminetetraacetic acid (**EDTA**; Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Samples were immediately placed in ice and transported to the laboratory within 3 h of collection. Blood tubes were centrifuged at 2,000 x g for 15 min in a refrigerated centrifuge (4°C) and plasma samples were frozen at -20 °C for later analyses.

Concentrations of progesterone were analyzed in all plasma samples by radioimmunoassay (**RIA**) using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.1 ng/mL calculated at 2 standard deviations below the mean counts per minute at maximum binding. Samples were analyzed in three assays. Moderate (1.5 ng/mL) and high (2.5 ng/mL) concentration samples were incorporated several times in each assay and used to calculate the intra- and inter-assay coefficient of variation (**CV**). The intra-assay CV were 6.3, 5.1 and 4.4% for the first, second and third assays, respectively; and the inter-assay CV was 7.0%. Concentrations of estradiol were analyzed in plasma samples collected from experimental d -2 to 1 by RIA using a commercial kit (Estradiol Double Antibody, Siemens Healthcare Diagnostics, Los Angeles, CA) validated previously for use in bovine samples (Siddiqui et al., 2009). The sensitivity of the assay was 0.16 pg/mL calculated at 2 standard deviations below the mean counts per minute at maximum binding. Samples were analyzed in duplicate and repeated when the coefficient of variation between duplicates was > 0.15. Samples were analyzed in three assays. Plasma from a cow exhibiting standing activity and charcoal-stripped plasma from a male calf were used as positive and negative quality controls, respectively. High

(10 pg/mL), moderate (5 pg/mL) and low (2.5 pg/mL) concentration samples were incorporated to each assay and used to evaluate the extraction. The intra-assay CV were 10.1, 8.0 and 9.7% for the first, second and third assays, respectively; and the inter-assay CV was 17.0%.

Collection of Conceptuses, Uterine and CL Samples

Cows were killed on d 17 after first insemination via insensibilization by captive bolt followed by exsanguination. The reproductive tract was collected, immediately placed on ice and processed within 1 h. The uterine horn ipsi-lateral to the CL was dissected free from the intercornual and broad ligaments, and isolated from the contra-lateral horn using a Doyen forceps. A transversal incision was performed approximately 2 cm caudal to the uterotubal junction. The horn was flushed with 30 mL of sterile Dulbecco's phosphate buffered saline (**DPBS**) solution containing 0.01% poly-vinyl-alcohol from the cervix towards the incision using a 14-gauge needle coupled to a 60 mL syringe. The fluid was collected into a petri dish and evaluated for the presence of a conceptus. Conceptuses were measured, snap-frozen in liquid N₂ and stored at -80°C for further analysis. Remaining fluid was clarified by centrifugation (2,000 x g for 30 min at 4°C), quantified and stored at -80°C until assayed. The horn was opened longitudinally and samples of inter-caruncular endometrium were dissected from the myometrium, snap-frozen in liquid N₂ and stored at -80°C. Additionally, two circular sections of the uterine wall (1 cm of diameter) were collected from the inter-caruncular region using a cork hole hand drill. One of the samples was fixed in 4% formaldehyde (prepared in DPBS; 16% Methanol-free Formaldehyde Solution, Thermo Scientific, Rockford, IL) for 16 min, washed in DPBS 3 times for 10 min, frozen into an embedding mold containing an optimal cutting temperature (**OCT**) compound (Tissue-Tek, Sakura

Finetek USA Inc., Torrance, CA), and stored at -80°C . The remaining sample was fixed in 4% formaldehyde for 20 h, washed in DPSB for 10 min, and stored in 70% ethanol at 4°C until embedding in paraffin. The CL was dissected from the ovary, measured using a caliper and weighted. Corpus luteum was divided into four semi hemispheres and processed as described for uterine samples. Briefly, two of the quarters were snap-frozen in liquid N_2 and stored at -80°C , one was fixed in 4% formaldehyde and frozen embedded OCT compound, and the remaining sample was fixed in 4% formaldehyde and embedded in paraffin. Double ovulation was observed in one cow from FW and one cow from FWP4 treatments. In both cases, ovulations were detected in separate ovaries; therefore, each horn and its respective ovary were processed individually.

Measurement of IFN- τ in Uterine Flush

Concentrations of IFN- τ in uterine flush samples were determined based on their antiviral activity as described previously (Rodina et al., 2009) with few modifications. Briefly, 100 μL of Dulbecco's Modified Eagle Medium (**DMEM**; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1% sodium pyruvate (Invitrogen, Grand Island, NY), 1% L-glutamine (Invitrogen, Grand Island, NY), and 1% penicillin/streptomycin (Invitrogen, Grand Island, NY) was added to each well of a 96-well plate. Serial 1:3 dilutions of either a recombinant human IFN- α solution (100 IU/ μL ; 3.84×10^8 IU/mg; EMD Biosciences Inc., Gibbstown, NJ) or the uterine flush samples were performed by adding 50 μL to the first well, mixing and transferring 50 μL to the subsequent well. Madin-Darby Bovine Kidney (**MDBK**) cells were plated and cultured at 37°C (95% air/5% CO_2) for 24 h. Cells were challenged with vesicular stomatitis virus (**VSV**) for 1 h and incubated in supplemented DMEM for 20 h. Cells

were fixed with 70% ethanol and stained with 0.5% gentian violet diluted in 70% ethanol.

Laboratory units of antiviral activity (**LU**) were calculated for each sample as the inverse of 1/3 of the dilution factor for the well that VSV-induced lysis of MDBK cells was prevented by 50%, which was accessed visually. Laboratory units were converted to international units (**IU**) by dividing the amount of IFN- α of the standard by its LU and multiplying by the LU of individual samples. The activity of bovine IFN- τ has been described (8.03×10^8 IU/mg; Rodina et al., 2009) and was used to calculate the concentration of IFN- τ present in individual samples. The calculations for a hypothetical sample that inhibited VSV-induced lysis of MDBK cells by 50% on the tenth well, considering that the same inhibition was achieved by the standard on the eighth well, would be performed as follows. The dilution factor on the first well is 1:3 (by default, 1 LU) and samples were diluted 3 times in each well in relation to the previous one. Therefore, the dilution factor on the eighth well is 1:6,561, which means that the standard has 2,187 LU. Because 5,000 IU of IFN- α were added to first well, 1 LU represents 2.29 IU. The dilution factor on the tenth well is 1:59,049; therefore, the unknown sample has 19,683 LU and a total of 45,000 IU. As the initial volume was 50 μ L, the concentration of IFN- τ in the sample is 900,000 IU/mL, which based in the activity of bovine IFN- τ determined previously represents 1,120 ng/mL.

The sensitivity of the assay was 0.06 ng/mL. All plates were duplicated. Samples were analyzed in a single assay and the intra-assay coefficient of variation was 9.9%. The accuracy with which concentrations of IFN- τ in uterine flush samples predicted the presence of a conceptus on d 17 after insemination was determined using receiver

operating characteristic (**ROC**) curves (Figure 4-2). All samples on which antiviral activity were not detected (0.0 ng/mL) were associated with nonpregnant cows. The false positive rate (proportion non-pregnant cows that yield concentrations of IFN- τ above the sensitivity of the assay) was 4.4%.

RNA isolation and Quantitative Real-Time Reverse Transcription PCR

The relative abundance mRNA was assessed for genes of interest using quantitative real-time reverse transcription polymerase chain reaction (**qRT-PCR**). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and purified (PureLink RNA Mini Kit, Invitrogen, Carlsbad, CA) according to the manufacturer's recommendation. Integrity and concentration of the RNA was analyzed using a micro-volume spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA).

Samples of RNA (250 ng/reaction) were treated with RNase-free DNase (Applied Biosystems Ambion Inc., Foster City, CA) for 30 min at 35°C, heat denatured at 75°C for 15 min. Total RNA was reverse-transcribed (**RT**) using a high capacity complementary DNA (**cDNA**) archive kit and random hexamers (Applied Biosystems Inc., Foster City, CA). Reverse transcription was performed at 25°C for 10 min, 37°C for 60 min, and 85°C for 5 min. Gene-specific primer sets for 3 β -HSD, CYP11A1, FLT-1, KDR, StAR, VEGFA (in the CL) and IFN- τ (in the conceptuses) were selected and GAPDH was used as internal control (Table 4-1). Quantitative real-time reverse transcription PCR was performed (ABI 7300 Sequence Detector; Applied Biosystems Inc., Foster City, CA) using a SybrGreen detector system (Applied Biosystems Inc., Foster City, CA). Reactions were run in triplicates using the RT positive product and 1 replicate was run using a RT negative control. After an initial activation/denaturation step (60°C for 2 min; 95°C for 10 min), 40 cycles of a two-step amplification protocol (60°C for 1 min; 95°C for

15 s) were completed and subsequent cooling (4°C) terminated the reaction. Primer efficiency ranged from 89 to 103%. The abundance of target genes mRNA was calculated relative to that of GAPDH using the comparative threshold (**Ct**) method as described previously (Pfaffl, 2001). Briefly, a ΔCt was calculated for each gene (target genes and internal control) as the sample's Ct for the referred gene minus the average Ct for the same gene within the reference group (reference groups for each comparison were indicated accordingly throughout the manuscript). The ΔCt was then corrected by raising the efficiency of the primer to the power of its ΔCt . The relative expression ratio was calculated by dividing the corrected ΔCt of the target gene by the corrected ΔCt of the internal control, and was used in the statistical analyses.

Experimental Design and Statistical Analysis

Cows were assigned to treatments in a randomized-block design. Cows were blocked by parity and body condition score at enrollment and, within each block, randomly assigned to one of three treatments. Tests for normality of residuals (Shapiro-Wilk test) and homogeneity of variances (F-Max test) were conducted for each variable. Data which did not fulfill the assumptions for analysis of variance (**ANOVA**) were transformed accordingly. For clarity, only untransformed data were presented. Continuous data were analyzed by ANOVA using the GLIMMIX procedure of SAS version 9.2 (SAS/STAT, SAS Institute Inc., Cary, NC) for normal distribution. The effects of treatment, parity and interaction between treatment and parity were included in the model as independent variables. Concentrations of estradiol and progesterone were analyzed as repeated measures by ANOVA using the GLIMMIX procedure of SAS for normal distribution. The effects of parity, treatment, day and interaction between treatment and day were included in the model as independent variables, whereas cow

nested within treatment was considered a random effect. The first order autoregressive was selected as covariance structure because it presented the smallest Schwarz's Bayesian information criterion value.

Correlations between the length of the conceptuses and production of IFN- τ and mRNA abundance were performed using the REG procedure of SAS. Binary responses were analyzed by multivariate logistic regression using the GLIMMIX procedure of SAS for binary distribution. The effects of treatment, parity and interaction between treatment and parity were included in the model as independent variables.

Evaluation of the individual effects of follicular wave (FWP4 vs. SW) and progesterone concentration during development of the ovulatory follicle (FW vs. FWP4 +SW) were conducted using a CONTRAST statement whenever suitable. Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered tendencies.

Receiver operating characteristic curves were generated using the ROC curve analysis option of MedCalc version 11.2.1 (MedCalc Software, Mariakerke, Belgium) to determine the accuracy with which concentrations of IFN- τ in uterine flush samples predict the presence of a conceptus on d 17 after insemination.

Results

Two cows were not included in the statistical analyzes because they had either an abnormal oviduct detected at slaughter (7187; SW) or a hyperechogenic mass in the ovary that was capable of producing progesterone during the growth of the ovulatory follicle (5708; FW).

Ovarian Responses and Hormonal Profiles

The concentration of progesterone during the development of the ovulatory follicle differed ($P < 0.0001$) among treatments (Figure 4-3). Cows that did not receive exogenous progesterone exhibited a constant increase in the concentration of progesterone throughout the treatment period. Moreover, progesterone concentration was consistently greater ($P < 0.01$) for SW than for FW. The incorporation of 3 CIDR inserts to the synchronization protocol increased concentrations of progesterone during the development of the first follicular wave. Following the insertion of the second and third CIDR inserts, the concentration of progesterone in plasma for FWP4 was greater ($P < 0.01$) than that of FW and similar ($P > 0.50$) to SW. The concentration of progesterone in FWP4 cows remained somewhat constant from d -6 to -2 whereas it increased in FW and SW cows. The proportion of cows bearing a CL at PGF_{2α} injection (d -2) tended ($P = 0.06$) to be less for FWP4 (Table 4-2); however, the pattern of progesterone concentration in plasma did not change when only cows bearing a CL were included in the statistical analysis. The luteal volume for cows bearing a CL on d -2 was similar ($P = 0.22$) between FWP4 and FW. Progesterone concentration was reduced to basal values following the treatment with PGF_{2α}, which indicates similar luteolysis among treatments.

The diameter of the ovulatory follicle was larger ($P \leq 0.05$) for cows with reduced concentrations of progesterone during follicular growth, which resulted in greater ($P = 0.05$) concentrations of estradiol on the day of the peak (Table 4-2). Conversely, the wave of the ovulatory follicle did not affect ($P > 0.10$) ovulatory diameter and maximum concentration of estradiol. An interaction between treatment and day ($P = 0.03$) was found to influence concentration of estradiol in plasma between d -1 and 1, because the

average concentration for FW increased earlier than for other treatments (Figure 4-4). On the day before the final GnRH, estradiol concentrations for FW were greater ($P < 0.001$) than for SW and tended ($P < 0.06$) to be greater than for FWP4. This can be partially explained by the fact that the incidence of premature peaks of estradiol was greater ($P = 0.01$) among FW cows (Table 4-2).

Progesterone concentration during the growth of the ovulatory follicle affected the size of the newly formed CL (Table 4-2). The greater luteal volume observed in FW cows was associated with increased concentrations of progesterone (Figure 4-5). Nevertheless, the capacity of the CL to produce progesterone, calculated as the ratio between the concentration of progesterone in plasma and CL volume or weight at slaughter, was not affected ($P > 0.10$) by concentration of progesterone during follicular growth or wave of the ovulatory follicle. None of the cows in the present study had their CL regressed before d 17 of the estrous cycle, based on visual evaluation on the day of slaughter and on concentrations of progesterone in plasma.

Expression of Steroidogenic and Angiogenic Factors in the CL

Luteal expression of mRNA encoding StAR on d 17 of the estrous cycle was greater ($P < 0.05$) for FW than for other treatments (Figure 4-6). The expression of P450scc was reduced ($P < 0.05$) for FWP4, whereas no difference between FW and SW was detected. Finally, the expression of 3β -HSD was greater ($P < 0.05$) for FW than for FWP4 and SW had an intermediate relative abundance. The expression of mRNA expression encoding VEGF tended ($P < 0.10$) to be greater for FW than for FWP4 and SW (Figure 4-7). Nonetheless, the expression of VEGF receptors, KDR and Flt-1, did not differ among treatments.

Pregnancy, Conceptus Development and IFN- τ Production

Conceptuses were recovered from 21 of the initial 31 cows (Table 4-3). One FW (5183) and one FWP4 cow (5448) ovulated one follicle from each ovary. On the day of slaughter, uterine horns were flushed individually and two conceptuses were recovered from each cow. The risk of pregnancy on d 17 after insemination tended ($P = 0.10$) to be less for cows exposed to low concentrations of progesterone during the development of the ovulatory follicle. Conceptus length was not affected ($P > 0.10$) by follicular wave or concentration of progesterone during follicular growth. Conceptuses were categorized in relation to the mean length observed in the present study (14 cm) as large (> 14 cm; $n = 12$) or small (≤ 14 cm; $n = 11$). Effectively, the average length for large conceptuses was greater ($P < 0.001$) than for small conceptuses (19.9 ± 1.0 vs. 7.5 ± 1.5 cm). The proportion of small conceptuses did not differ ($P > 0.01$) among treatments.

Ovulation of a SW dominant follicle tended ($P < 0.10$) to reduce the concentration of IFN- τ in the uterine flush of pregnant cows and the total amount of IFN- τ recovered. The ratio between IFN- τ concentration in the uterine flush or total amount of IFN- τ recovered and conceptus length indicated that conceptus from SW cows were less ($P \leq 0.05$) capable of producing IFN- τ . Nevertheless, the relative abundance of mRNA encoding for IFN- τ in elongated conceptuses did not differ ($P = 0.31$) among treatments.

The linear regression of both, the concentration of IFN- τ in the uterine flush and the total amount of IFN- τ recovered per uterus, and conceptus length demonstrated a positive relationship ($P < 0.001$; Figure 4-8). Large conceptuses were associated with greater ($P < 0.001$) concentration of IFN- τ in uterine flush and total amount of IFN- τ recovered (Figure 4-9). The increase in uterine concentration and total amount of IFN- τ per centimeter of conceptus was greater ($P < 0.05$) for large conceptuses. However, the

difference in capacity to synthesize IFN- τ was not associated with a correspondent increase in mRNA relative abundance (Figure 4-10). Nonetheless, a positive correlation between IFN- τ mRNA relative abundance and conceptus length was detected (Figure 4-11).

Surprisingly, concentrations of progesterone in plasma from d -5 to -3 relative to AI were greater for cows that produced small conceptuses (Figure 4-12). Concentrations of progesterone during the first 5 d of gestation did not differ ($P = 0.71$) between cows that had small and large conceptuses; however, they were greater ($P = 0.004$) from d 6 to 17 of gestation for cows that had large conceptuses compared with cows that produced small conceptuses (Figure 4-13).

Discussion

Results from the current study confirmed our initial hypotheses that ovulation of FW dominant follicles reduces fertility in dairy cows and that this effect is mediated by suboptimal concentrations of progesterone during ovulatory follicle growth. Nonetheless, the present results indicate that the underlying mechanisms are related with the final maturation of the oocyte, fertilization and embryonic development before the elongation period. The reduction on the risk of pregnancy for cows induced to ovulate a FW dominant follicle that grew under reduced concentrations of progesterone was evident on d 17 after AI. However, the average length and the proportion of conceptus classified as small in relation to the overall mean were similar for FW compared to SW and FWP4. Furthermore, the capability of conceptuses to produce IFN- τ was either similar or superior for FW compared to other treatments. These results are supported by previous studies on which dairy cows were superstimulated during the FW or SW of follicular development (Rivera et al., 2009). Embryo viability on d 6 after AI

was markedly reduced following superstimulation of the FW in comparison to the SW. Likewise, the negative effects were mediated by reduced concentration of progesterone during growth of the ovulatory follicle, since no differences on embryo quality were found between cows that ovulated SW dominant follicles and cows that were supplemented with progesterone during the development of the FW. Furthermore, no differences in pregnancies per embryo transfer or in pregnancy loss between d 21 and 63 of gestation were reported for viable embryos derived from superstimulation of the FW, FW supplemented with progesterone, and SW (Rivera et al., 2009). These observations further support the present conclusion that follicular wave of the ovulatory follicle and progesterone concentrations during follicular growth affects the final maturation of the oocyte, fertilization and/or embryonic development before the elongation period.

Post-hatching development of bovine embryos is highly dependent on growth factors and nutrients secreted by the endometrium in response to progesterone (Bazer et al., 2008). The rise in progesterone concentration after AI occurred more rapidly for FW than for SW and FWP4. The augmentation in progesterone production observed for FW was supported by the ovulation of a larger follicle followed by the development of a larger CL (Vasconcelos et al., 2001). The relationship between the concentration of progesterone in plasma and luteal volume indicates a similar steroidogenic capacity of the CL among treatments. Nonetheless, an increase in CL relative abundance of mRNA encoding for StAR was detected for FW compared to SW and FWP4. This result agrees with the finding that progesterone output per cell differed between in vitro luteinized cells from FW and SW dominant follicles (Wolfenson et al., 1999). Luteinized theca cells

derived from FW dominant follicle produced 2 to 3 times more progesterone than the SW-derived counterparts. Nonetheless, no difference was found for luteinized granulosa cells. Because granulosa cells give rise to large luteal cells after the pre-ovulatory surge of luteinizing hormone (**LH**) and become responsible for 80 to 90% of the production of progesterone by the CL (Fitz et al., 1982; Farin et al., 1986), the overall improvement in steroidogenic capacity per cell is expected to be secondary to the increase in luteal volume in the present study. Taken together, results from luteal dynamics after induced ovulation supports the results regarding conceptus morphology and IFN- τ production (Mann and Lamming, 2001; Robinson et al., 2006).

Because of the experimental design presented here, the lifespan of the ovulatory follicle is expected to be similar among treatments. Nonetheless, ovulatory follicles from the FW were significantly larger than those derived from the SW. This is in accordance with previous studies that reported a faster rate of growth and a greater maximum diameter for FW compared with SW dominant follicles (Sirois and Fortune, 1988). The presence of a fully mature CL and greater concentrations of progesterone are negatively correlated with LH pulse frequency, which diminishes follicular growth during the SW (Evans et al., 1997). In fact, supplementation with exogenous progesterone reduced growth of the FW dominant follicle and resulted in ovulatory diameters similar to those observed for SW follicles in the present study. On account of these changes in follicular development, the greatest concentration of estradiol in plasma and the proportion of cows that had their greatest concentration detected on the day before of the final GnRH were greater for FW compared to other treatments. It is possible that some of these cows had endogenous surge of LH and ovulated shortly after AI; thus,

the reduction in fertility observed for FW might have been, at least in part, due to an asynchrony between ovulation and AI. Nevertheless, estradiol concentrations in plasma are expected to decrease by 50% within 5 h of the peak and to return to basal values during the following 9 h (Chenault et al., 1974). Estradiol concentrations 32 h after the maximum concentration had decreased by less than 50% in 2 of 8 cows, and were greater than those observed on the day before the peak in 5 of 8 cows in the present study. The present scheme of blood sampling does not allow for the determination of the highest concentration of estradiol; however, for the majority of the cows that had a premature peak of estradiol, it is expected that the maximum concentration occurred within 14 h of the final GnRH. Therefore, the effect of spontaneous ovulation in the present study is expected to be of minor importance.

The oocyte enclosed in FW dominant follicles were exposed to greater concentrations of estradiol for a longer period of time than oocytes from SW and FWP4 dominant follicles. It has been shown that reduction of circulating concentrations of progesterone during early diestrus increased concentrations of estradiol and induced maturation-related changes in the cumulus-oocyte complex (Inskeep, 2004). These changes were detected within 48 h after lowered progesterone and included degeneration of cumulus cell processes, irregularity of oocyte's nuclear membrane and hastening of stage II of meiosis. Similar alterations were described in persistent follicles (Revah and Buttler, 1996), and have been linked to decreased embryo quality and increased mortality before the 16-cell stage (Ahmad et al., 1995; Cerri et al., 2009). Nonetheless, further studies are needed to accurately determine the effects of wave

and concentration of progesterone during follicular development on parameters of oocyte competence in dairy cows.

The concentration of IFN- τ in the uterine flush and the total amount of protein recovered were highly correlated with the length of the conceptus. This response agrees with previous literature regarding IFN- τ production by elongating bovine conceptuses (Mann and Lamming, 2001; Robinson et al., 2006). Nevertheless, the classification of conceptuses into small and large showed that conceptuses that were longer than the mean for the present study produced more IFN- τ per centimeter than those that were shorter than the mean length. This finding contradicts the dogma that modulation of IFN- τ production by bovine conceptuses relies only on differences in length (Robinson et al., 2006). It has been shown that IFN- τ mRNA abundance is developmentally regulated in the bovine embryo (Bertolini et al., 2002). Nonetheless, similar IFN- τ gene expression was reported for conceptuses between d 14 and 18 of gestation and among conceptus that measured less than 5, from 5 to 10, and more than 10 cm (Robinson et al., 2006). The latter is consistent with our finding that the relative IFN- τ mRNA abundance was similar between conceptuses classified as small and large. Simple linear regression analyses demonstrated a weak correlation between IFN- τ mRNA relative abundance and conceptus length; however, length of conceptuses significantly accounted for part of the fluctuation in relative abundance of IFN- τ mRNA.

Conclusion

Ovulation of FW dominant follicles reduces fertility in dairy cows and this negative effect is mediated by suboptimal concentrations of progesterone during ovulatory follicle growth. The results presented here suggest that the underlying mechanisms are related with oocyte maturation, fertilization and embryonic development before the elongation

period. Furthermore, our results indicate that luteal function during early gestation and capability of elongated conceptuses to produce IFN- τ are not involved with the poorer fertility observed in cows induced to ovulate FW dominant follicles.

Table 4-1. Primer sequences used for quantitative real-time reverse transcription PCR

Target gene	Primer	Sequence (5' to 3') ¹
3 β -HSD	Sense (5') primer	GCCCAACTCCTACAGGGAGAT
	Antisense (3') primer	TTCAGAGCCCACCCATTAGCT
CYP11A1	Sense (5') primer	CTGCAAATGGTCCCCTTCT
	Antisense (3') primer	CACCTGGTTGGGTCAAACCTT
FLT-1	Sense (5') primer	GCCTGAAATCTACCAGATCATGTTG
	Antisense (3') primer	AAGATTCGTGGAGCTTGTGGAA
GAPDH	Sense (5') primer	ACCCAGAAGACTGTGGATGG
	Antisense (3') primer	CAACAGACACGTTGGGAGTG
IFN- τ	Sense (5') primer	GCCCGAATCAACAGACTCCC
	Antisense (3') primer	GATCCTTCTGGAGCTGGCTG
KDR	Sense (5') primer	ACTGCAGTGATGGCGTCTT
	Antisense (3') primer	TGGAAATGATACTGGAGCCTACAAG
StAR	Sense (5') primer	CCCATGGAGAGGCTTTATGA
	Antisense (3') primer	TGATGACCGTGTCTTTTCCA
VEGFA	Sense (5') primer	GCCCACTGAGGAGTTCAACAT
	Antisense (3') primer	GATCAAACCTCACCAAAGCCAG

Table 4-2. Effect of follicular wave and concentration of progesterone during the development of the ovulatory follicle on ovarian responses and hormonal profile

Variables	Treatment ¹			P ²		
	FW	FWP4	SW	Treat.	Wave	P4
Ovulatory follicle, cm						
at PGF _{2α} injection (d -2) ³	1.29 ± 0.07	1.04 ± 0.09	1.16 ± 0.08	0.10	0.29	0.05
at GnRH injection (d 0)	1.79 ± 0.06	1.53 ± 0.07	1.47 ± 0.06	< 0.01	0.47	< 0.01
Premature peak of estradiol, % (n/n) ⁴	58.3 (7/12)	12.5 (1/8)	0.0 (0/11)	0.04	0.77	0.01
Estradiol concentration at peak, pg/mL	7.98 ± 0.58	6.98 ± 0.71	5.87 ± 0.61	0.06	0.25	0.05
CL volume at PGF _{2α} (d -2), cm ³						
All cows	8.30 ± 1.04	4.62 ± 1.27	10.95 ± 1.08	< 0.01	< 0.01	0.70
Only cows with CL	8.30 ± 0.99	6.15 ± 1.40	10.95 ± 1.03	0.03	0.01	0.85
Cows with a CL at PGF _{2α} , % (n/n)	100.0 (12/12)	75.0 (6/8)	100.0 (11/11)	0.06	0.16	0.51
CL on d 7 after the final GnRH						
Volume, cm ³	5.23 ± 0.46	3.55 ± 0.56	4.20 ± 0.50	0.07	0.39	0.03
Progesterone in plasma, ng/mL/cm ³	0.70 ± 0.09	0.86 ± 0.11	0.72 ± 0.10	0.63	0.38	0.63
CL on d 13 after the final GnRH						
Volume, cm ³	6.81 ± 0.67	5.44 ± 0.80	7.03 ± 0.73	0.30	0.15	0.51
Progesterone in plasma, ng/mL/cm ³	1.08 ± 0.11	0.91 ± 0.14	0.93 ± 0.12	0.50	0.83	0.26
CL on d 17 after the final GnRH						
Volume, cm ³	11.15 ± 0.99	7.62 ± 1.15	9.00 ± 1.15	0.08	0.41	0.04
Progesterone in plasma, ng/mL/cm ³	0.65 ± 0.11	0.90 ± 0.13	0.78 ± 0.12	0.37	0.58	0.19
Weight, g	6.33 ± 0.45	4.94 ± 0.52	6.30 ± 0.55	0.11	0.08	0.24
Progesterone in plasma, ng/mL/g	1.06 ± 0.12	1.30 ± 0.14	1.06 ± 0.14	0.46	0.24	0.67

¹ FW = ovulation of a first wave dominant follicle; FWP4 = ovulation of a first wave dominant follicle and supplementation with progesterone during follicular growth; SW = ovulation of a second wave dominant follicle. Responses are indicated as LS-means ± SEM unless specified.

² Statistical significance for the effect of treatment, follicular wave (FWP4 vs. SW) and concentration of progesterone during the development of the ovulatory follicle (FW vs. FWP4 + SW).

³ Day 0 = final injection of GnRH of the synchronization protocol administered concurrently with the first insemination.

⁴ Proportion of cows in which the peak of estradiol was detected on the day before the final injection of GnRH (d -1).

Table 4-3. Effect of follicular wave and concentration of progesterone during the development of the ovulatory follicle on conceptus development and production of IFN- τ

Variables	Treatment ¹			P ²		
	FW	FWP4	SW	Treat	Wave	P4
Pregnant cows, % (n/n)	50.0 (6/12)	87.5 (7/8)	72.7 (8/11)	0.24	0.45	0.10
Length of the conceptus, cm	17.5 \pm 2.8	13.7 \pm 2.6	11.2 \pm 2.6	0.29	0.52	0.15
Range	4.40 – 24.00	4.20 – 23.00	0.35 – 22.00	---	---	---
Small conceptuses ³ , % (n/n)	28.6 (2/7)	50.0 (4/8)	62.5 (5/8)	0.45	0.62	0.25
Concentration of IFN- τ , ng/mL ⁴	299.5 \pm 91.9	211.0 \pm 85.9	60.1 \pm 91.8	0.09	0.06	0.22
Per centimeter of conceptus, ng/mL/cm	16.1 \pm 4.9	13.1 \pm 4.6	3.7 \pm 4.9	0.04	0.31	0.02
Total amount of IFN- τ recovered, μ g ⁵	4.6 \pm 1.3	3.0 \pm 1.2	1.5 \pm 1.3	0.14	0.09	0.25
Per centimeter of conceptus, μ g/cm	0.22 \pm 0.05	0.21 \pm 0.05	0.09 \pm 0.05	0.05	0.02	0.37
IFN- τ mRNA relative expression, dCt ratio ⁶						
All conceptuses	1.83 \pm 0.29	2.03 \pm 0.28	1.27 \pm 0.28	0.15	0.06	0.62
Elongated conceptuses ⁷	1.47 \pm 0.23	1.64 \pm 0.22	1.15 \pm 0.23	0.31	0.13	0.78

¹ FW = ovulation of a first wave dominant follicle; FWP4 = ovulation of a first wave dominant follicle and supplementation with progesterone during follicular growth; SW = ovulation of a second wave dominant follicle. Responses are indicated as LS-means \pm SEM unless specified.

² Statistical significance for the effect of treatment, follicular wave (FWP4 vs. SW) and concentration of progesterone during the development of the ovulatory follicle (FW vs. FWP4 + SW).

³ Conceptuses were classified as small or large in relation to the mean length (small \leq 14 cm, and large $>$ 14 cm).

⁴ Concentration of interferon-tau in the uterine flush.

⁵ Calculated by multiplying concentration of interferon-tau in the uterine flush by the total amount of uterine flush recovered.

⁶ Expression of interferon-tau mRNA relative to GAPDH. The treatment SW was used as reference for comparisons.

⁷ One SW cow (5686) was excluded from the analysis because its conceptus was not elongated.

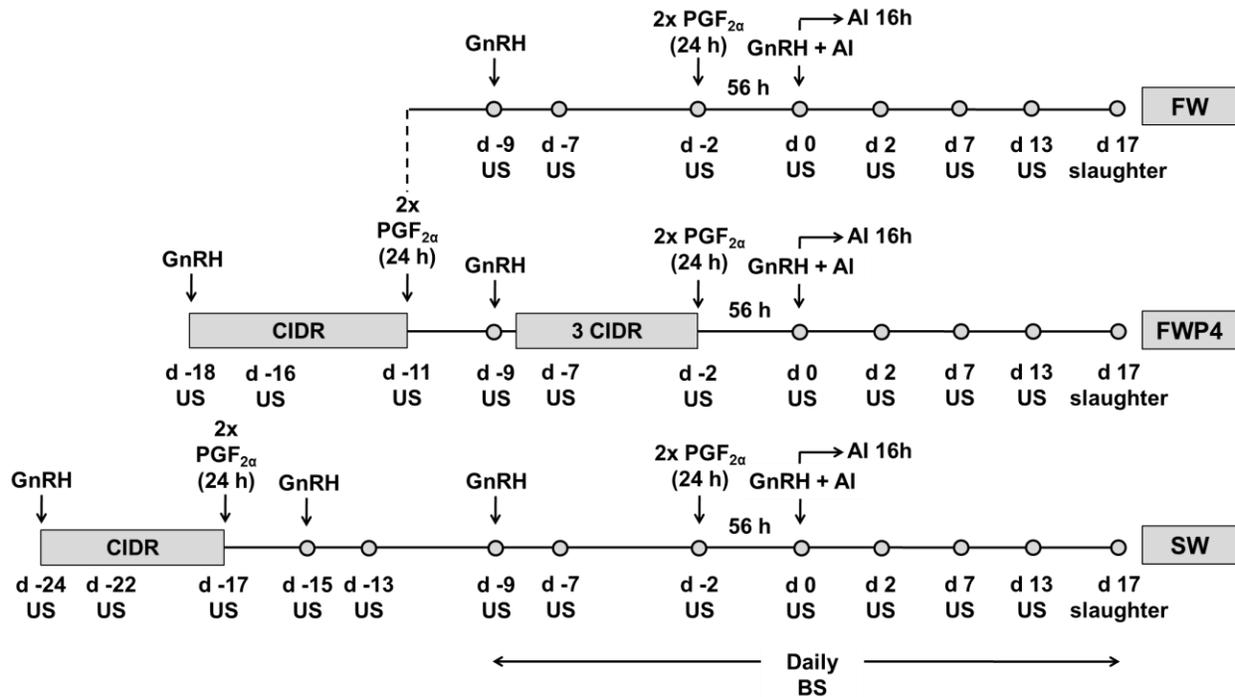


Figure 4-1. Diagram of activities during experiment. AI = artificial insemination; BS = blood sampling for analysis of concentration of estradiol and/or progesterone in plasma; Slaughter = cows were killed via insensibilization by captive bolt followed by exsanguinations; US = ultrasonography of ovaries.

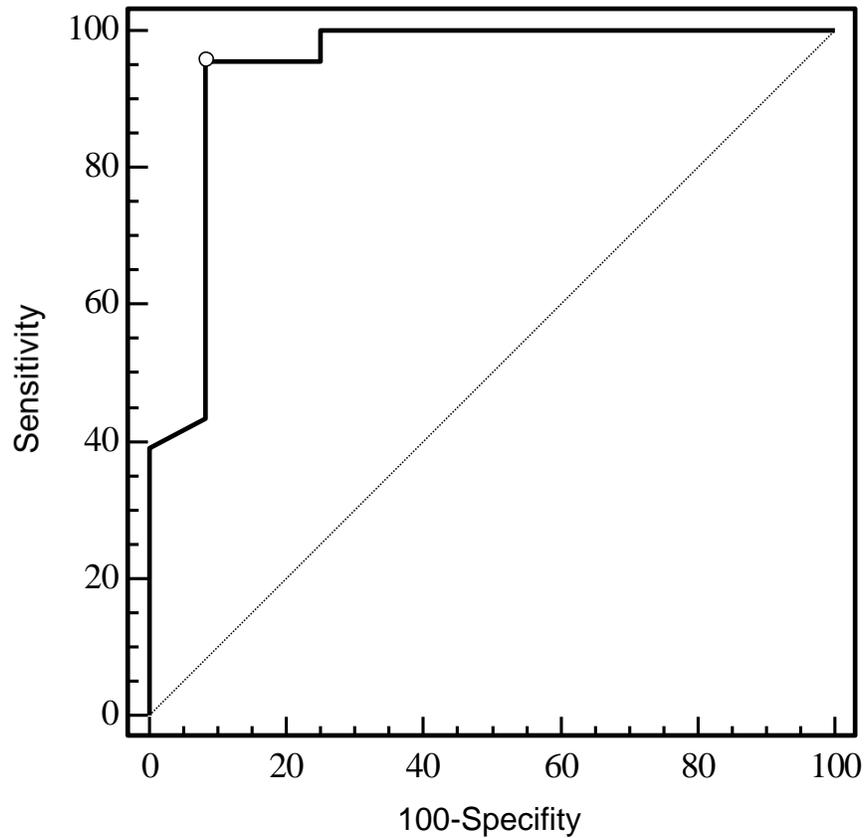


Figure 4-2. Receiver operating characteristic curve representing the accuracy with which concentrations of IFN- τ in uterine flush samples (ng/mL) predicted the presence of a conceptus on d 17 after insemination. Highest combined sensitivity (95.7%; 95% confidence interval = 78.1 to 99.9%) and specificity (91.7%; 95% confidence interval = 61.5 to 99.8%) was detected at 0.34 pg/mL (open circle). The area under the curve was 0.94 (95% confidence interval = 0.81 to 0.99).

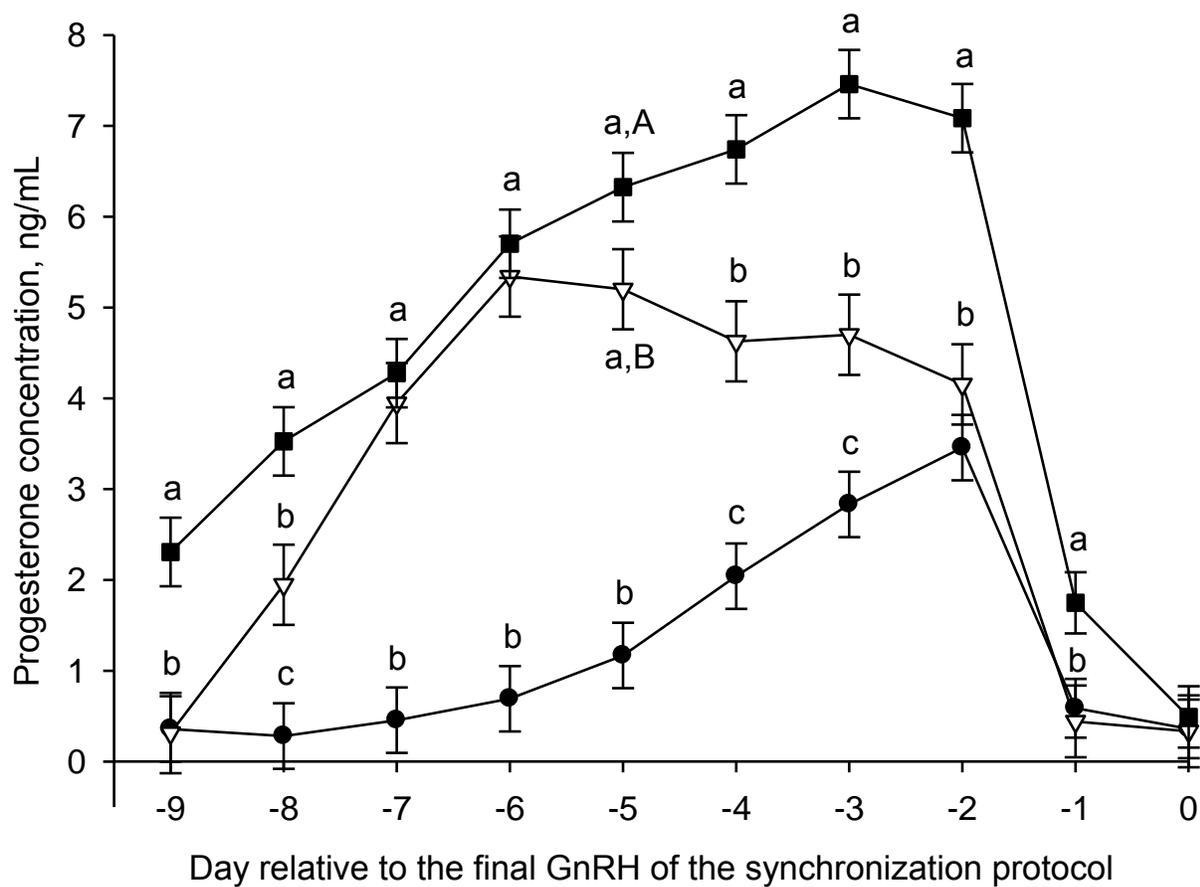


Figure 4-3. Concentrations of progesterone in plasma during treatment period in cows induced to ovulate either the dominant follicle of the first wave (FW, solid circles), first wave concurrent with supplemental progesterone (FWP4, open triangles), or second wave (SW, solid squares). ^{a,b,c} Means with different superscripts differed ($P < 0.05$) among treatments within the same day. ^{A,B} Means with different superscripts tended ($0.05 < P \leq 0.10$) to differ among treatments within the same day.

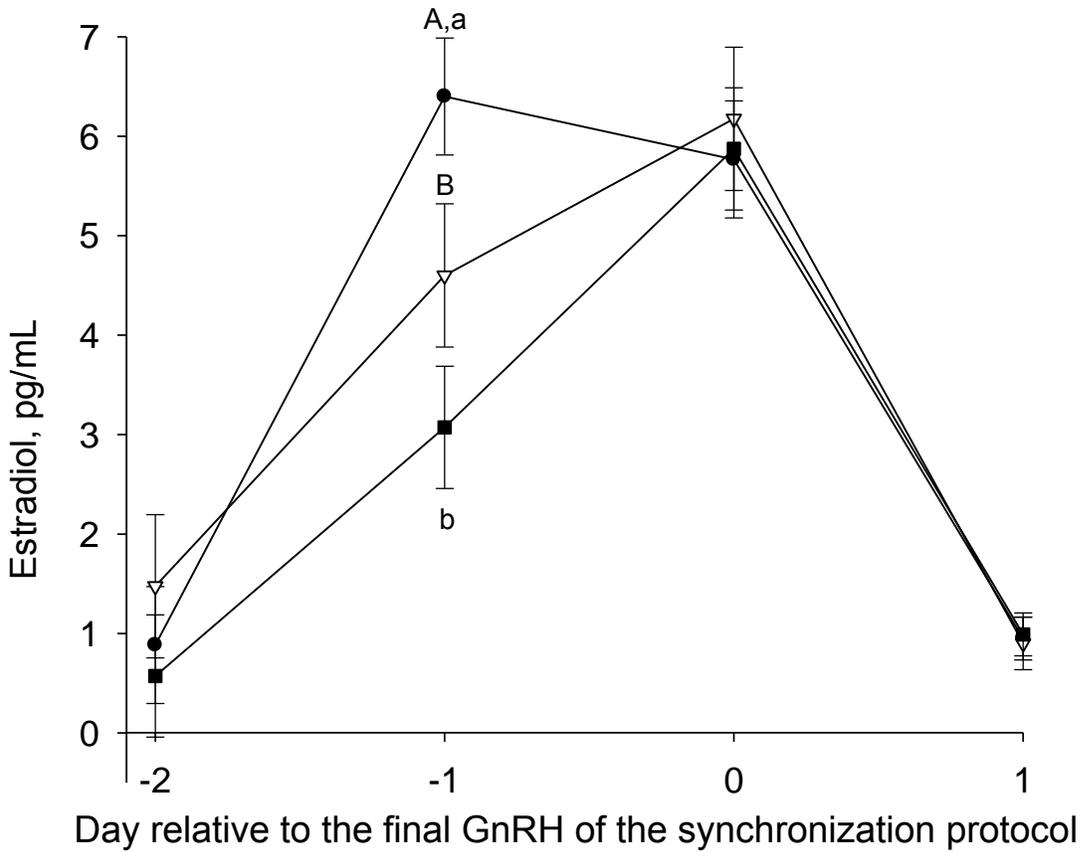


Figure 4-4. Concentrations of estradiol in plasma of cows induced to ovulate the dominant follicle of the first wave (FW, solid circles), first wave concurrent with supplemental progesterone (FWP4, open triangles), or second wave (SW, solid squares). ^{a,b} Means with different superscripts differed ($P < 0.05$) among treatments within the same day. ^{A,B} Means with different superscripts tended ($0.05 < P \leq 0.10$) to differ among treatments within the same day.

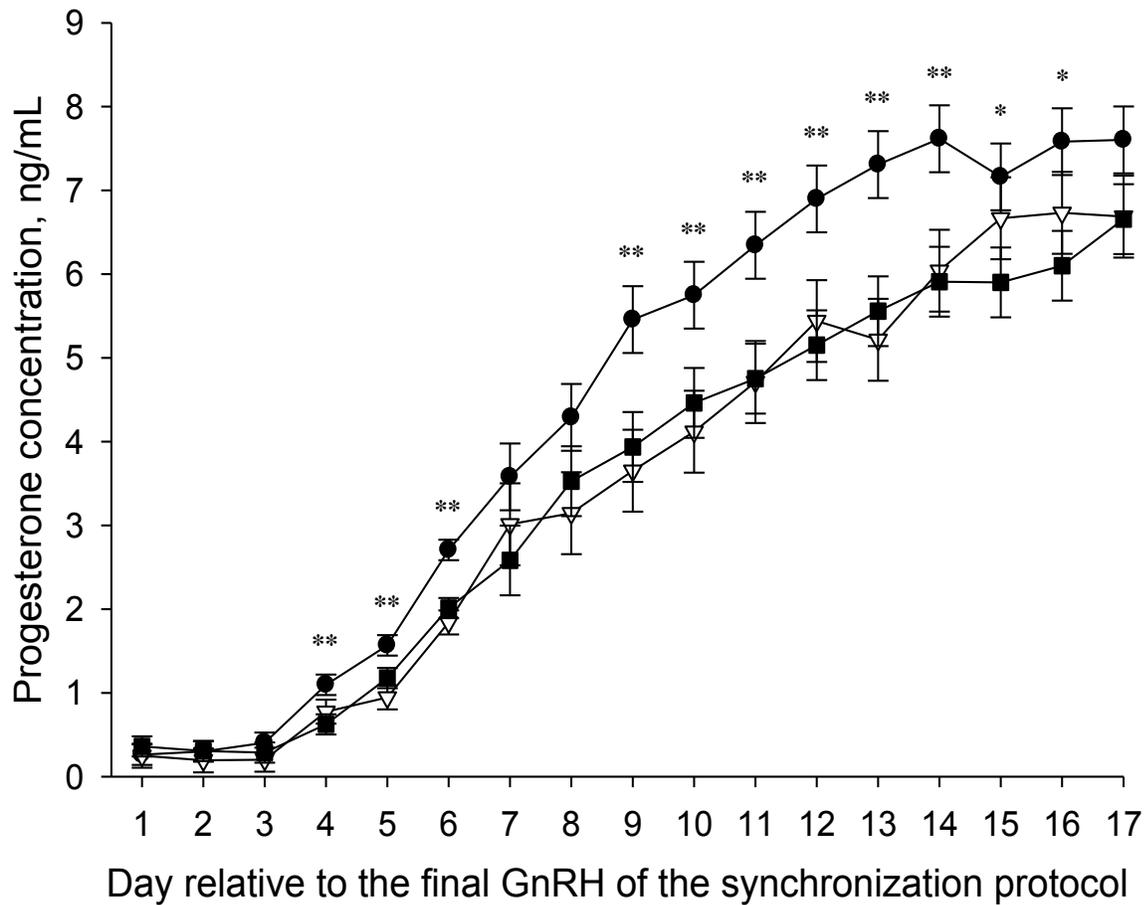


Figure 4-5. Concentrations of progesterone in plasma after the final GnRH in cows induced to ovulate either the dominant follicle of the first wave (FW, solid circles), the dominant follicle of the first wave concurrent with supplemental progesterone (FWP4, open triangles), or the dominant follicle of the second wave (SW, solid squares). * Daily means are greater ($P < 0.05$) for FW than for SW and FWP4 has an intermediate value. ** Daily means are greater ($P < 0.05$) for FW; however, did not differ ($P > 0.05$) between FWP4 and SW.

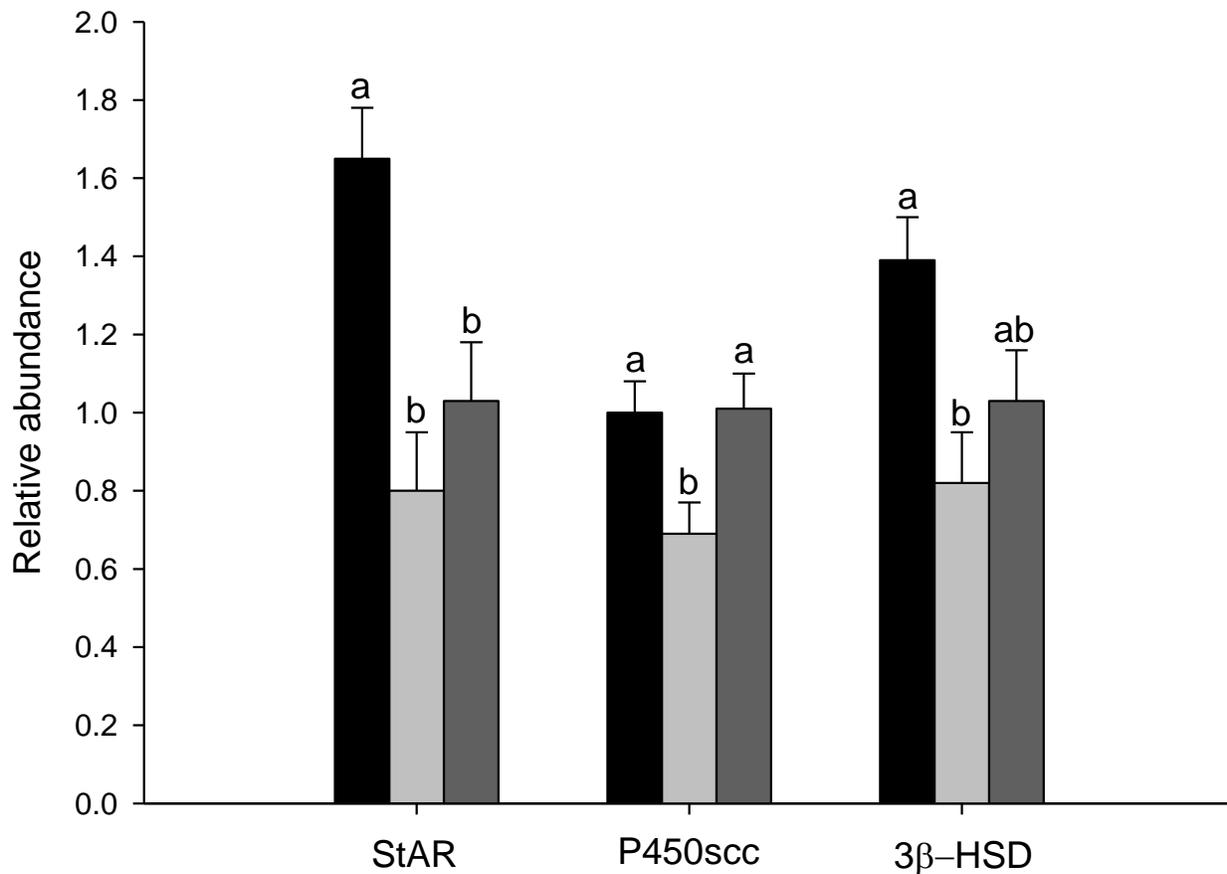


Figure 4-6. Fold change expression from qRT-PCR analysis for steroidogenic acute regulatory protein (StAR), cytochrome *P*-450 side-chain cleavage enzyme (*P*-450scc), and 3β-hydroxysteroid dehydrogenase/Δ5, Δ 4 isomerase (3β-HSD) for FW (black bars), FWP4 (light gray), and SW cows (dark gray). ^{a,b} Means with different superscripts differed ($P < 0.05$) among treatments.

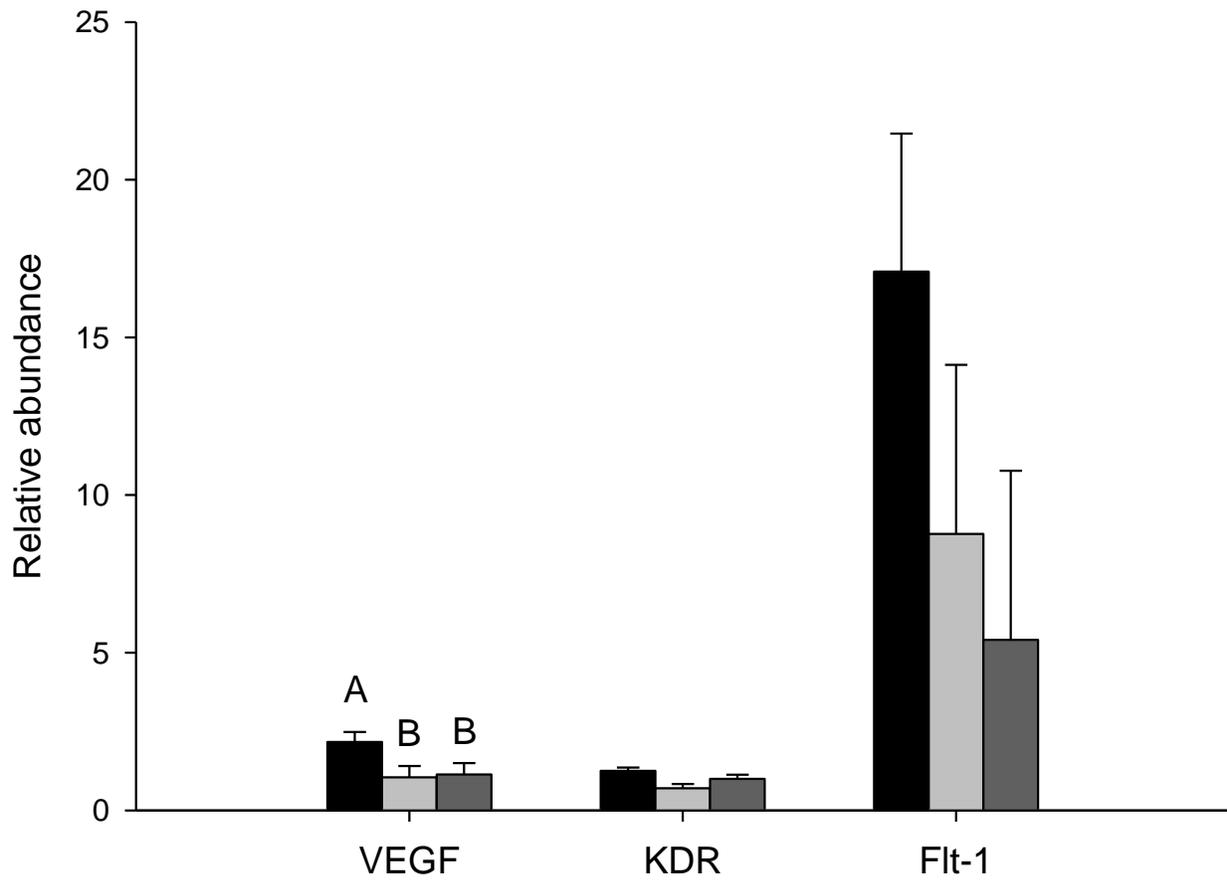


Figure 4-7. Fold change expression from qRT-PCR analysis for vascular endothelial growth factor (VEGF), fms-like tyrosine kinase receptor 1(Flt-1), and kinase insert domain containing region (KDR) for FW (black bars), FWP4 (light gray), and SW cows (dark gray). ^{A,B} Means with different superscripts tended ($0.05 < P \leq 0.10$) to differ among treatments.

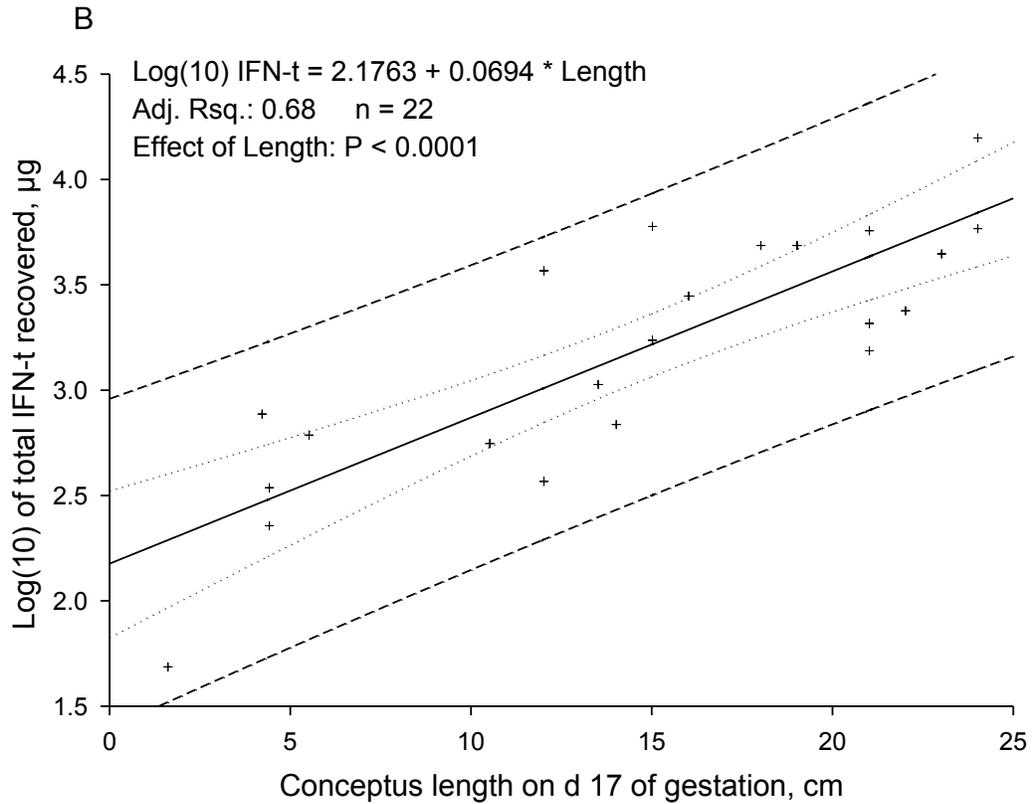
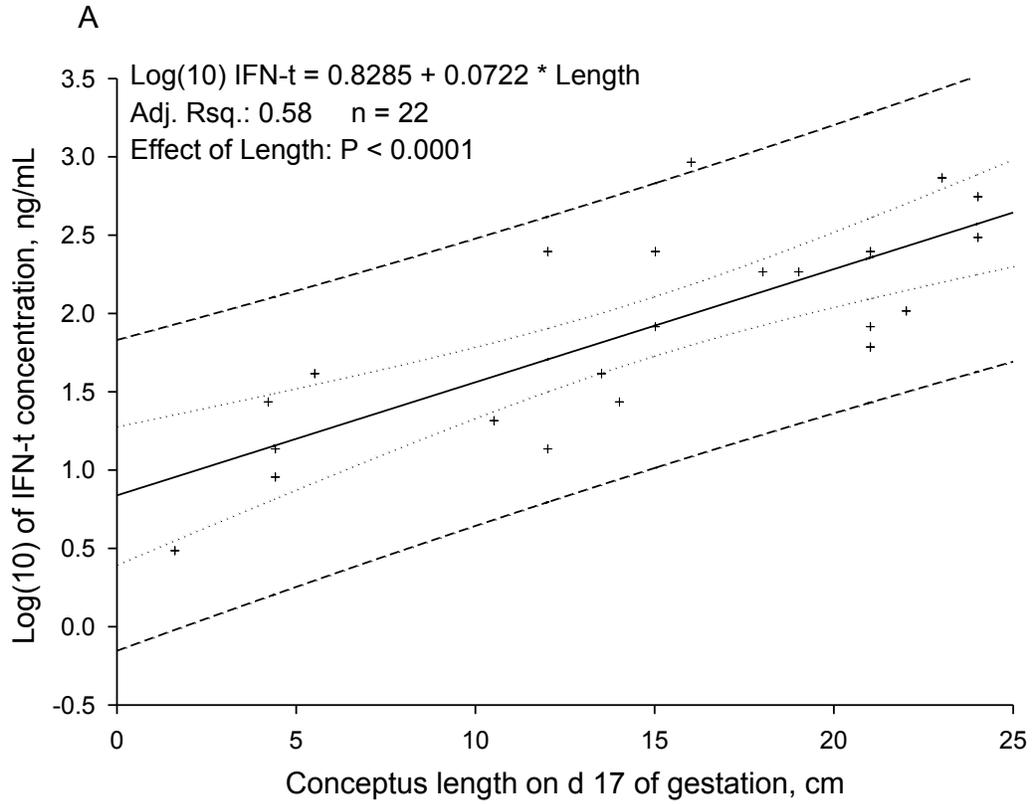


Figure 4-8. Regression of conceptus length and concentration of interferon-tau in uterine flush samples (ng/mL; panel A) and total amount of interferon-tau recovered (μg ; panel B). One SW cow (5686) was considered an outlier and was removed from the statistical analyses. Solid lines represent the value predicted by the regression equation. Dotted lines represent the 95% confidence interval for the mean. Dashed lines represent the 95% confidence interval for a predicted value.

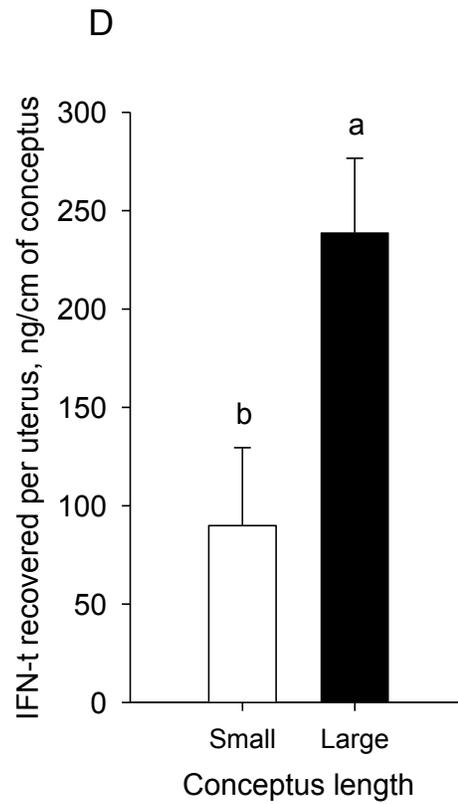
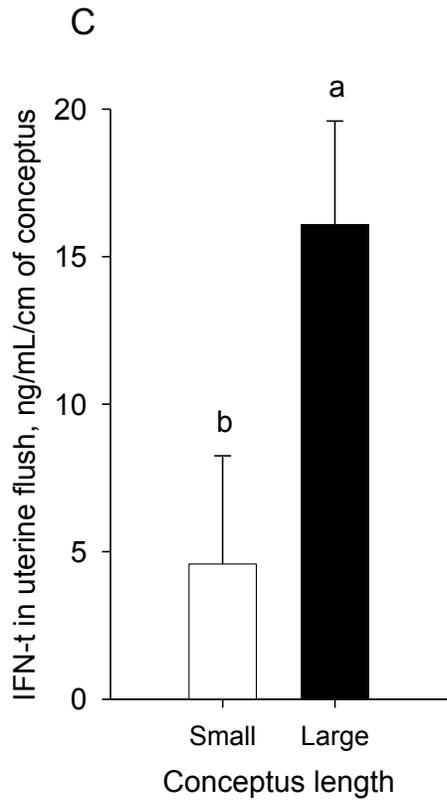
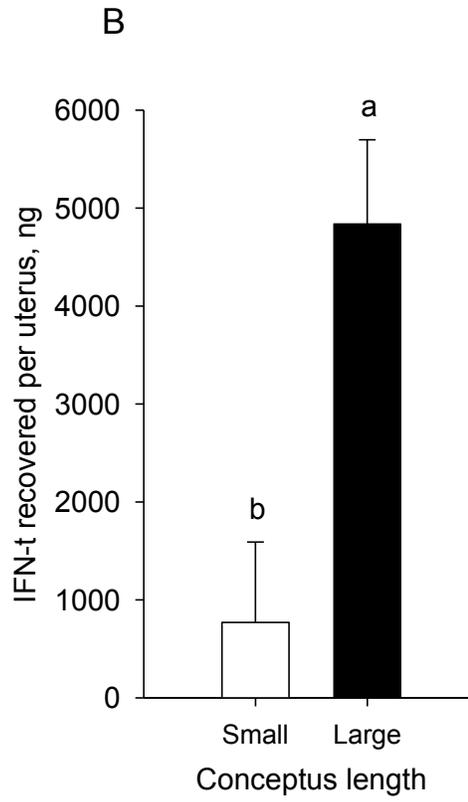
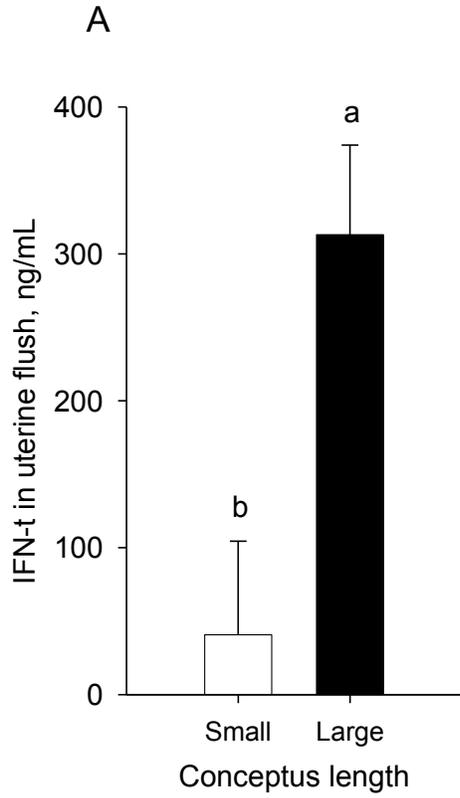


Figure 4-9. Concentration and total amount of interferon-tau recovered by post-mortem flushing of the uterus on d 17 of gestation. Absolute values (panels A and B) and values per centimeter of conceptus (panels C and D) are presented. Conceptuses were classified as small or large in relation to the mean length (small ≤ 14.0 cm, and large > 14.0 cm). ^{a,b} Means with different superscript letters differ ($P < 0.05$).

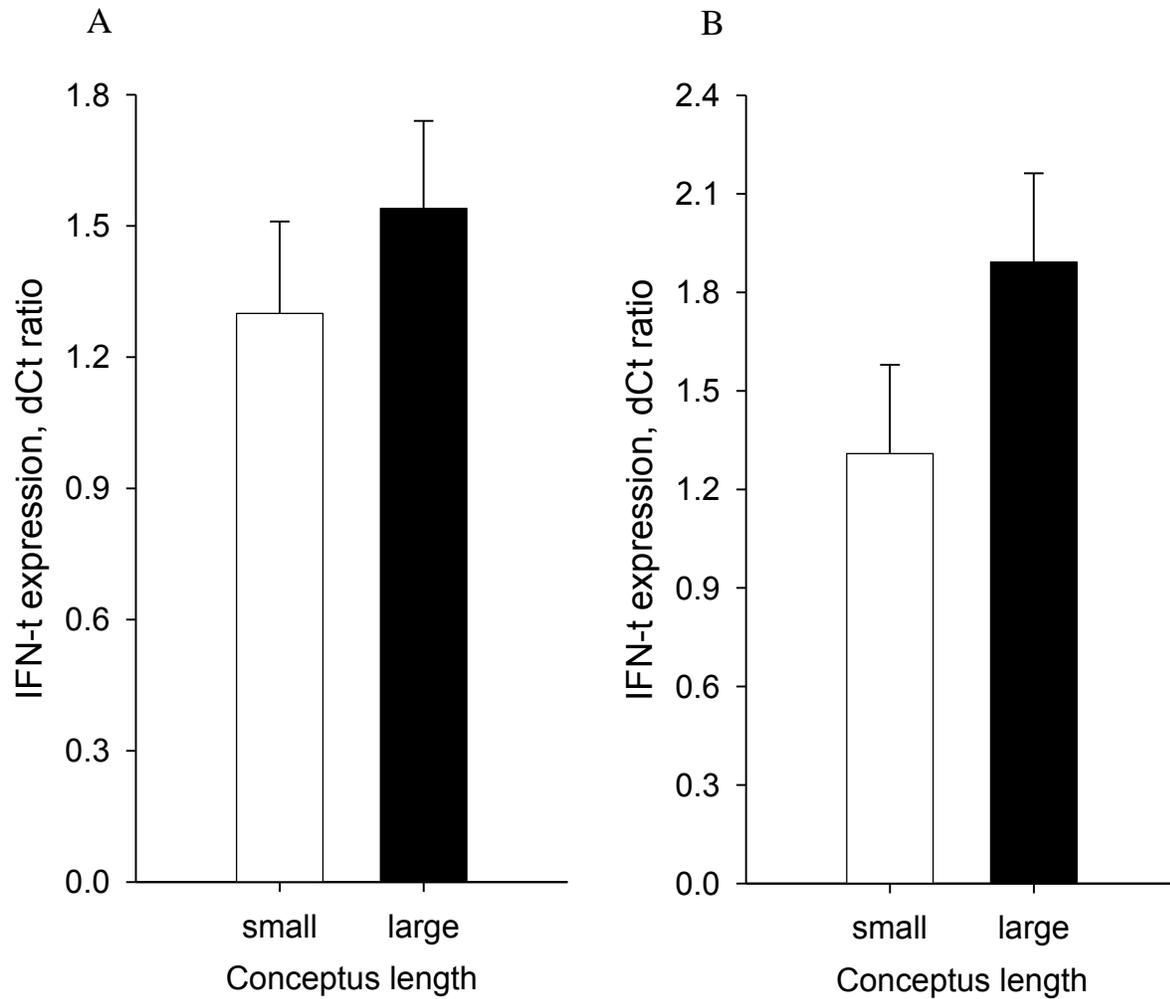


Figure 4-10. Expression of interferon-tau in relation to GAPDH in conceptuses on d 17 of gestation. Data from all conceptuses (panel A) and only intact conceptuses (panel B) are presented. Conceptuses were classified as small or large in relation to the mean length (small \leq 14.0 cm, and large $>$ 14.0 cm).

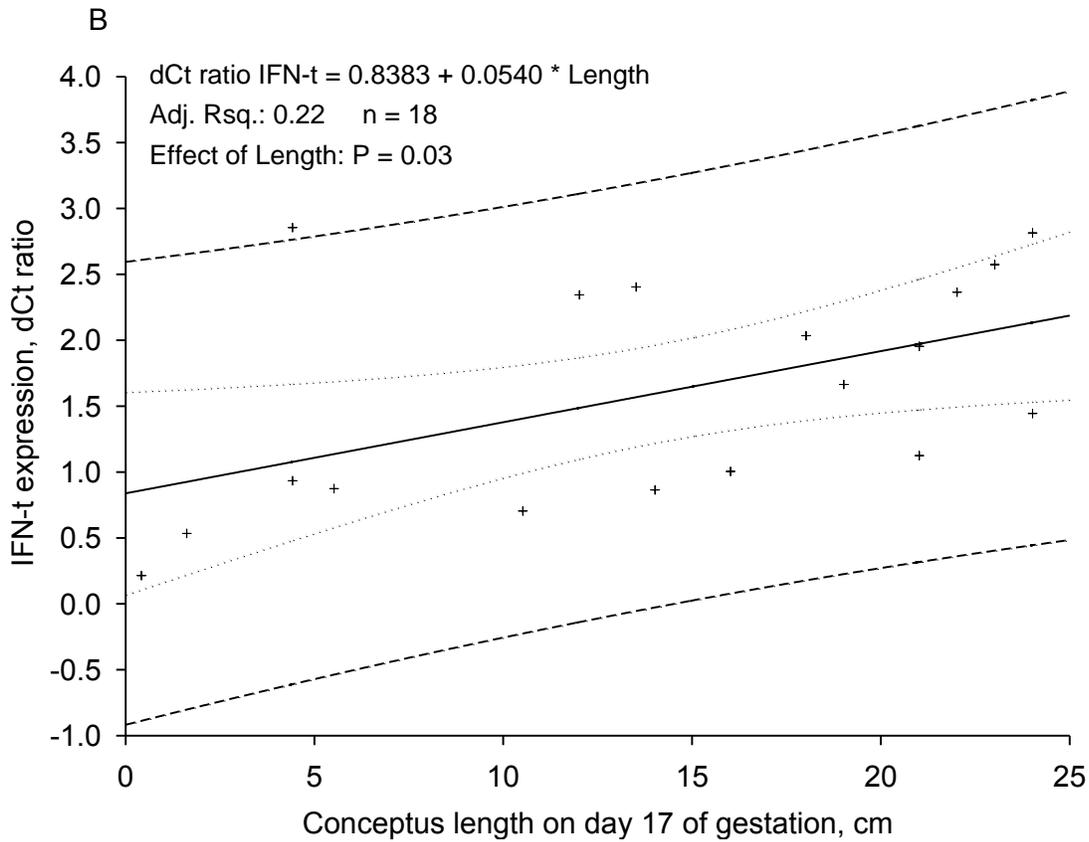
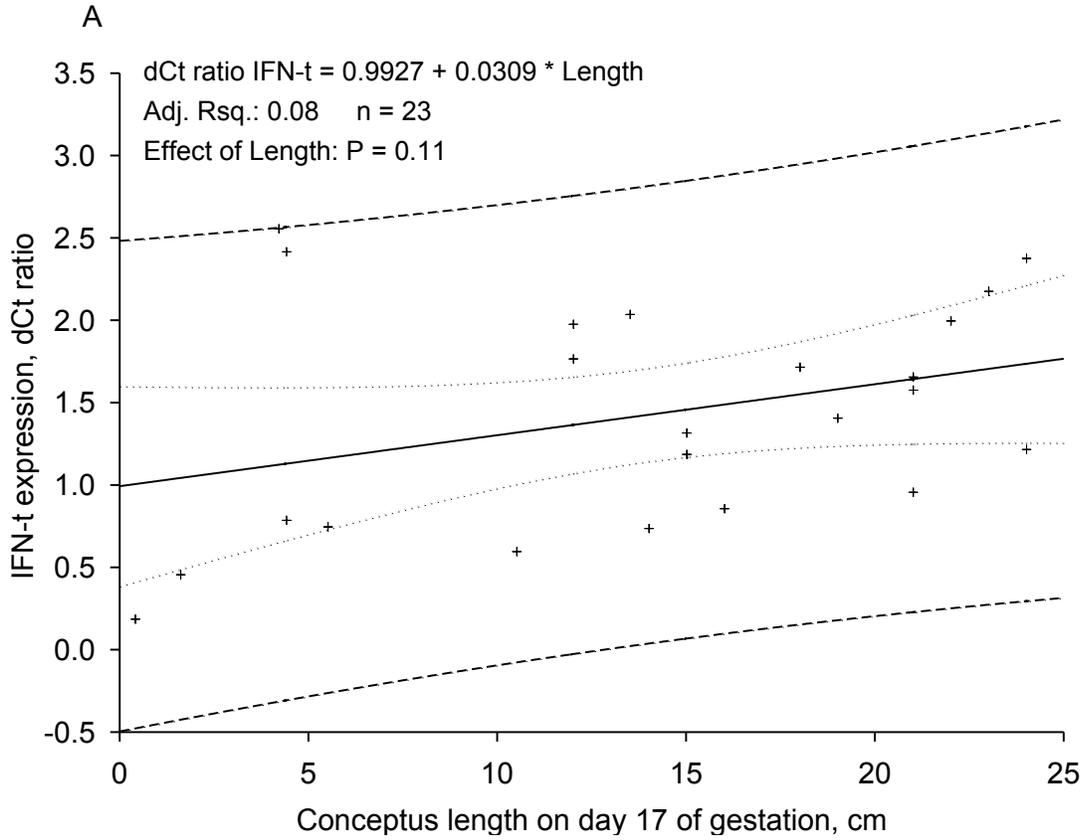


Figure 4-11. Regression of conceptus length and expression of interferon-tau in conceptuses on d 17 of gestation. Data from all conceptuses (panel A) and intact conceptuses (panel B) are presented. Solid lines represent the value predicted by the regression equation. Dotted lines represent the 95% confidence interval for the mean. Dashed lines represent the 95% confidence interval for predicted value.

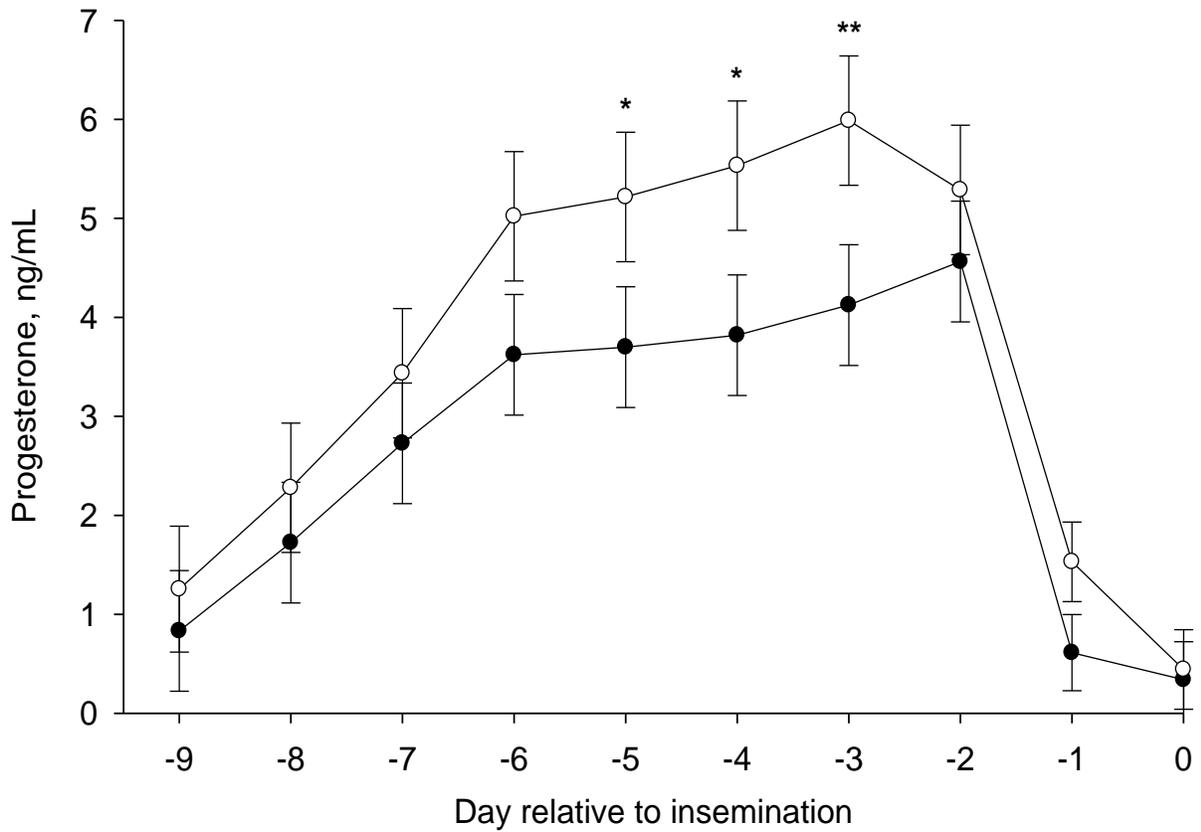


Figure 4-12. Concentrations of progesterone in plasma of cows that had small (open circles) or large (solid circles) conceptuses on d 17 of gestation. ** Means differed ($P < 0.05$) between categories within the same day. * Means tended ($0.05 < P \leq 0.10$) to differ between categories within the same day.

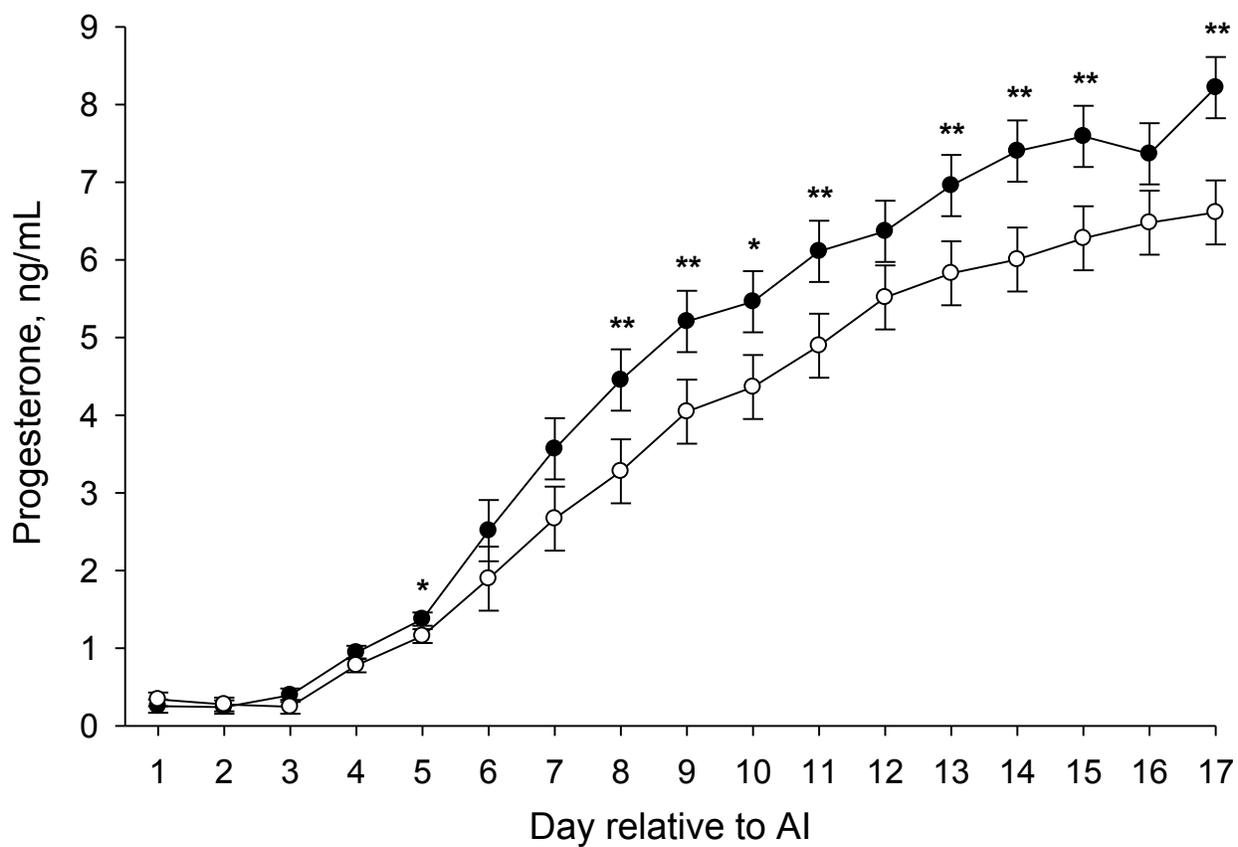


Figure 4-13. Concentrations of progesterone in plasma of cows that had small (open circles) or large (solid circles) conceptuses on d 17 of gestation. ** Means differed ($P < 0.05$) between categories within the same day. * Means tended ($0.05 < P \leq 0.10$) to differ between categories within the same day.

CHAPTER 5
EFFECT OF INTERVAL BETWEEN INDUCTION OF OVULATION AND AI AND
SUPPLEMENTAL PROGESTERONE FOR RESYNCHRONIZATION ON FERTILITY
OF DAIRY COWS SUBJECTED TO A 5-D TIMED AI PROGRAM

Objectives were to investigate 2 intervals from induction of ovulation to artificial insemination (**AI**) and the effect of supplemental progesterone for resynchronization on fertility of lactating dairy cows subjected to a 5-d timed AI program. In experiment 1, 1,227 Holstein cows had their estrous cycle presynchronized with 2 injections of prostaglandin (**PG**) $F_{2\alpha}$ at 46 and 60 days in milk (**DIM**). The timed AI protocols were initiated with gonadotropin-releasing hormone (**GnRH**) at 72 DIM, followed by 2 injections of $PGF_{2\alpha}$ at 77 and 78 DIM and a second injection of GnRH at either 56 (**OVS56**) or 72 h (**COS72**) after the first $PGF_{2\alpha}$ of the timed AI protocols. All cows were timed inseminated at 72 h after the first $PGF_{2\alpha}$ injection. Pregnancy was diagnosed on d 32 and 60 after AI. In experiment 2, 675 nonpregnant Holstein cows had their estrous cycle resynchronized starting at 34 d after the first AI. Cows received the OVS56 with (**RCIDR**) or without (**RCON**) supplemental progesterone, as an intravaginal insert, from the first GnRH to the first $PGF_{2\alpha}$. Pregnancy diagnoses were performed on d 32 and 60 after AI. Subsets of cows during the experiment 2 had their ovaries scanned by ultrasonography at the first GnRH, the first $PGF_{2\alpha}$, and second GnRH injections of the resynchronization protocol. Blood was sampled on the day of AI and 7 d later, and concentrations of progesterone were determined in plasma. Cows were considered to have a resynchronized ovulation if they had progesterone < 1 ng/mL and > 2.26 ng/mL on the day of AI and 7 d later, respectively, and if no ovulation was detected between the first $PGF_{2\alpha}$ and second GnRH injections during resynchronization. In experiment 1, the proportion of cows detected in estrus at AI was greater for COS72 than OVS56

(40.6 vs. 32.4%). Pregnancy per AI (**P/AI**) did not differ between OVS56 (46.4%) and COS72 (45.5%). In experiment 2, cows supplemented with progesterone had greater P/AI compared with unsupplemented cows (51.3 vs. 43.1%). Premature ovulation tended to be greater for RCON than RCIDR cows (7.5 vs. 3.6%), although synchronization of the estrous cycle after timed AI was similar between treatments. In conclusion, timing of induction of ovulation with GnRH relative to insemination did not affect P/AI of dairy cows enrolled in a 5-d timed AI program. Furthermore, during resynchronization starting on d 34 after the first AI, supplementation with progesterone improved P/AI in cows subjected to the 5-d timed AI protocol.

Introduction

Reproductive programs based on GnRH and PGF_{2α} for timed AI maximize the proportion of cows inseminated early after the end of the voluntary waiting period and typically result in pregnancy per AI similar to that observed in breeding programs based on estrous detection (Chebel et al., 2006). From the first report of the Ovsynch protocol (d 0 GnRH, d 7 PGF_{2α}, d 9 GnRH, d 10 timed AI; Pursley et al., 1995), synchronization programs have been modified to improve fertility responses of inseminated lactating dairy cows. Recently, the reduction of the interval between the initial GnRH and the PGF_{2α} injections from 7 to 5 d (5-d Cosynch program) was shown to improve P/AI in lactating dairy cows (Santos et al., 2010). Nevertheless, all cows in that particular experiment were inseminated simultaneously with the final GnRH, which was administered 72 h after the injection of PGF_{2α}. Pursley et al. (1998) suggested that maximum P/AI in the conventional Ovsynch protocol was achieved when the interval between the final GnRH and AI was 16 h. In fact, when the interval between synchronization of follicular wave with GnRH and injection of PGF_{2α} to promote

luteolysis was 7 d, induction of ovulation with the final GnRH injection 16 h before AI improved fertility of dairy cows compared with GnRH concurrent with AI (Brusveen et al., 2008).

When cows are subjected to the 5-d program, the period of ovulatory follicle development is reduced by approximately 2 d compared with conventional programs (Santos et al., 2010). This reduction resulted in ovulatory follicles of smaller diameter, reduced concentration of estradiol in plasma, and a smaller proportion of cows in estrus at AI compared with cows in the conventional 7-d program (Santos et al., 2010). Although insemination of dairy cows 4 to 16 h after the onset of estrus (Dransfield et al., 1998) or 16 h after induction of ovulation (Pursley et al., 1998; Brusveen et al., 2008) improved P/AI, this response might not be the same in cows subjected to a shorter period of ovulatory follicle maturation in the 5-d program. The additional 16 h of follicle development might result in increased concentrations of estradiol in plasma and proportion of cows in estrus at AI, which has been linked with fertility of cows in timed AI programs (Santos et al., 2010). In a series of experiments with beef cows subjected to the 5-d program, extending proestrus from 60 to 72 h was beneficial to fertility (Bridges et al., 2008). Therefore, it is possible that the improved synchrony between ovulation/oocyte competence and numbers of viable sperm available for fertilization (Saacke, 2008) might be offset by the shortened proestrus and reduced proportion of cows in estrus when GnRH is given 56 compared with 72 h after luteolysis. The hypothesis for experiment 1 was that induction of ovulation concurrent with insemination would not compromise P/AI of lactating dairy cows subjected to a 5-d synchronization protocol compared with the administration of the final GnRH 16 h before AI.

After the first postpartum AI, 55 to 65% of the cows remain nonpregnant. These cows should receive a new insemination soon after the nonpregnancy diagnosis; however, the stage of the estrous cycle at which the resynchronization protocol is initiated affects ovarian responses to hormonal treatments, synchrony of ovulation and, consequently, P/AI (Vasconcelos et al., 1999). During resynchronized inseminations, the stage of the estrous cycle at the beginning of the protocol is generally unknown. This can influence synchrony of ovulation when cows are inseminated. The use of a controlled internal drug release (**CIDR**) containing progesterone improved synchrony of the estrous cycle when used in a timed AI protocol (Lima et al., 2009) and increased fertility of dairy cows in the first postpartum AI in some (Chebel et al., 2010) but not all studies (Lima et al., 2009). The hypothesis for experiment 2 was that supplementation with progesterone during the resynchronization with the 5-d protocol would enhance synchrony of the estrous cycle and improve fertility responses in lactating dairy cows.

The objectives of this study were to investigate 2 intervals between induction of ovulation relative to AI and supplemental progesterone during resynchronization on fertility when lactating dairy cows are subjected to a 5-d timed AI program.

Materials and Methods

Experiment 1

Cows, housing and diets

Lactating Holstein cows, 498 primiparous and 769 multiparous, from a single dairy herd in Central Florida, were assigned to the study. Primiparous and multiparous cows were housed separately in free-stall barns equipped with sprinklers and fans.

Inseminations were performed from January 1st to April 9th, 2009. Primiparous and multiparous cows received the same total mixed ration (**TMR**) to meet or exceed the

nutrient requirements for a lactating Holstein cow producing 45 kg of milk per day with 3.5% fat and 3.2% true protein (NRC, 2001). Cows were fed twice daily and milked thrice daily. Diet consisted of rye grass silage, corn silage, alfalfa hay, ground corn, citrus pulp, soybean meal, molasses, minerals and vitamins.

Treatments

Cow enrollment lasted 15 weeks, and in a given week all cows at 46 ± 3 DIM were blocked by parity such that each block had either 2 primiparous or 2 multiparous cows. Within each block, a cow was assigned randomly to 1 of 2 treatments (Figure 5-1). Cows had their estrous cycle presynchronized with 2 i.m. injections of 25 mg of $\text{PGF}_{2\alpha}$ (Lutalyse, 5 mg/mL dinoprost tromethamine sterile solution, Pfizer Animal Health, New York, NY) given 14 d apart, at 46 ± 3 and 60 ± 3 DIM. At 72 ± 3 DIM, cows received an i.m. injection of 100 μg of GnRH (Cystorelin, 50 $\mu\text{g}/\text{mL}$ of gonadorelin diacetate tetrahydrate, Merial Ltd., Duluth, GA) followed by injections of $\text{PGF}_{2\alpha}$ 5 and 6 d later (Santos et al., 2010). Cows then received either 1) a second injection of GnRH 56 h after the first $\text{PGF}_{2\alpha}$ of the synchronization protocol and timed AI 16 h later (**OVS56**, $n = 634$; 241 primiparous and 393 multiparous); or 2) a second injection of GnRH and timed AI at 72 h after the first $\text{PGF}_{2\alpha}$ of the synchronization protocol (**COS72**, $n = 593$; 236 primiparous and 357 multiparous). Cows were painted with chalk on their tailheads daily after the first GnRH of the timed AI protocol and removal of chalk was used as indication of estrus. Forty of the original 1,267 cows were observed in estrus and inseminated before the scheduled timed AI; therefore, they did not receive any of the treatments evaluated in the present experiment. These cows were not included in the statistical analyses and a total of 1,227 cows remained in the study.

Pregnancy diagnosis and calculation of reproductive outcomes

Pregnancy was diagnosed by transrectal ultrasonography (Easi-Scan equipped with 7.5 MHz linear transducer, BCF Technology, Livingston, UK) on d 32 after the timed AI. Presence of an amniotic vesicle with a live embryo (i.e., with heart beat) was used as determinants of pregnancy. Pregnant cows on d 32 were re-examined for pregnancy by transrectal palpation 4 wk later, on d 60 of gestation.

Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at 32 or 60 d after AI by the number of cows receiving AI. Proportion of pregnancy loss was calculated as the number of cows that lost a pregnancy between d 32 and 60 after AI divided by the number of cows diagnosed pregnant on d 32 after AI.

Milk yield and BCS

Body condition score (**BCS**) of all cows was recorded (Ferguson et al., 1994) on the day of the first PGF_{2α} of the presynchronization protocol at 46 ± 3 DIM. For analysis of effect of BCS on reproductive outcomes, BCS was categorized as low (≤ 2.50) or moderate (≥ 2.75). Yields of milk were recorded for individual cows once monthly, using an on-farm milk meter (Tru-Test Ltd., Manukau, New Zealand). The average for the first 3 mo postpartum was used in the statistical analysis. Cows were categorized according to milk production above or below the mean value within parity.

Experiment 2

Cows, housing, and diets

Lactating Holstein cows, 285 primiparous and 390 multiparous, from a single site in Central Florida were assigned to the study. Cows were housed and fed as described in experiment 1. Inseminations were performed from February 2nd to June 4th, 2009.

Treatments

Cows diagnosed as nonpregnant by transrectal ultrasonography on d 32 after the first AI postpartum (112 ± 3 DIM) were enrolled in experiment 2. Cows were blocked by parity and method of synchronization at first AI (from experiment 1: AI at estrus, OVS56, or COS72) at 112 ± 3 DIM. Within each block of 2 cows, they were assigned randomly to 1 of 2 treatments (Figure 5-2). All cows received an injection of GnRH 2 d after the nonpregnancy diagnosis (d 34 after the previous AI), followed by an injection of PGF_{2 α} 5 and 6 d after the first GnRH. A second injection of GnRH was administered 56 h after the first PGF_{2 α} and insemination was performed 16 h later. Cows assigned to control (**RCON**, n = 334) did not receive any further treatment, whereas cows assigned to supplemental progesterone (**RCIDR**, n = 341) received an intravaginal insert containing 1.38 g of progesterone (Eazi-Breed CIDR Cattle Insert, Pfizer Animal Health, New York, NY) from the first GnRH to the first PGF_{2 α} of the resynchronization protocol. Cows were painted with chalk on their tailheads daily after the first GnRH of the resynchronization protocol and removal of chalk was used as an indication of estrus. Thirty-nine of the 675 cows were observed in estrus after the first PGF_{2 α} of the resynchronization and before the day of timed AI. Because they received the treatments and display of estrus might have been influenced by supplemental progesterone, these cows remained in the statistical analyses.

Ultrasonography of ovaries

A subset of 576 cows had their ovaries examined on the day of the first GnRH of the resynchronization by transrectal ultrasonography and the presence of a CL was recorded to determine if responses to resynchronization treatments differed according to ovarian status of cows. Another subset of cows from both RCON (n = 173) and

RCIDR (n = 167) treatments were scanned during the resynchronization protocol on the days of the first PGF_{2α} and second GnRH injections (Figure 5-2). A map of each ovary was drawn and the position of follicles ≥ 5 mm in diameter and CL were recorded.

Ovulation in response to the first GnRH of the resynchronization protocol was determined as the presence of a newly formed corpora lutea (**CL**) on the day of the first PGF_{2α} injection; therefore it was considered only for cows evaluated at both injections.

Premature ovulation was characterized by the disappearance of one or more follicles ≥ 8 mm in diameter present at the first PGF_{2α} and absent at the second GnRH injection.

Blood sampling and analysis of progesterone in plasma

Blood was sampled from 398 cows by puncture of the coccygeal vein or artery into evacuated tubes containing K₂ ethylenediaminetetraacetic acid (**EDTA**; Vacutainer; Becton Dickinson, Franklin Lakes, NJ). Blood was sampled on the day of AI and 7 d later. Samples were immediately cooled by placing them on ice and they arrived at the laboratory within 6 h of collection. Blood tubes were centrifuged at 3,000 x g for 15 min in a refrigerated centrifuge at 4°C for plasma separation. Plasma samples were frozen at -20°C for later analyses.

Concentration of progesterone was analyzed in all plasma samples by radioimmunoassay (**RIA**) using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.1 ng/mL calculated at 2 SD below the mean counts per minute at maximum binding. Samples were analyzed in 3 assays. A moderate (1.5 ng/mL) and a high (2.5 ng/mL) concentration sample was incorporated several times in each assay and used to calculate the intra-assay coefficient of variation (**CV**). The intra-assay CV were 5.3, 5.2 and 5.7% for the first, second and third assays, respectively. The inter-assay CV was 7.1%.

Evaluation of protocol synchronization

Synchronization of the estrous cycle after treatments was evaluated in 409 cows based on two different criteria. In both cases, cows that had premature ovulation (between PGF_{2α} and final GnRH) were considered to not have a synchronized estrous cycle. First, the standard 1 ng/mL concentration of progesterone was used to determine if progesterone was low on the day of AI (< 1 ng/mL) and high 7 d later (≥ 1 ng/mL). Cows with low and high progesterone on the day of AI and 7 d later, respectively, were considered to have a synchronized estrous cycle.

A second criterion was used based on the cut-point for concentrations of progesterone in plasma that best predicted pregnancy on d 32 after AI. Receiver operator characteristic (**ROC**) curves were used to determine the concentrations of progesterone at AI (≤ 0.41 ng/mL) and on d 7 after AI (> 2.26 ng/mL) that resulted in highest accuracy for prediction of pregnancy on d 32 after insemination. Cows with progesterone ≤ 0.41 ng/mL on the day of AI and progesterone > 2.26 on d 7 after AI were considered to have a synchronized estrous cycle.

Pregnancy diagnosis and calculation of reproductive outcomes

Pregnancy diagnosis and calculation of reproductive outcomes were performed as described in experiment 1.

Milk yield

Milk production during the first 3 mo postpartum considered for experiment 1 were used in experiment 2. Cows were categorized according to milk production as described for experiment 1.

Experimental design and statistical analyses

Experiment 1

Cows were assigned to treatments in a randomized block design. Weekly, cows in the range of 43 to 49 DIM were blocked by parity (primiparous or multiparous) and randomly assigned to 1 of 2 treatments within each block. A sample size calculation was performed (Minitab ver. 15, Minitab Inc., State College, PA) to allow for sufficient experimental units to detect a difference of 6 percentage units between treatments when P/AI range from 35 to 45% ($\alpha = 0.05$; $\beta = 0.20$). Under these assumptions, a total of 518 to 534 experimental units per treatment were calculated to be needed to detect a statistical effect under the range of expected P/AI. More cows were added to secure that potential attrition during the study would not limit the number of experimental units initially planned.

Binary responses were analyzed by logistic regression using the LOGISTIC procedure of SAS version 9.2 (SAS/STAT, SAS Institute Inc., Cary, NC, USA). A backward stepwise regression model was used and variables were removed continuously from the model by the Wald statistic criterion if the significance was > 0.10 . Initial models included the effects of treatment, parity, BCS, milk yield, and interactions of treatment and parity, treatment and BCS, and treatment and milk yield. Treatment was forced into the final model in all analyses. Effects of inseminator and sire, and their interactions with treatment were included in the models for P/AI and pregnancy loss. Additional covariates and their interactions with treatment were added to the models if univariate analyses identified them as significant ($P < 0.10$). Adjusted odds ratio (**AOR**) and 95% confidence interval (**CI**) were reported.

Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered as a tendency.

Experiment 2

Cows were assigned to treatments in a randomized block design. Weekly, cows diagnosed as nonpregnant after the first AI were blocked by method of insemination at first insemination (from experiment 1: AI on estrus, OVS56, or COS72) and parity (primiparous or multiparous) and, within each block, they were randomly assigned to 1 of 2 resynchronization treatments.

Binary responses were analyzed as described in experiment 1. Initial models included the effects of treatment, parity, milk yield, and interactions between treatment and parity, and treatment and milk yield. Effects of inseminator and sire, and their interactions with treatment were included in the models for P/AI and pregnancy loss.

Concentrations of progesterone at AI and on d 7 after AI were analyzed by analysis of variance (**ANOVA**) using the GLIMMIX procedure of SAS for normal distribution. The models included the effects of treatment, parity, milk yield, and interactions between treatment and parity.

Receiver operating characteristic curve were generated using the ROC curve analysis option of MedCalc version 11.2.1 (MedCalc Software, Mariakerke, Belgium) to determine the optimal concentration of progesterone at AI and on d 7 after AI that results in greatest sensitivity and specificity to predict pregnancy in dairy cows.

Treatment differences with $P \leq 0.05$ were considered significant and $0.05 < P \leq 0.10$ were considered as a tendency.

Results

Experiment 1

Average BCS and milk yield did not differ between OVS56 (2.81 ± 0.01 and 44.1 ± 0.43 kg/d) and COS72 (2.79 ± 0.01 and 43.6 ± 0.43 kg/d).

Cows in COS72 were more likely ($P < 0.001$) to be detected in estrus on the day of AI than cows in OVS56 (Table 5-1). A tendency ($P = 0.08$) for an interaction between treatment and milk yield was detected, and most of the effect was observed in cows with milk yield below the mean (45.1 vs. 31.7% for COS72 and OVS56, respectively), and less so for cows with milk above the mean (36.2 vs. 32.9% for COS72 and OVS56, respectively). Primiparous cows were more likely ($P = 0.03$) to be detected in estrus at AI than multiparous cows (40.3 vs. 33.8%; AOR = 1.32; 95% CI = 1.03 to 1.68), but no interaction between treatment and parity was observed. Expression of estrus did not differ between cows classified as low or moderate BCS.

Treatment did not affect P/AI on either d 32 or 60 after AI (Table 5-1). Expression of estrus at AI was not associated with P/AI on either d 32 (estrus = 45.6 vs. no estrus = 46.2%) or d 60 after AI (estrus = 39.6 vs. no estrus = 39.9%). Parity and milk yield did not influence P/AI, but cows with moderate BCS had greater ($P < 0.001$) P/AI than those with low BCS (43.1 vs. 31.0%; AOR = 1.68; 95% CI = 1.29 to 2.19). The risk of pregnancy loss between d 32 and 60 of gestation did not differ between treatments (Table 5-1). Pregnancy loss was not influenced by expression of estrus at AI, milk yield, and BCS, or by their interactions with treatment. Although pregnancy loss did not differ between multiparous (14.9%) and primiparous (10.0%), an interaction ($P = 0.02$) between treatment and parity was observed. For multiparous cows, pregnancy loss tended ($P = 0.06$) to increase when receiving COS72 compared with OVS56 (19.5 vs.

10.7%; AOR = 1.82; 95% CI = 0.99 to 3.34); however, for primiparous cows no difference was observed for cows receiving COS72 and OVS56 (7.1 vs. 12.8%; AOR = 0.52, 95% CI = 0.20 to 1.37).

Experiment 2

The average milk yield in the first 3 mo postpartum did not differ between RCON (42.8 ± 0.56 kg/d) and RCIDR cows (42.8 ± 0.59 kg/d). On the day of the first GnRH at 34 d after previous AI, 72.2% of the cows had a visible CL present in the ovaries and the proportion was not different between treatments (RCON = 71.6 vs. RCIDR = 72.8%).

Ovarian responses and progesterone concentrations in plasma

Ovulation to the first GnRH of the resynchronization on d 34 after the previous AI was low, and it did not differ between treatments (Table 5-2). Cows without a CL had greater ($P < 0.001$) ovulation in response to GnRH than those with a CL (77.4 vs. 20.8%; AOR = 16.40; 95% CI = 8.62 to 31.25), but no interaction between method of resynchronization and presence of CL was observed for ovulation rate. The proportion of cows with a visible CL at the first PGF_{2α} of the resynchronization treatments did not differ between RCON and RCIDR. Similarly, the diameter of the largest follicle on the day before AI was not affected by treatment.

A small proportion of cows ovulated between the first PGF_{2α} and second GnRH injections of the resynchronization protocol (5.6%; 19/340). The risk of premature ovulation tended ($P = 0.08$) to be less for cows supplemented with progesterone during the resynchronization protocol than for non-supplemented cows (Table 5-2).

Occurrence of premature ovulation was not affected by the presence of a CL at the beginning of the resynchronization protocol and no interaction between treatment and

the presence of a CL was found to affect occurrence of premature ovulation. The proportion of cows that ovulated between the first PGF_{2α} and second GnRH injections of the resynchronization protocol tended ($P = 0.08$) to be greater for primiparous than for multiparous cows (7.7 vs. 3.5%; AOR = 2.65; 95% CI = 0.91 to 7.75).

Concentration of progesterone in plasma on the day of AI and 7 d later did not differ between treatments (Table 5-2). Parity did not affect plasma concentration of progesterone at AI. Primiparous cows, however, had a greater ($P = 0.02$) concentration of progesterone in plasma on d 7 after AI than multiparous cows (2.97 ± 0.11 vs. 2.64 ± 0.10 ng/mL). Concentration of progesterone in plasma was similar between cows with milk production above and below the average on both the day of AI and 7 d later.

Receiver operating characteristic curves were generated to determine the concentration of progesterone in plasma sampled on the day of AI and 7 d later with greatest accuracy to predict pregnancy on d 32 after insemination. On the day of AI, the concentration that resulted in the greatest combined sensitivity (94.6%; 95% CI = 90.3 to 97.4%) and specificity (14.6%; 95% CI = 10.0 to 20.1%) was ≤ 0.41 ng/mL. On day 7 after AI, the best cut-off concentration was > 2.26 ng/mL, resulting in a sensitivity of 83.2% (95% CI = 77.1 to 88.3) and specificity of 47.6% (95% CI = 40.6 to 54.6).

The proportions of cows with progesterone concentrations in plasma ≤ 0.41 or < 1.0 ng/mL on the day of AI were not affected by treatment (Table 5-2). Similarly, the proportions of cows with progesterone concentrations in plasma ≥ 1.0 or > 2.26 ng/mL 7 d after AI did not differ between treatments. Synchronization of estrous cycle after treatments either using progesterone concentration of 1 ng/mL at AI and 7 d later, or

progesterone ≤ 0.41 at AI and > 2.26 ng/mL 7 d after AI as cut-points were not influenced by supplementation with progesterone during resynchronization.

Estrus at AI, pregnancy per AI, and pregnancy loss

The incorporation of a CIDR insert to the resynchronization protocol did not affect expression of estrus at AI (Table 5-3). The presence of a visible CL at the beginning of the resynchronization protocol did not influence expression of estrus at AI and no interaction between presence of a CL and treatment was observed. Milk production was not associated with expression of estrus. Nonetheless, an interaction ($P = 0.04$) between treatment and milk yield was observed for detection of estrus because supplementation with progesterone tended ($P = 0.10$) to increase the proportion of cows in estrus at AI for those with production above the mean (39.1 vs. 29.8%; AOR = 1.52; 95% CI = 0.92 to 2.49), but not for cows with production below the mean (RCIDR = 26.8 vs. RCON = 33.1%; AOR = 0.74; 95% CI = 0.45 to 1.21). Expression of estrus at AI was not affected by parity or by the interaction between treatment and parity.

Supplementation with progesterone during the resynchronization protocol increased ($P \leq 0.05$) P/AI on d 32 and 60 after the second AI postpartum (Table 5-3). Interestingly, the benefit from the CIDR was observed only in cows that had a CL when the resynchronization protocol was initiated, and these differences were 8 and 9 percentage units greater on d 32 and 60 after AI, respectively (Figure 5-3). Pregnancies per AI on d 32 and 60 after insemination were not affected by detection of estrus at AI, and no interaction between expression of estrus and supplementation with progesterone on P/AI was observed. Primiparous had greater ($P = 0.03$) P/AI than multiparous cows on d 60 after AI (46.8 vs. 37.9%; AOR = 1.43; 95% CI = 1.05 to 1.95). No interactions between treatment and parity were observed on P/AI. Cows with a CL

on the day of the first PGF_{2α} of the resynchronization had greater ($P < 0.05$) P/AI on d 32 (47.7 vs. 29.7%; AOR = 2.25; 95% CI = 1.07 to 4.76) and on d 60 after AI (41.4 vs. 24.3%; AOR = 2.36; 95% CI = 1.06 to 5.24).

The risk of pregnancy loss was not affected by supplementation with progesterone during the resynchronization protocol (Table 5-3). Cows detected in estrus at AI tended ($P = 0.09$) to have less risk of pregnancy loss than those not in estrus (6.5 vs. 12.8%; AOR = 0.45; 95% CI = 0.17 to 1.15). Parity and milk yield were not associated with risk of pregnancy loss in the first 60 d of gestation.

Discussion

The success of programs for synchronization of the estrous cycle and ovulation depends on an orchestrated manipulation of physiological processes such as recruitment of a new follicular wave, control of follicular dominance (Cerri et al., 2009), length of proestrus (Bridges et al., 2008), and the interval between induction of ovulation with GnRH and AI (Pursley et al., 1998). Reduction in the period of follicular dominance was shown to improve embryo quality (Cerri et al., 2009). Accordingly, shortening the interval between the initial GnRH and the injection of PGF_{2α} from 7 to 5 d increased P/AI in lactating dairy cows (Santos et al., 2010).

No differences in P/AI were observed when induction of ovulation using GnRH was performed either 16 h before or concurrent with timed AI at 72 h after the first PGF_{2α} injection in the 5-d program. This is contrary to previous research with cows subjected to a standard 7-d (from GnRH to PGF_{2α} injections) Ovsynch program in which optimal P/AI was achieved when AI was performed 16 h after GnRH-induction of ovulation, and it was significantly reduced when cows were inseminated simultaneously with induction of ovulation (Pursley et al., 1998; Brusveen et al., 2008). In the present

experiment, delaying the final GnRH to 72 h allowed for a longer period of proestrus for COS72 than for OVS56, which resulted in a greater proportion of cows detected in estrus on the day of AI. The longer proestrus period for the COS72 treatment occurred with a comparable period of low progesterone from luteolysis to AI in both treatments.

The advantage of performing AI 16 h after the final GnRH has been attributed to the synchrony between oocyte and numbers of viable sperm available for fertilization (Saacke, 2008). Cows subjected to the 7-d program are expected to ovulate approximately 28 h after the final GnRH (Pursley et al., 1995), which is similar to the time of ovulation relative to the onset of estrus described for lactating dairy cows (27.6 h; Walker et al., 1996). Insemination of dairy cows at the onset of estrus reduced fertilization rate (Dalton et al., 2001) and P/AI (Dransfield et al., 1998) compared with AI 12 h later. This reduction was associated with a decrease in the number of accessory spermatozoa per embryo/ovum, suggesting a continuous loss of functional sperm reservoirs in the additional 12 h preceding ovulation (Dalton et al., 2001). Therefore, insemination of cows 16 h after the final GnRH might increase the number of sperm available for fertilization compared with insemination concurrent with the final GnRH.

Although administration of the final GnRH 16 h before the AI has been shown to benefit fertility of cows in the standard 7-d timed AI program, the additional 16 h of proestrus for cows in COS72 would allow for further growth of the ovulatory follicle (Peters and Pursley, 2003), greater concentrations of estradiol, and more cows in estrus at the time of AI (Hillegass et al., 2008). This might be particularly important in a program with a reduced period of follicle development. Fertility of dairy cows has been positively associated with the diameter of the ovulatory follicle (Vasconcelos et al.,

2001) and concentrations of estradiol in plasma (Lopes et al., 2007). In fact, P/AI in response to timed AI programs are generally superior for cows detected in estrus on the day of insemination compared with cows that do not exhibit signs of estrus (Hillegass et al., 2008; Santos et al., 2010). In the 5-d program, the period of follicular growth is shortened by 2 d compared with the 7-d programs, which results in smaller ovulatory follicles, reduced concentrations of estradiol and a lesser proportion of cows in estrus at AI (Santos et al., 2010). Although administration of their final GnRH concurrently with timed AI reduced P/AI in a 7-d program (Pursley et al., 1998; Brusveen et al., 2008), it is possible that the additional 16 h of proestrus might benefit fertility of dairy cows in the 5-d program and may have offset the better synchrony between induction of ovulation and AI offered by the OVS56. Indeed, experiments conducted with beef cows have shown that reducing the interval from the initial GnRH to the PGF_{2α} increased P/AI only when the period of proestrus was extended from 60 to 72 h (Bridges et al., 2008). This might be even more important for high-producing dairy cows considering the extensive metabolism of steroids (Sangsrivong et al., 2002) and the generally low concentrations of estradiol in plasma during proestrus (Lopes et al., 2007; Hillegass et al., 2008).

Intravaginal inserts for controlled release of progesterone have been used to improve synchrony of ovulation and P/AI in response to timed AI protocols with variable success (Chebel et al., 2006; Lima et al., 2009). After the first postpartum AI, approximately 55 to 65% of the cows are expected to be diagnosed as nonpregnant 28 to 35 d after insemination. The stage of the estrous cycle on which the first GnRH of resynchronization protocol is administered influenced the subsequent P/AI in some

(Fricke et al., 2003; Sterry et al., 2006), but not all studies (Silva et al., 2009). In experiment 2, cows received the first GnRH of the resynchronization protocol 34 d after the previous AI. The length of the estrous cycle in lactating dairy cows averages 22.9 ± 0.7 d (Sartori et al., 2004); therefore, on average, it is expected that most of the cows would be on d 11 of the estrous cycle when the resynchronization protocol was initiated. This is important because approximately 50% of the cows treated with GnRH between d 10 and 16 of the estrous cycle failed to ovulate (Vasconcelos et al., 1999), which in association with spontaneous regression of the CL before the injection of $\text{PGF}_{2\alpha}$, resulted in increased premature ovulation at the end of the synchronization program and reduced fertility (Vasconcelos et al., 1999). In the present study, 11.6% of the cows did not bear a CL at the injection of $\text{PGF}_{2\alpha}$, which reduced P/AI. A greater than anticipated proportion of cows did not have a visible CL by ultrasonography on the day of the first GnRH of the resynchronization (27.8%). This is similar to results reported by Bartolome et al. (2009), but also indicates that a large proportion of the resynchronized cows were not on the expected days of the new estrous cycle as one might expect.

Although ovulation to the first GnRH has been shown to decline in cows that receive a CIDR (Galvão et al., 2004; Stevenson et al., 2006), this response has not been consistent (Lima et al., 2009). A possible reason for lack of effect of CIDR on ovulation to GnRH in the current study was the day when the protocol was initiated. Overall, the ovulatory response was low, only 35.6%, which indicates that most cows did not have a follicle responsive to a GnRH/luteinizing hormone (**LH**) surge, thereby limiting a potential effect of progesterone blocking ovulation. Progesterone released by the CIDR insert acts to reduce LH pulsatility and to block the preovulatory LH surge

(Rathbone et al., 2001); therefore, it potentially reduces the occurrence of premature ovulations during a timed AI protocol. In fact, the odds of ovulating before the final GnRH injection tended to be lesser for cows supplemented with progesterone during the resynchronization protocol in experiment 2. It is possible that a portion of cows that did not ovulate prematurely as assessed here had an endogenous surge of LH before the final injection of GnRH, which would result in ovulation early after AI and reduce P/AI (Saacke, 2008). The supplementation with progesterone is also expected to reduce the incidence of endogenous surges of LH, which could account for some of the increased fertility observed in RCIDR cows in the present study.

High producing dairy cows have reduced plasma concentrations of progesterone during follicular development (Sartori et al., 2004), which may increase LH pulsatility and reduce oocyte competence and embryo quality (Inskeep, 2004). In fact, greater concentrations of progesterone during development of the ovulatory follicle have been associated with improved fertility in dairy cows (Bisinotto et al., 2010; Chapters 3 and 4). Use of a single CIDR insert increased the concentration of progesterone in plasma by approximately 0.9 ng/mL (Lima et al., 2009); therefore, it is inadequate to increase progesterone in cows not bearing a CL to concentrations comparable to those of cows in diestrus (Lima et al., 2009). Nonetheless, it is possible that the additional progesterone from the CIDR insert complemented luteal synthesis of progesterone and allowed for adequate follicular maturation, which resulted in increased P/AI. Similar to our findings, supplementation with progesterone tended to improve P/AI only in cows that had an active CL at the initiation of the Ovsynch protocol (Bartolome et al., 2009).

Conclusion

Fertility of lactating dairy cows subjected to a 5-d timed AI protocol was not affected by the time of administration of the final GnRH relative to AI. As opposed to standard timed AI programs with an interval of 7 d from the first GnRH to induction of luteolysis, in the 5-d program induction of ovulation with GnRH administered concurrently with AI resulted in similar fertility compared with inducing ovulation 16 h before AI. During resynchronization, starting on d 34 after the previous AI, the incorporation of a CIDR insert into the 5-d timed AI program improved P/AI, primarily in cows with a CL at the initiation of the resynchronization. The benefits of the CIDR were observed despite major changes in synchronization of the estrous cycle after AI.

Table 5-1. Effect of moment of administration of the final GnRH relative to timed AI on fertility responses of dairy cows – Experiment 1

	Treatment ¹		AOR (95% CI) ²	P
	OVS56	COS72		
 % (n/n)			
Estrus at timed AI ³	32.4 (199/615)	40.6 (235/579)	1.44 (1.13-1.83)	<0.001
Pregnant				
Day 32	46.4 (294/634)	45.5 (270/593)	1.00 (0.78-1.26)	0.99
Day 60	40.7 (256/629)	38.6 (228/591)	0.94 (0.75-1.18)	0.60
Pregnancy loss ⁴	11.4 (33/289)	14.9 (40/268)	1.37 (0.84-2.25)	0.21

¹ All cows had their estrous cycle presynchronized with 2 injections of PGF_{2α} given 14 days apart and the timed AI protocols initiated 12 days later. COS72 = day 0 GnRH, days 5 and 6 PGF_{2α}, day 8 GnRH + timed AI; OVS56 = day 0 GnRH, days 5 and 6 PGF_{2α}, day 7.3 GnRH, day 8 timed AI.

² AOR = adjusted odds ratio; CI = confidence interval. OVS56 is the reference for comparison.

³ Evaluated based on removal of tail chalk at timed AI.

⁴ Number of pregnant cows on day 32 that were not pregnant on day 60 divided by the number of pregnant cows on day 32. Five OVS56 and two COS72 cows left the study before reconfirmation of pregnancy on day 60 after AI.

Table 5-2. Effect of supplementation with progesterone during the resynchronization protocol on fertility responses of dairy cows – Experiment 2

	Treatment ¹		AOR (95% CI) ²	P
	RCON	RCIDR		
Ovulation to 1 st GnRH, % (n/n)	36.5 (58/159)	34.8 (56/161)	1.07 (0.62-1.86)	0.81
CL at PGF _{2α} , % (n/n)	86.8 (138/159)	90.1 (145/161)	1.34 (0.66-2.71)	0.42
Diameter of largest follicle ³ , mm	16.4 ± 0.4	15.5 ± 0.4	---	0.13
Premature ovulation ⁴ , % (n/n)	7.5 (13/173)	3.6 (6/167)	0.38 (0.13-1.12)	0.08
Progesterone, ng/mL				
Day of AI	0.24 ± 0.04	0.31 ± 0.04	---	0.12
7 days after AI	2.88 ± 0.11	2.73 ± 0.11	---	0.35
Progesterone ⁵ , % (n/n)				
< 1.00 ng/mL at AI	96.4 (186/193)	95.1 (195/205)	0.73 (0.27-1.97)	0.54
≥ 1.00 ng/mL 7 days after AI	94.3 (182/193)	90.2 (185/205)	0.56 (0.26-1.20)	0.14
≤ 0.41 ng/mL at AI	89.1 (172/193)	90.2 (185/205)	1.13 (0.59-2.16)	0.71
> 2.26 ng/mL 7 days after AI	66.3 (128/193)	67.3 (138/205)	1.04 (0.68-1.58)	0.87
Synchronization ⁶ , % (n/n)				
Progesterone at 1 ng/mL	84.9 (169/199)	84.3 (177/210)	0.95 (0.56-1.63)	0.86
Greatest accuracy	52.8 (105/199)	59.1 (124/210)	1.29 (0.87-1.91)	0.20

¹ Nonpregnant cows had their estrous cycle resynchronized 34 days after the first postpartum AI. RCON = day 0 GnRH, days 5 and 6 PGF_{2α}, day 7.3 GnRH, day 8 timed AI; RCIDR = day 0 GnRH + CIDR insertion, day 5 PGF_{2α} + CIDR removal, day 6 PGF_{2α}, day 7.3 GnRH, day 8 timed AI.

² AOR = adjusted odds ratio; CI = confidence interval. RCON is the reference for comparison.

³ Measured on the day before AI.

⁴ Disappearance of one or more follicles ≥ 8 mm in diameter present at the first PGF_{2α} and absent at the second GnRH injection.

⁵ Concentration of progesterone in plasma on the day of AI and 7 d later using conventional cut-off values (1 ng/mL) or optimized values for luteolysis (≤ 0.41 ng/mL) and luteal function on day 7 (> 2.26 ng/mL) according to greatest accuracy from the receiver operator characteristic curves to predict pregnancy on day 32 after AI.

⁶ Progesterone at 1 ng/mL = Cows without premature ovulation and progesterone < 1 ng/mL at AI and progesterone ≥ 1 ng/mL on day 7 after AI; Greatest accuracy = cows without premature ovulation and with progesterone ≤ 0.41 ng/mL at AI and progesterone > 2.26 ng/mL on day 7 after AI.

Table 5-3. Effect of supplementation with progesterone during the resynchronization protocol on fertility responses of dairy cows – Experiment 2

	Treatment ¹		AOR (95% CI) ²	P
	RCON	RCIDR		
 % (n/n)			
Estrus at timed AI ³	31.5 (90/286)	32.5 (96/295)	1.05 (0.74-1.48)	0.80
Pregnant				
Day 32	43.1 (144/334)	51.3 (175/341)	1.38 (1.02-1.88)	0.04
Day 60	37.8 (126/333)	45.5 (153/336)	1.37 (1.00-1.87)	0.05
Pregnancy loss ⁴	11.9 (17/143)	10.0 (17/170)	0.82 (0.40-1.68)	0.59

¹ Nonpregnant cows had their estrous cycle resynchronized 34 days after the first postpartum AI. RCON = day 0 GnRH, days 5 and 6 PGF_{2α}, day 7.3 GnRH, day 8 timed AI; RCIDR = day 0 GnRH + CIDR insertion, day 5 PGF_{2α} + CIDR removal, day 6 PGF_{2α}, day 7.3 GnRH, day 8 timed AI.

² AOR = adjusted odds ratio; CI = confidence interval. RCON is the reference for comparison.

³ Evaluated based on removal of tail chalk at timed AI.

⁴ Number of pregnant cows on day 32 that were not pregnant on day 60 divided by the number of pregnant cows on day 32. One RCON and 5 RCIDR cows left the study before reconfirmation of pregnancy on day 60 after AI.

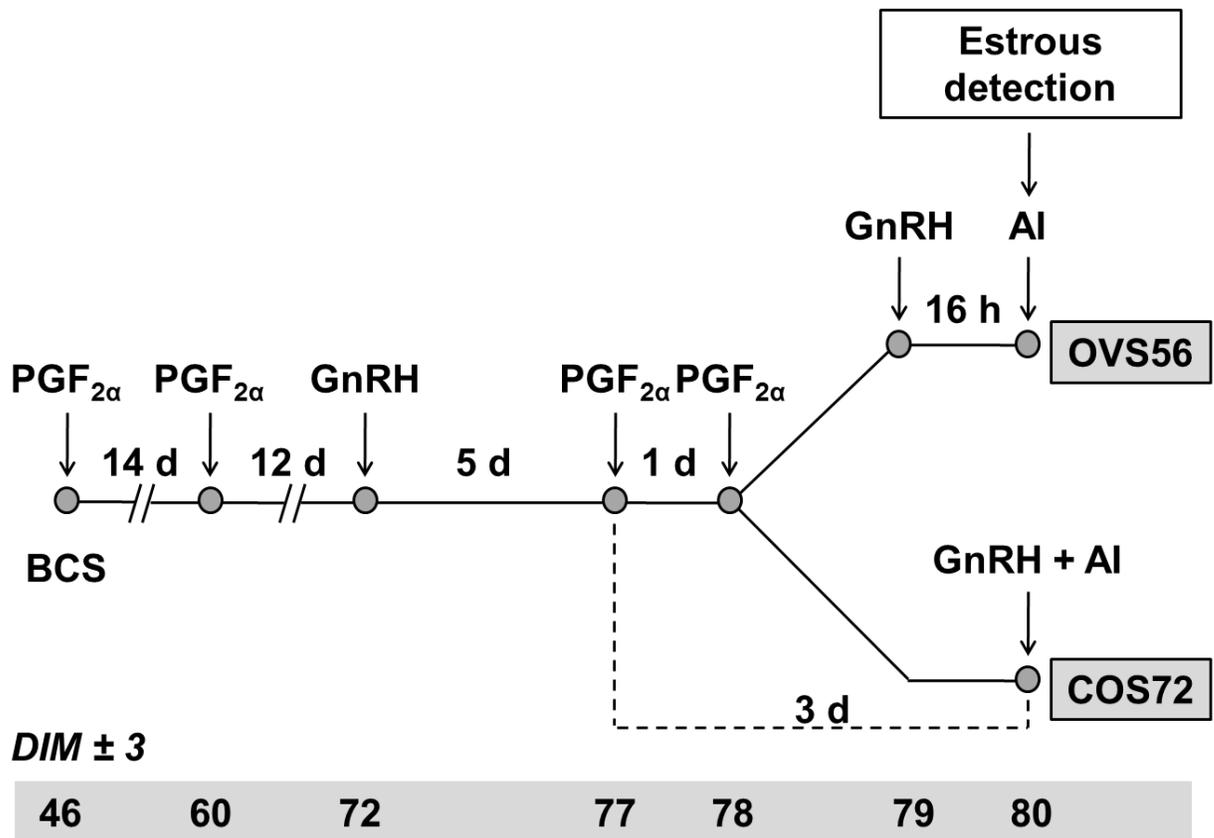


Figure 5-1. Diagram of activities during experiment 1. COS72 = Cosynch-72, induction of ovulation with GnRH concurrent with AI; OVS56 = Ovsynch-56, induction of ovulation with GnRH 16 hours before AI.

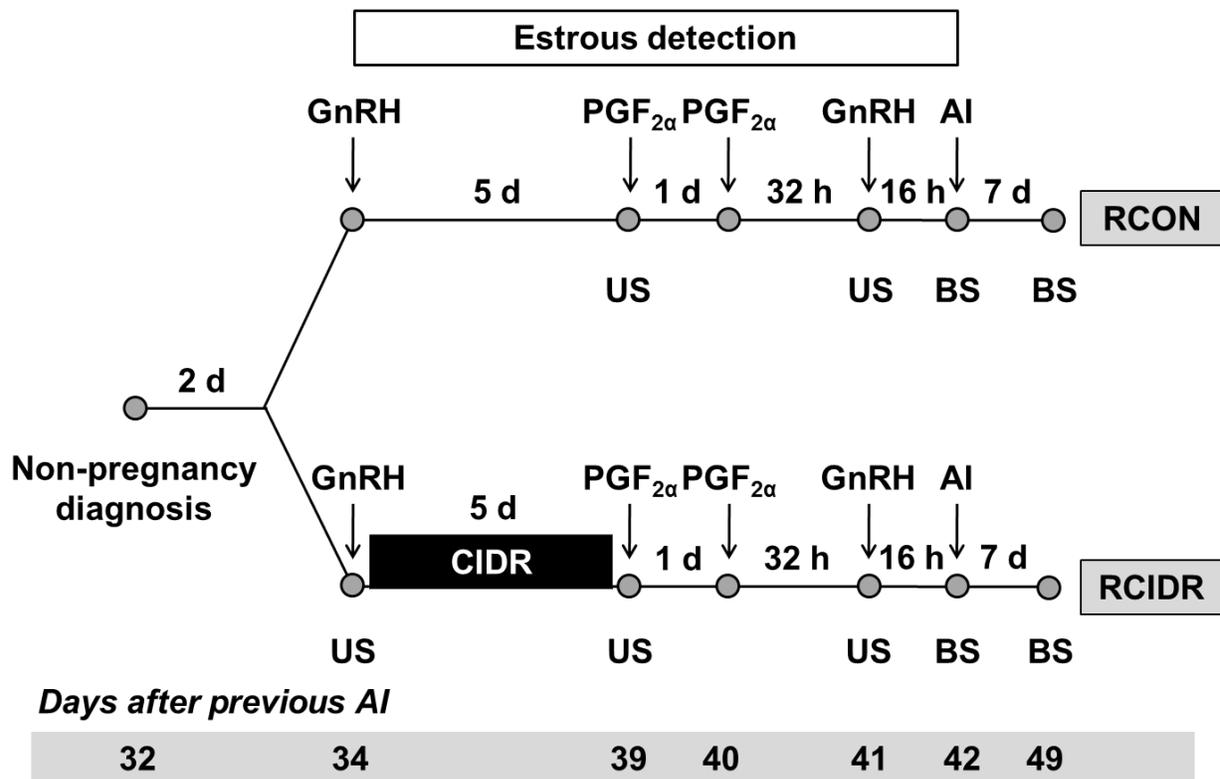


Figure 5-2. Diagram of activities during experiment 2. BS = blood samples for analysis of progesterone concentration in plasma; CIDR = controlled internal drug release containing 1.38 g of progesterone; RCIDR = treatment with CIDR; RCON = no treatment with CIDR; US = ultrasonography of ovaries.

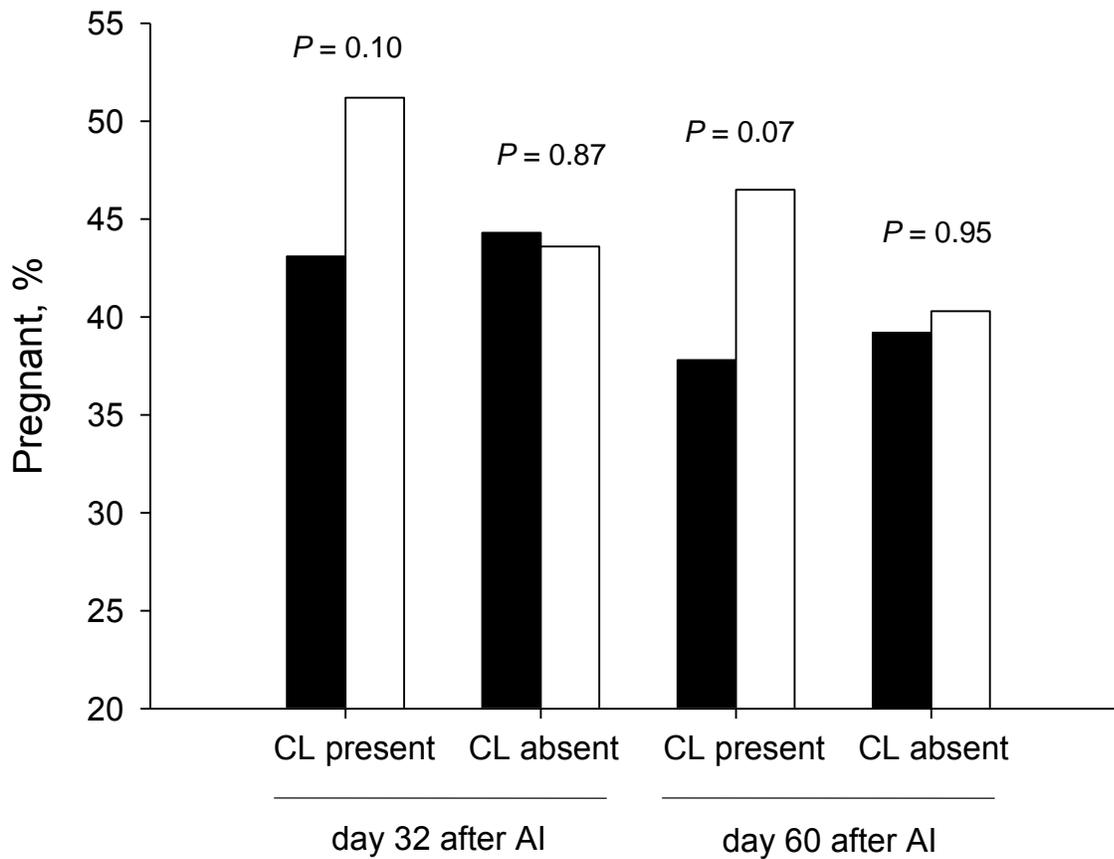


Figure 5-3. Pregnancy per AI according to presence of CL at the first GnRH of the resynchronization protocol (experiment 2). Solid bars = RCON (day 0 GnRH, days 5 and 6 PGF_{2α}, day 7.3 GnRH, day 8 timed AI); Open bars = RCIDR (day 0 GnRH + CIDR insertion, day 5 PGF_{2α} + CIDR removal, day 6 PGF_{2α}, day 7.3 GnRH, day 8 timed AI). Number of cows were: RCON with CL = 204; RCIDR with CL = 215; RCON without CL = 79; and RCIDR without CL = 78.

CHAPTER 6 GENERAL DISCUSSION AND CONCLUSION

According to reports from the USDA, the US produced a total of 190,396 millions of pounds of milk in the year of 2009. The yearly milk production per cow has grown approximately 1.3% per year during the last decade, which resulted in an increment of 16,500 lbs/cow/year from the average cow in the 1940s to the current American dairy cow. This remarkable boost was obtained through advances in genetic selection and spreading of superior lineages, nutrition plans for both dry and lactating cows, sanitary control of herds, and systematic reproductive management. Reproductive performance exerts a major impact in dairy herds' profitability. The average value of a pregnancy has been estimated in \$ 278.00, whereas the cost of losing a pregnancy is \$ 555.00 (De Vries, 2006). This is because submission rate, pregnancy per AI (**P/AI**) and risk of pregnancy loss have a direct effect on time to conception, which in turns determine the average days in milk (**DIM**) of the herd and, consequently, the average milk yield. Accordingly, a deeper understanding of dairy cows' biology would allow for further improvement in reproductive efficiency.

Delayed resumption of ovulation postpartum is an endemic condition among dairy herds. Reports from Canada and the US have shown that, within herds, the proportion of anovular cows between 60 and 65 DIM might be greater than 40% (Walsh et al., 2007; Santos et al., 2009). This becomes critical because, when subjected to timed artificial insemination (**AI**) programs, anovular cows were 40.1% less likely to conceive and twice as likely to lose their pregnancies compared to cyclic cows (Santos et al., 2009,2004). Consequently, the interval from calving to pregnancy was 30 days longer for anovular cows (Walsh et al., 2007). Ovulation in response to the first injection of

gonadotropin-releasing hormone (**GnRH1**) was greater for anovular cows, whereas corpora lutea (**CL**) regression in response to prostaglandin (**PG**) $F_{2\alpha}$ and ovulation following the final GnRH did not differ between anovular and cyclic cows (Gümen et al., 2003). Therefore, the response to hormonal treatments is unlikely to explain the negative effect of anovulation on fertility. The results from the first experiment presented in Chapter 3 also showed a reduction in P/AI for anovular cows; however, when cyclic cows were classified according to their concentration of progesterone at the GnRH1, it became clear that the difference was between anovular cows and cyclic cows with a functional CL at the initiation of the synchronization protocol. Anovular cows and cyclic cows with low progesterone at the GnRH1 had similar P/AI. Cyclic cows with progesterone ≥ 1 ng/mL at the GnRH1 likely in diestrus, which would likely result in the ovulation of a SW dominant follicle after a GnRH-PGF $_{2\alpha}$ based synchronization protocol. Conversely, cyclic cows with progesterone < 1 ng/mL at GnRH1 would likely be in proestrus, estrus, or metestrus and they would likely ovulate a first wave (**FW**) dominant follicle at AI. Similarly, anovular cows that successfully respond to a GnRH-PGF $_{2\alpha}$ based synchronization protocol are expected to ovulate the dominant follicle from the FW at AI, which suggests that the ovulation of a FW dominant follicle might be one of the components of the poor fertility observed in anovular dairy cows. Results from the second experiment confirmed that cows induced to ovulate FW dominant follicles were less fertile than those induced to ovulate follicles from the second wave (**SW**).

The mechanisms that underlie the reduction in fertility observed in anovular cows have been associated with lack of exposure to progesterone after calving (Crowe, 2008). Nonetheless, results from the first study presented here suggest that, if mediated

by low concentrations of progesterone, the negative effects of anovulation require a much shorter period on which progesterone is reduced. The lack of progesterone priming in anovular cows was suggested to reduce CL lifespan during subsequent estrous cycle (Crowe, 2008); however, supplementation with progesterone 20 to 15 days before ovulation was unable to extend subsequent interestrus interval (Kyle et al., 1992). Conversely, using a model that induces low concentrations of progesterone via treatment with PGF_{2α} early after ovulation, others have shown an increase in oxytocin-induced release of PGF_{2α} (Shaham-Albalancy et al., 2001; Cerri et al., 2008a) associated with an increase in the proportion of cows with shortened luteal phases (Cerri et al., 2008a). In beef cows, this phenomena has been attributed to a premature downregulation of progesterone receptors and an early and increased expression of receptors for oxytocin in the endometrium (Zollers et al., 1989, 1993). Current results regarding luteal function and CL lifespan following the ovulation of FW and SW dominant follicles are somewhat controversial. In Chapter 3, cows on which reduced concentrations of progesterone during development of the ovulatory follicle were expected (anovular and CLOW cows) had increased incidence of short cycles. On the other hand, in a controlled study (Chapter 4), none of the cows had regressed their CL before day 17 of the estrous cycle, in disregard of wave of the ovulatory follicle and concentration of progesterone during follicular growth. Indeed, luteal function was enhanced in cows subjected to reduced concentrations of progesterone. Cows that ovulated FW follicles and were not supplemented with progesterone ovulated a larger follicle and produced more estradiol (Chapter 4), presumably caused by enhanced luteinizing hormone (**LH**) pulse frequency (Evans et al., 1987), which resulted in a larger

CL and greater circulating concentrations of progesterone after ovulation. This is in agreement with previous studies that reported that ovulation of larger follicles resulted in the formation of larger CL and greater concentration of progesterone in plasma (Vasconcelos et al., 2001). Furthermore, Wolfenson et al. (1999) reported that circulating concentrations of progesterone following ovulation of FW follicles was either greater (1st replicate) or similar (2nd replicate) to those observed following ovulation of SW follicles. Furthermore, no difference on the day of CL regression was reported (Wolfenson et al., 1999). A greater capability of theca cells, but not granulosa cells, to produce progesterone after luteinization *in vitro* was reported for FW follicular cells (Wolfenson et al., 1999). In the present study (Chapter 4), messenger ribonucleic acid (**mRNA**) abundance for steroidogenic acute regulatory protein (**StAR**) was greater in FW derived CL when no progesterone was supplemented. This might partially explain the increase in steroidogenesis per cells described by Wolfenson et al. (1999). Nonetheless, the ratio between circulating progesterone and CL measures (Chapter 4) indicates that the greater total mass of cells producing progesterone is more important than the increase in progesterone production per cell.

The results presented in the current thesis (Chapters 3 and 4) clearly show that 1) ovulation of FW dominant follicles reduces fertility of dairy cows; 2) that this negative effect is mediated by reduced concentrations of progesterone during the development of the ovulatory follicle; and 3) that ovulation of FW that grow under low concentrations of progesterone is likely a protagonist of anovular cows' infertility. Nonetheless, further evaluation of the effects on endometrium and oocyte are needed to precisely determine the underlying mechanisms. It has been shown that cows induced to ovulate FW dominant

follicles had less P/AI (Chapter 3) and that a tendency for reduced P/AI was detected as early as day 17 of gestation (Chapter 4). One of our initial hypotheses was that the hormonal milieu that precedes ovulation of FW follicles would modulate endometrial gene expression during subsequent cycle. Particularly, that exposure to reduced circulating progesterone would reduce endometrial expression of progesterone receptors and enhance that of oxytocin receptors (Zollers et al., 1989, 1993). The first obvious consequence of that would be the hastening of luteolysis (Cerri et al., 2008a). This hypothesis was somewhat supported in Chapter 3, but could not be confirmed based on results presented in Chapter 4. Nonetheless, the action of progesterone on its receptors is crucial for endometrial secretion of the histotroph (Bazer et al., 2008). Therefore, it is possible that a slight modulation of endometrial receptors would have differential effects, impacting embryo development without changing luteal function and lifespan. A simplistic interpretation of the data presented here might lead to erroneous conclusions, and further evaluation of endometrial gene expression and protein abundance, as well as oocyte maturation-related events, ought to be performed before final inferences. Nevertheless, results suggest that changes in fertilization and/or pre-elongation processes, and not related with conceptus elongation, are triggering differences in fertility. No indications of impaired elongation in conceptus generated from FW follicles were found. Indeed, the average length and the proportion of conceptus classified as small in relation to the overall mean were similar for FW compared to SW and FWP4 (Chapter 4). Furthermore, the capability of conceptuses to produce IFN- τ was either similar or superior for FW compared to other treatments (Chapter 4). These results are supported by previous studies on which dairy cows were superstimulated during the FW

or SW of follicular development (Rivera et al., 2009). Embryo viability on d 6 after AI was markedly reduced following superstimulation of the FW in comparison to the SW. Likewise, the negative effects were mediated by reduced concentration of progesterone during growth of the ovulatory follicle, since no differences on embryo quality were found between cows that ovulated SW dominant follicles and cows that were supplemented with progesterone during the development of the FW. Furthermore, no differences in pregnancies per embryo transfer or in pregnancy loss between d 21 and 63 of gestation were reported for viable embryos derived from superstimulation of the FW, FW supplemented with progesterone, and SW (Rivera et al., 2009). It has been shown previously that reduction of circulating concentrations of progesterone during early diestrus induced maturation-related changes in the cumulus-oocyte complex in the existing follicles (Inskeep, 2004). These changes included degeneration of cumulus cell processes, irregularity of oocyte's nuclear membrane and hastening of stage II of meiosis. Similar alterations were described in persistent follicles (Revah and Buttler, 1996), and have been linked to decreased embryo quality and increased mortality before the 16-cell stage (Ahmad et al., 1995; Cerri et al., 2009).

The fine control of length of proestrus and exposure of the oocyte to estradiol is crucial for enhancement of fertility. At the same time that prolonged dominance (Ahmad et al., 1995; Revah and Buttler, 1996; Cerri et al., 2009) and exposure to reduced progesterone and increased estradiol (Inskeep, 2004) might compromise oocyte and embryo quality, inadequate follicular maturation was shown to reduce P/AI (Bridge et al., 2008). Results from Chapter 5 suggest that, in order to benefit from shorter periods of dominance and ovulation of younger follicles (Cerri et al., 2009; Santos et al., 2010),

additional time for follicular maturation during proestrus is needed. Our attempt in the first experiment presented in Chapter 5 was not to establish the optimal interval between induction of CL regression, induction of ovulation and AI during a 5-day protocol as it was done for the 7-day protocol (Pursley et al., 1998). Conversely, our hypothesis was that younger follicles would benefit from longer period of proestrus and allow for the administration of the final GnRH concurrently with timed AI. From the management standpoint, this modification would save the handling of cows in the afternoon before AI, which is undesirable by dairy farmers. No differences in P/AI were observed when induction of ovulation using GnRH was performed either 16 hours before or concurrent with timed AI at 72 hours after the first PGF_{2α} injection in the 5-day program. This is contrary to previous research with cows subjected to a standard 7-day Ovsynch protocol in which optimal P/AI was achieved when AI was performed 16 hours after GnRH-induction of ovulation (Pursley et al., 1998; Brusveen et al., 2008).

Administration of the final GnRH 16 hours before the AI is expected to result in a better synchrony between ovulation and semen availability (Walker et al., 1996; Dransfield et al., 1998; Dalton et al., 2001; Saacke, 2008). Nonetheless, in a 5-day program allowing additional 16 hours for the dominant follicle to finish its maturation overcame a possible asynchrony and resulted in similar fertility.

The stage of the estrous cycle on which the GnRH1 of resynchronization protocol is administered is expected to influence synchrony of the estrous cycle in response to hormonal treatments (Vasconcelos et al., 1999). Consequently, it modulated subsequent P/AI in some (Fricke et al., 2003; Sterry et al., 2006), but not all studies (Silva et al., 2009). In the second experiment presented in Chapter 5, cows received the

GnRH1 34 days after the previous AI. Considering that the average length of the estrous cycle in lactating dairy cows is 23 days (Sartori et al., 2004), it is expected that most of the cows would be on day 11 of the estrous cycle when the resynchronization protocol was initiated. This is important because approximately 50% of the cows treated with GnRH between days 10 and 16 of the estrous cycle failed to ovulate (Vasconcelos et al., 1999), which in association with spontaneous regression of the CL before the injection of PGF_{2α}, resulted in increased premature ovulation at the end of the synchronization program and reduced fertility (Vasconcelos et al., 1999). The benefit from supplemental progesterone on overall synchrony was minor. It tended to decrease premature ovulation; however, the occurrence of cows ovulating ahead of time *per se* was unimportant (5.6%). Strikingly, the greatest increment in fertility came from supplementation of cows that had a visible CL at the GnRH1. High producing dairy cows have reduced plasma concentrations of progesterone during follicular development (Sartori et al., 2004), which may increase LH pulsatility and reduce oocyte competence and embryo quality (Inskeep, 2004). Use of a single CIDR insert increased the concentration of progesterone in plasma by approximately 0.9 ng/mL (Lima et al., 2009); therefore, it is inadequate to increase progesterone in cows not bearing a CL to concentrations comparable to those of cows in diestrus (Lima et al., 2009). Nonetheless, it is possible that the additional progesterone from the CIDR insert complemented luteal synthesis of progesterone and allowed for adequate follicular maturation, which resulted in increased P/AI. Similar to our findings, supplementation with progesterone tended to improve P/AI only in cows that had an active CL at the initiation of the Ovsynch protocol (Bartolome et al., 2009).

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BIOGRAPHICAL SKETCH

Rafael Sisoneto Bisinotto was born in Campinas, state of São Paulo, Brazil. In 2001, he was an intern at the Agricultural Institute of Campinas and worked with biotechnologies applied to the potato cropping. In the spring of 2003, he began his studies in veterinary medicine in the School of Veterinary Medicine and Animal Sciences at the University of São Paulo, São Paulo, Brazil. From January of 2005 to December of 2006, he worked under the supervision of Dr. Mário Binelli in the Laboratory of Molecular Physiology and Endocrinology. During this time, he studied the effects of follicular estradiol on luteal lifespan and length of the estrous cycle in beef cows. In the fall of 2007, he came to the United States to work with nutrition and reproduction of dairy cows under the supervision of Dr. José Eduardo P. Santos at the Veterinary Medicine Teaching and Research Center, University of California Davis, Tulare, CA. He graduated in veterinary medicine on December 2007. He returned to the United States in the fall of 2008 to begin his Master of Science program in Animal Molecular and Cellular Biology under the supervision of Dr. José Eduardo P. Santos at the University of Florida with emphasis on dairy cattle reproduction.