INTEGRATION OF METABOLIC AND HORMONAL RESPONSES WITH CONCEPTUS/ENDOMETRIAL GENE EXPRESSION FOR THE OPTIMIZATION OF POSTPARTUM REPRODUCTIVE MANAGEMENT OF DAIRY COWS

By

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To my family and my husband Bryan for their endless support

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LIST OF ABBREVIATIONS

BCS	body condition score
BHBA	beta-hydroxy butyric acid
BUN	blood urea nitrogen
BW	body weight
CIDR	controlled internal drug releasing
CL	corpus luteum
CP	crude protein
CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1
DCAD	dietary cation-anion difference
DKK1	dickkopf homolog 1
DIM	days in milk
DM	dry matter
DMI	dry matter intake
DPBS	Dulbecco’s phosphate buffered saline
ELISA	enzyme-linked immunosorbent assay
GnRH	gonadotropin releasing hormone
HPGD	hydroxyprostaglandin dehydrogenase
INF-γ	interferon gamma
INF-*τ*	interferon tau
IGF	insulin-like growth factor
IgG	immunoglobulin G
LEM	late embryo mortality
LH	luteinizing hormone
FSH	follicle stimulating hormone

NEFA	non esterified fatty acid
MHC	major histocompatibility complex
NE_L	net energy of lactation
PAG	pregnancy associated glycoprotein
PG	prostaglandin
PGE_2	prostaglandin E_2
PGES	prostaglandin E synthase
PGES3	prostaglandin E synthase 3
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
$PGF_{2\alpha}R$	prostaglandin $F_{2\alpha}$ receptor
PGH2	prostaglandin H2 synthase (Cox-2)
PGRMC2	progesterone receptor membrane component 2
PIBF1	progesterone induced blocking factor 1
PP	postpartum
PSPB	pregnancy specific protein B
PSP60	pregnancy serum protein of M_r 60 kDa
PTGDS	prostaglandin D2 synthase
PTX3	pentraxin-related gene
TAI	timed artificial insemination
TKDP1	trophoblast Kunitz domain protein 1
TP1	trophoblast protein 1
TNF-α	tumor necrosis factor alpha
TMR	total mixed ratio
US	ultrasound

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INTEGRATION OF METABOLIC AND HORMONAL RESPONSES WITH CONCEPTUS/ENDOMETRIAL GENE EXPRESSION FOR THE OPTIMIZATION OF POSTPARTUM REPRODUCTIVE MANAGEMENT OF DAIRY COWS

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Experiments with dairy cows were conducted to further elucidate the mechanisms involved in embryo development and endometrium remodeling during early pregnancy, to improve dairy cattle fertility, and to evaluate alternative reproductive management tools for early pregnancy diagnosis.

In the first study, pregnant heifers were assigned randomly after calving to a lactating group (L) and a nonlactating group (NL). Lactating cows were fed a TMR (1.65Mcal NEL/kg, 16.5% CP) ad libitum. Nonlactating cows were fed a maintenance ration (1.45 Mcal NEL/kg, 12.2% CP) once per day. Rectal temperatures and blood, for analyses of progesterone and metabolites, were collected thrice weekly. Ovarian ultrasonography and BW measurements were performed weekly. Body condition score was measured every 14 d. All cows were pre-synchronized and enrolled in a TAI protocol (Presynch/5 d CIDRSynch), but only 10 in the L and 12 in the NL were TAI. On d 17 after GnRH/TAI, all cows were slaughtered and endometrial, conceptus, oviductal and ovarian tissues collected. The bovine Affymetrix GeneChip was used to assess conceptus and endometrial gene expression. Standard and partial correlation analyses

were used to evaluate associations of conceptus and endometrium expression of Pregnancy Associated Glycoproteins (PAGs) with other genes of interest, including genes expressed exclusively by trophoblast cells and genes expressed in the endometrium of both cyclic and pregnant cows. Lactation\diet altered metabolic status even though BW and BCS were the same between L and NL. Lactation delayed initiation of cyclicity and lowered concentrations of progesterone in pregnant L cows during a programmed period following an induced ovulation. Early expressions of PAG genes within the conceptus and endometrium of pregnant cows and its association with other genes infer a possible role of PAGs in pregnancy maintenance and implantation by regulation of prostaglandin metabolism.

In the second study, cows were enrolled in a presynchronization and Ovsynch protocol for first service and assigned to two resynchronization programs for second service. A treatment Resynch group received a controlled internal drug releasing (CIDR) insert containing progesterone from d 18 to 25, GnRH at d 25, ultrasound pregnancy diagnosis at d 32 whereby nonpregnant cows received an injection of $PGF_{2\alpha}$, followed by GnRH 56h after $PGF_{2\alpha.}$ and TAI 16 h later (d 35). Cows in the control group were diagnosed for pregnancy at d 32 and nonpregnant cows received GnRH followed by $PGF_{2\alpha}$ on d 39 and GnRH 56 h after $PGF_{2\alpha}$ and TAI 16 h later (d 42). Blood samples were collected for analyses of progesterone and PAGs. Resynch and Control protocols had comparable pregnancy per AI for first and second TAI services, but pregnancy occurred 3.3 d earlier in the Resynch group. Plasma progesterone was greater for pregnant cows in the Resynch group in which CIDR and/or GnRH increased

progesterone during pregnancy. Dynamics of PAGs were indicative of pregnancy status and pregnancy loss.

CHAPTER 1
INTRODUCTION

The transition period, characterized by a series of complex physiological changes in both endocrine and nutritional statuses to accommodate parturition and lactogenesis, is the most challenging period in the life of a dairy cow. The onset of lactation is associated with a prolonged period of negative energy balance (NEB) during which rate of DMI lags behind the rapid increase in milk production causing a subsequent mobilization of fat and protein from body reserves. The extended NEB experienced by dairy cows in early lactation has significant carry-over effects on reproductive performance. Low energy availability is associated with extended anovulatory periods and decreased fertility (Beam and Butler, 1997; Beam and Butler, 1999; Butler, 2003).

Despite progress in synchronizing estrus/ovulation and artificial insemination, the reproductive performance of dairy cows has not improved substantially (López-Gatius, 2003). Butler (2005) reported pregnancy per AI in large dairy herds of 35-40% for mature cows compared with 51% in first lactation cows or 65+% in virgin heifers. Although increases in herd size and milk yield per cow have been associated with a decline in fertility, reasons for decline in reproductive performance of lactating dairy cows are multifactorial (Lucy, 2001a). Negative energy balance, nutritional management, changes in BCS, herd health, poor expression and detection of estrus, low pregnancy per AI and increased embryo loss play a pivotal role in determining fertility of lactating cows.

Adequate reproductive management of dairy cattle is essential to optimize fertility in current intensive production systems. Development of synchronization systems to control and coordinate ovarian follicular dynamics, corpus luteum regression and

induction of ovulation has provided the ability to improve pregnancy per AI and embryo survival. Acceptable pregnancy per AI per AI has been achieved with the use of ovulation synchronization programs for TAI to maximize submission rates for insemination at the designated voluntary waiting period (Chebel et al, 2006). Moreover, accurate and early detection of nonpregnant cows combined with a program for resynchronization of ovulation will improve reproductive efficiency of dairy herds and reduce the interval between AI (Fricke. 2002). A reduction in the interval between TAI can be achieved by optimization of the stage for resynchronization through physiological approaches (Bartolome et al., 2005a; Bartolome et al 2005b; Bartolome et al., 2005c).

In cattle, the measurement of pregnancy associated glycoproteins (PAGs) as an indicator of non-pregnancy or, alternatively, early pregnancy in cattle can serve as a tool to help optimize reproductive management (Sasser et al., 1986; Green et al, 2005; Silva et al., 2007). In addition, monitoring the circulating concentration of PAGs can also assist in identification of embryo/fetal well being and pregnancy loss (Patel et al., 1995, Kornmatitsuk et al, 2002; Szenci et al, 2003).

In ruminants, the PAG gene family is comprised of at least 22 transcribed genes, temporally expressed throughout pregnancy and spatially distributed into two distinct groups: those PAG genes expressed predominantly in trophoblast binucleated cells (i.e., modern PAG genes) and those expressed throughout the trophectoderm (i.e., ancient PAG genes) (Green et al., 2000). The functional role (s) of these molecules is still unclear. PAG family genes may be involved in adhesion, implantation and remodeling of the fetal-maternal unit during placenta development (Wooding et al., 2005), immune regulation (Dosogne et al., 1999; Hoeben et al., 1999; Austin et al.,

1999) and prostaglandin synthesis and regulation (Del Vecchio et al., 1990, 1995 and 1996; Weems et al., 1998a, 1998b, 1999 and 2003).

Early embryo mortality accounts for major reproductive wastage in dairy farms. Up to 40% of total embryonic losses are estimated to occur between days 8 and 17 of pregnancy (Thatcher et al., 1994). A critical event on d 16 to 17 after estrus is the maintenance of the corpus luteum (**CL**). This process is established by the ability of the conceptus to secrete sufficient amounts of IFN-*τ*, which regulates secretion of $PGF_{2\alpha}$ in the uterine endometrium (Thatcher et al., 2001). Adequate communication between conceptus and endometrium is essential for pregnancy maintenance. Understanding early pregnancy development of the fetal-maternal unit is important in the development of strategies to reduce early pregnancy loss and improve reproductive efficiency of dairy cattle. Development of microarray analyses of the bovine transcriptome has provided an opportunity to identify genes involved in regulation of early pregnancy. Moreover, the microarray technique allows the study of molecular mechanisms that regulate early embryo development and endometrium remodeling.

This thesis literature review (Chapter 2) will summarize: 1) the transition period and the factors involved in reestablishment of postpartum fertility, 2) reproductive physiology of dairy cows, 3) strategies to synchronize and improve fertility of dairy cattle and 4) the current understanding of PAG genes expression and their possible functions throughout pregnancy. Chapter 3 consists of an experiment that describes the effect of lactation on postpartum metabolic responses and evaluates the effect of lactation on early pregnancy conceptus and endometrial expression of genes. Moreover, Chapter 3 describes potential associations of conceptus and endometrial PAG gene expression

with both trophoblast and endometrial specific genes expressed by both cyclic and pregnant dairy cows. Chapter 4 describes the use of a resynchronization protocol to improve dairy cattle fertility and the measurement of plasma PAG concentrations as an alternative reproductive management tool for early pregnancy diagnosis and embryo/fetal loss.

CHAPTER 2
LITERATURE REVIEW

The Transition Period

The transition period, defined as 3 weeks prepartum until 3 weeks postpartum (Grummer, 1995), is a critical time in the life cycle of a dairy cow. It is characterized by a series of complex physiological changes in both endocrine and nutritional statuses to accommodate parturition and lactogenesis. The dry period consists of about 60 days and has typically been seen as a period of recovery after the last lactation. Feeding dairy cows different diets during the dry period has resulted in the evolution of two-group nutritional management strategies for dry cows (Contreras et al., 2004). During the far-off period (generally the first 4 to 6 weeks), cows are offered modest amounts of feed followed by an increased specialization of the ration in terms of energy and protein during the close-up period (i.e., generally the last 3 wk before expected parturition) (Beever, 2006). Besides changes in energy and protein demands around the time of calving, transition cows also experience dynamic changes in mineral mobilization and utilization. A decrease in the dietary cation-anion difference (DCAD; milliequivalents [(Na + K) − (Cl + S)]/100 g of DM) during the last 3 to 4 weeks before calving may be beneficial to systemic acid-base status, Ca metabolism, peripartum health, and postpartum productive and reproductive performance. Moore et al. (2000) reported that adjustment of the rate of anionic salt supplementation in dry cow diets may support proper functioning of both the Ca and energy regulatory systems and enhance overall health during the peripartum period.

During late gestation the increase in nutritional demand to support fetal growth and initiation of lactation also is associated with a gradual decline in DMI. Feed intake is

conditioned by several factors including changes in body condition score, environmental conditions, diet composition, feeding management and metabolic disorders. After parturition, the onset of lactation is associated with a prolonged period of negative energy balance (NEB) during which rate of DMI lags behind the rapid increase in milk production causing a subsequent mobilization of fat and protein from body reserves. The severity and duration of NEB varies among cows and is related primarily to differences in DMI and its rate of increase during early lactation.

Metabolites and metabolic hormones also are modified with a reduction in circulating insulin-like growth factor I (IGF-I) and rise in growth hormone (GH) concentrations, associated with down-regulation in liver growth hormone receptor (GHR) (Lucy, 2001). Mobilization of body fat results in elevated plasma concentrations of nonesterified fatty acids (NEFA), β-hydroxy butyrate (BHBA) and triacylglycerols (TAGs). Nonesterified fatty acids (NEFA) released from lipid stores are taken up by the liver, where they may be oxidized to provide energy or partially oxidized to produce ketone bodies, which are transported for use elsewhere in the body. NEFAs may also be esterified to triacylglycerols (TAGs) which accumulate in the liver and compromise liver function (i.e., fatty liver). Beta-hydroxybutyrate (BHBA), the predominant form of ketone bodies in blood is an index of fatty acid oxidation. Impaired liver function also reduces the metabolic clearance of urea (Wathes et al., 2007). In addition, plasma concentrations of glucose and insulin are decreased in early lactation. (Grummer, 1995).

The extended period of NEB experienced by dairy cows in early lactation has significant carry-over effects on reproductive efficiency. Peripartum ovulation is

dependent on the re-establishment of pulsatile LH secretion and estradiol production by the dominant follicle. Low energy availability suppresses LH secretion and reduces ovarian responsiveness to LH stimulation, increasing the interval to the first ovulation (Butler, 2003). Reduction in concentrations of metabolic hormones like insulin and IGF-I also influence follicular development and peripartum ovulation. Cows that experience an early postpartum rise in IGF-1 concentrations in plasma have enhanced ovarian follicular development coupled with increased estradiol secretion (Beam and Butler, 1997, 1999).

Most infectious diseases and metabolic disorders such as milk fever, ketosis, mastitis, retained fetal membranes, metritis and displaced abomasums occur during the periparturient period. The prevalence of these diseases and disorders has negative impacts on milk yield, energy balance, and causes extensive loss of body condition during the periparturient period that affects subsequent reproductive performance (Gröhn and Rajala-Schultz, 2000; Chebel et al., 2004; LeBlanc, 2008; Garbarino et al., 2004; Schrick et al., 2001 and Opsomer et al., 2000). The success of the transition period determines the profitability of the cow during that lactation (Drackley, 1999).

Fertility

High Milk Production

The increased genetic capability of cows for milk production, coupled with improvements in nutrition and management, has made it possible to produce >10,000 kg of milk per cow in a 305 day lactation (Shrestha et al., 2005). In the past few decades, the shift toward more productive cows and larger herds has been associated with a decline in fertility. Despite progress in synchronizing estrus and artificial insemination, the reproductive performance of dairy cows has not improved

substantially (López-Gatius, 2003). On the contrary, Butler (1998) documented a decline in first-service pregnancy per AI from approximately 65% in 1951 to 40% in 1996. It also has been shown that fertility declines with each calving until the lowest level at maturity. Pregnancy per AIs in large commercial dairy herds are at only 35-40% for mature cows compared with 51% in first lactation cows or 65+% in virgin heifers (Butler, 2005). Intensive genetic selection for milk production without attention to reproductive performance has contributed to an inverse relationship between milk production and pregnancy to first artificial insemination (AI). Inclusions of productive life, daughter pregnancy per AI, and more recently the availability of sire pregnancy per AI, as a measurement of phenotypic service-sire fertility, appear to have reduced the rate of decline in fertility in the USA (Norman et al., 2009). Sartori et al. (2002) developed two different experiments to compare fertilization and early embryo development of nonlactating and lactating dairy cows. The experiments compared lactating dairy cows with nulliparous heifers during the summer and lactating with dry cows during cool weather. All animals were bred and embryos collected at d 6 (d 0 = day before ovulation). Results of both experiments showed poorer embryonic development for lactating dairy cows. In the first experiment, a higher percentage of degenerated embryos were observed for cows (62%) compared with heifers (6%). Moreover, a reduction in fertilization rate was observed, where 100% of the structures recovered from heifers on d 6 were fertilized, whereas only 55.3% of structures recovered from lactating cows were considered fertilized. In the second experiment, dry cows had a greater percentage of viable embryos recovered compared with lactating cows. Furthermore, more degenerated embryos were observed in lactating than dry cows

(42% vs 18%). De la Sota et al. (1993) reported increased progesterone and estradiol and fewer large follicles in nonlactating cows compared with lactating cows. Sartori et al. (2002a) compared the size of follicles and CL and circulating progesterone and estradiol concentrations between lactating cows and heifers and between lactating and dry cows of the two experiments previously described (Sartori et al., 2002). After embryo collection (d 6), all animals received a $PGF_{2\alpha}$ injection (d 7 and 8). Blood samples were collected from the day of $PGF_{2\alpha}$ until the next ovulation. In the first experiment, greater peak estradiol concentrations were observed in heifers compared with lactating cows (12.1 ± 0.5 vs 8.6 ± 0.6 pg/mL), however, no differences in size of the ovulatory follicles was detected. Moreover, on d 7, lactating cows had greater luteal tissue volume but heifers had greater serum progesterone concentrations . In addition, results of the second experiment showed greater luteal tissue volume for lactating cows, even though, serum progesterone concentrations were similar between the two groups. Furthermore, even though peak serum estradiol was similar between lactating and dry cows, lactating cows had larger ovulatory follicles (18.6 ± 0.4 vs 16.2 ± 0.4 mm). Lactating cows of both experiments had an increased time for follicular growth demonstrated by the greater time from $PGF_{2\alpha}$ injection to ovulation. Average days from $PGF_{2\alpha}$ to ovulation for the first experiment was 4.6 ± 0.1 for lactating cows and 3.8 ± 0.1 for heifers respectively. Lactating cows of the second experiment had an average of 4.8 ± 0.1 compared with 4.2 ± 0.1 for dry cows . Another study described the differences between lactating cows and heifers in ovarian dynamics and reproductive hormones during the estrous cycle (Sartori et al., 2004). Results showed that on average, after luteolysis, lactating cows ovulated later than heifers (5.2 ± 0.2 vs 4.6 ± 0.1d). In

addition, maximal serum estradiol concentration preceding ovulation was lower in cows than in heifers (7.9 ± 0.8 vs 11.3 ± 0.6 pg/mL), despite the occurrence of larger follicles in cows (16.8 ± 0.5 vs 14.9 ± 0.2 mm). Maximal volume of luteal tissue was larger for cows than heifers, even though maximal serum progesterone concentration was lower for cows compared with heifers (5.6 ± 0.5 vs 7.3 ± 0.4 ng/mL). Discrepancies between size of ovarian structures and serum steroid concentrations between lactating cows and heifers and lactating cows and dry cows may be due to greater steroid metabolism observed in lactating dairy cows, which have potential physiological consequences that may compromise reproduction performance of lactating dairy cows. Sangsritavong et al. (2002) showed that the continuous high plane of nutrition of lactating cows appears to chronically increase liver blood flow and metabolic clearance rate of progesterone and estradiol, which may affect reproduction outcome due to lower circulating steroid concentrations.

The modern high producing dairy cow partitions a greater proportion of available nutrients towards milk production. Nutritional strategies that provide regulatory signals to stimulate reproductive processes without compromising the partitioning of energy into lactation are needed to improve reproductive function while maintaining high productivity (Chagas et al., 2007). These strategies vary with cow genotype, production and management systems. Strategies include management of body reserves during the transition period and inclusion of specific dietary nutrients such as starch, protein, minerals and lipids, which may directly or indirectly alter reproductive efficiency.

During the past few decades, milk production has increased and reproductive efficiency has declined. However, reasons for the decline in fertility are multifactorial

and not associated entirely with an increase in milk production (Lucy, 2001a). Negative energy balance, nutritional management, changes in BCS and herd health play a pivotal role in determining fertility of postpartum cows.

Body Condition Score

The period of NEB begins about one week before calving and worsens over the next 2-3 weeks, reaching its nadir about 2 weeks postpartum (Butler and Smith, 1989; Bell, 1995). NEB results in mobilization of body fat and protein and becomes visible during early lactation as a loss in body condition. Body condition score (BCS) is a major cow factor related to decreased dietary intake during the transition period. Kim and Suh (2003) investigated the effects of BCS loss from the dry period to near parturition to subsequent occurrence of postpartum metabolic and reproductive disorders. Cows were categorized based on BCS loss into moderate or marked loss groups. The authors concluded that cows with severe BCS loss from 35±16 days prepartum to day 5±4 postpartum had a higher incidence of postpartum metabolic and reproductive disorders and a longer interval to first breeding, which will likely lead to subsequent decrease in fertility. Shrestha et al (2005) reported that poor nutritional status of cows after calving, as evident from low BCS, was an important cause for delayed first ovulation. Furthermore, cows with more severe NEB lost more body condition (≥1 unit) during the first 30 days of lactation and experienced longer intervals to first ovulation. The variation in degree of NEB among individual cows is explained by differences in energy intake rather than milk yield (Staples and Thatcher, 1990; Zurek et al., 1995). A number of studies have showed the carryover effects of early lactation NEB and BCS loss on fertility. Staples and Thatcher (1990) examined the relationship of energy balance and ovarian activity of early postpartum dairy cows. Cows were classified according to their

time of return to normal ovarian activity as early responders (ER) (i.e., cows that showed CL activity within 40 d after parturition), late responders (LR) (i.e., cows showing CL activity between 40 and 60 d postpartum) and anestrus or non responders (NR) (i.e., cows with no CL activity during the first 63 d postpartum). Results showed that ER and LR cows increased their postpartum feed intake more rapidly than NR cows. Moreover, ER and LR groups consumed more feed than the NR group. In addition, cows in the ER and LR groups produced more milk than cows in the NR group, showing that daily milk yields were positively correlated with day of first ovulation. Furthermore, NR cows lost more body weight during the first two weeks of lactation, mobilized the largest amount of body reserves and were in the greatest energy deficit. It was concluded that the severe NEB in early lactation influenced ovarian activity later in lactation.

López-Gatius et al. (2003a) reanalyzed the results of 11 large studies and reported that low BCS (< 2.5) at first AI extended the days open by 12 days. Comparison of differences in BCS loss during early lactation on average days open showed that 0.5 to 1 unit of BCS loss = +3.5 days open and >1 unit of BCS loss = +11 days open. Roche et al (2007) documented that the effect of BCS changes on reproduction in early lactation is further supported by the effect of a BW change. Animals calving at a higher BW lost more BW during the post partum period at a greater rate and for a longer period of time. The same was true for BCS. Moreover, BCS and BW measurements influenced cyclicity, frequency of cows mated in the first 21 days from the planned start of mating (PSM), pregnancy to first service, and pregnancy per AI for the first 21, 42 and 84 days of the breeding season.

Interval to First Ovulation

The first ovulation postpartum reflects the recovery from the hormonal conditions of late pregnancy and resumption and completion of preovulatory ovarian follicular development. The drop in plasma concentrations of estradiol following delivery of calf and fetal membranes terminates the inhibition of FSH secretion, and increases in plasma FSH begin to stimulate follicle development as early as 7 days after parturition (Thatcher et al., 2006). Low concentrations of follicle growth factors such as luteinizing hormone, insulin and IGF-I during early lactation altered the interval to first ovulation (Butler, 2003).

Peripartum follicular development may follow three different patterns, characterized by either ovulation of an estrogen-active dominant follicle during the first follicular wave, the development of a first wave non ovulatory dominant follicle with low estradiol output followed by one or more waves of dominant follicle development, or the development of an estrogen-active, non ovulatory dominant follicle that becomes cystic (Beam and Butler, 1997). The fate of the first-wave dominant follicle has a significant impact on the postpartum anovulatory interval and influences the reproductive performance of lactating cows.

Resumption of postpartum ovarian activity and adequate reproductive performance is associated with postpartum uterine involution, which involves endometrial tissue repair, myometrial contraction, and bacterial clearance. The impact of early postpartum spontaneous estrus on uterine involution and subsequent fertility is debatable. Thatcher and Wilcox (1973) documented more favorable reproductive performance for cows that experienced earlier and frequent occurrences of postpartum estrus (Thatcher and Wilcox, 1973). In contrast, other studies showed that resumption

of post partum ovulation before complete uterine involution, characterized by an early rise of progesterone, affects subsequent fertility by increasing the risk of having prolonged luteal phase and uterine infections (Opsomer et al., 1998; Opsomer et al., 2000). Another important link between NEB and fertility is the carryover effects on concentrations of progesterone in the blood. The rate of the increase in progesterone is reduced or moderated by early lactation NEB. Severe NEB during the first 9 days postpartum reduced luteal function during second and third postpartum luteal phases (Villa-Godoy et al., 1988). Reduced or sub-optimal concentrations of progesterone might influence fertility through alteration of cyclicity, uterine function and early embryo development.

Metabolic and Other Health Disorders

Gröhn and Rajala-Schultz (2000) and Chebel et al (2004) associated reproductive success with periparturient diseases. Excessive lipid mobilization and suppression of the immune system coupled with other factors linked with peripartum NEB result in greater incidences of health disorders. Several studies reported reductions in fertility for cows affected by disorders of the reproductive tract (LeBlanc, 2008). Lower pregnancy per AI was experienced by cows with retained placenta (14%), metritis (15%) and ovarian cysts (21%) (Gröhn and Rajala-Schultz, 2000). Lameness (Garbarino et al., 2004), mastitis (Schrick et al., 2001) and metabolic disorders (Opsomer et al., 2000) were also linked to a decrease in fertility. Moreover, Opsomer et al. (1998) evaluated postpartum resumption of cyclicity and ovarian dysfunction by measuring milk fat progesterone profiles starting 10 days after calving until first insemination. Abnormal progesterone profiles and ovarian examinations showed that postpartum delayed cyclicity and prolonged luteal phase were the two most common ovarian disturbances

and were associated with inactive ovaries and abnormal uterine environment. Therefore, occurrence of postpartum ovarian dysfunctions is associated with longer interval from calving to pregnancy and decline in fertility.

Embryo Loss

A major reason for reduced fertility and farm profitability in lactating dairy cows is increased embryonic mortality. It is not yet known whether this increased loss is due to ovulation of an incompetent oocyte or due to reduced embryonic development as a result of oviduct-uterine environmental abnormalities (Roche, 2006). Santos et al (2004) documented that lactating dairy cows are vulnerable to reproductive failure in part due to low fertilization rates (~ 76%) and embryo viability in the first few days of gestation, but also extensive embryonic and fetal death (~ 60%). Moreover, Humblot (2001) reported significant embryonic loss of 20.5- 43.6%, with luteolysis occurring before day 24 (early embryo mortality) and 8.0–17.5% with return to estrus after 24 days (late embryo mortality). Another study documented that high rates of embryonic loss were a result of inadequate concentrations of progesterone, which altered LH and estradiol secretion and negatively affected oocyte quality, embryo development and uterine environment (Inskeep, 2004).

The Estrous Cycle

The average length of the bovine estrous cycle is 21 days, ranging from 17 to 26 days (Olds and Smith, 1951). The estrous cycle is regulated by ovarian steroid hormones via feedback mechanisms associated with neuroendocrine regulation of the hypothalamus and pituitary. The balance of interactions between the hypothalamic-pituitary-gonadal hormones regulates gonadotropin secretion and promotes development of ovarian follicles and regression of the corpus luteum (CL). The

hypothalamus secretes gonadotropin releasing hormone (GnRH) in response to circulating estradiol or inhibits GnRH secretion in response to progesterone. Release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary occurs in response to GnRH.

The estrous cycle consists of two major phases, follicular and luteal. The follicular phase, subdivided into proestrous and estrus, is characterized by elevated concentrations of estradiol, linear increases in mean concentrations of LH, and an acute preovulatory surge of LH occurring at the onset of estrus (Chenault et al., 1976). The follicular phase encompasses the period from regression of the corpus luteum (CL) to ovulation. The luteal phase, subdivided into metestrus and diestrus, is defined as the period from ovulation to CL regression. In general, it is characterized by a shift from estrogen to progesterone dominance following ovulation, and structural and functional maturation of the corpus luteum. During this phase, progesterone, secreted by the newly formed CL, conditions the uterus for a possible pregnancy.

Estrus demarcates the beginning of the estrous cycle and is distinguished by the LH surge and receptivity to mounting (estrous behavior). The surge release of LH is a result of high concentrations of estradiol being secreted from the preovulatory follicle (Walters and Schanllenberger, 1984). An increase in estradiol concentrations beginning about 3 days before estrus with an average of 3.6 pg/ml during the luteal phase of the cycle (days 2 to 11), reaches a peak of 9.7 pg/ml at 0.5 days before estrus, and remains high on the day of estrus (8.4 pg/ml) (Wettemann et al., 1972). The average duration of bovine estrus is 11.8 ±4.4 h and ovulation occurs 24.7 ± 5h following the onset of estrus (Roelofs et al, .2005).

Metestrus follows estrus and ovulation and is characterized by formation of a fully functional CL and secretion of progesterone. This phase of the estrous cycle consists of cellular and structural remodeling (luteinization) of the ovulated follicle to form the CL and a rise in FSH associated with emergence of a new follicular wave (Adams et al., 1992). Walters et al. (1984) reported that progesterone concentrations are low during early luteal phase (day 4) and increase during the mid-luteal phase (day 11±1). During the bovine estrous cycle, secretion of progesterone from the CL appears to occur in a pulsatile fashion during the early to mid-luteal phases (Schallenberger et al., 1984; Walters and Schallenberger, 1984), but the frequency of progesterone pulses is greater during the early luteal phase and declines during the mid-luteal phase. Moreover, pulsatile secretion of LH has been documented during the estrous cycle (Rahe et al., 1980; Schallenberger et al., 1984; Walters and Schanllenberger, 1984; Walters et al. 1984). During early luteal phase, LH pulses are characterized by low amplitude and high frequency, while at mid-luteal phase LH pulses are distinguished by high amplitude and low frequency (Rahe et al., 1980). Walters et al. (1984) reported a two fold increase in frequency of LH pulses during the early luteal period. Furthermore, pulsatile release of LH stimulates secretion of progesterone (Foster et al., 1980). Suppression of LH release during luteal development impairs normal function of the CL, indicating that pulsatile secretion of LH is necessary for adequate CL development (Peters et al., 1994).

The diestrus stage is distinguished by increased concentrations of progesterone. It has been reported that an average plasma concentration during diestrus is of 5.4 ± 0.16 ng/ml (Shemesh et al., 1971). In a more recent study, Mann (2008) documented that

non lactating dairy cows had an average plasma progesterone concentrations of 2.01±0.21, 5.82 ± 0.39 and 7.36 ± 0.30 ng/ml at days 5, 8 and 16 of the estrous cycle respectively. Moreover, Sartori et al. (2004) reported that lactating dairy cows have lower serum progesterone concentrations from d 1 to d 14 of the estrous cycle compared with heifers (5.6 ± 0.5 vs 7.3 ± 0.4 ng/mL) . The duration of the diestrus is related directly to the functionality of the CL (i.e. production of progesterone). High concentrations of progesterone prepare the uterine environment for early embryo development and subsequent conceptus attachment. Relative high concentrations of progesterone are maintained throughout the luteal phase, but if pregnancy does not occur, the endometrium releases $PGF_{2\alpha}$ causing luteolysis. The process of CL regression occurs between days 16 and 19 of the estrous cycle and is characterized by functional luteolysis which consists of a decline in progesterone secretory capacity of the CL and structural regression, which is manifested as tissue degradation.

Proestrus begins at the time of CL regression and ends with the LH surge and onset of estrus. Luteolysis removes the progesterone negative feedback towards GnRH release and promotes LH secretion. The consequent increase in LH pulse frequency promotes the development of the preovulatory follicle, rise in estrogen levels and initiation of a new estrous cycle (Schallenberger et al., 1984).

Follicular Development

Initiation of Follicular Growth

In ruminants, follicular growth is a dynamic process that begins during fetal development (Fortune, 1994; Fortune et al., 2000). Immature follicles, named primordial follicles consist of a layer of flat pre-granulosa cells and an oocyte, and exist as non-growing pools in the ovaries. These immature follicles are the source from which

follicles will gradually be recruited to grow throughout life. In vitro activation of primordial follicles has been achieved in different species. Eppig and O'Brien (1996) documented the development of primordial follicles to the primary and secondary stages in serum cultures of whole newborn rodent ovaries without gonadotropins. Moreover, Wandji et al. (1996) developed a serum-free culture system that supported the activation and development of primordial follicles from ovarian cortical explants of cattle. Another in vitro study showed that during early follicular growth in the bovine, primordial follicles become primary follicles by changing the morphology of their granulosa cells from flattened to cuboidal and by increasing the size of the oocyte (Braw-Tal and Yossefi, 1997). In this study, it also was shown that primordial follicles entered the growth phase in the absence of gonadotropins. In addition, Fortune et al. (1998) cultured pieces of ovarian cortex obtained from fetal bovine and baboon ovaries and observed that as early as 2 days of culture, the number of primordial follicles had decreased, whereas the number of primary follicles had increased. The addition of FSH to the culture had no effect on follicle development. These results confirm that primordial follicles from cattle and baboons can be activated to grow in a serum-free medium and in the absence of gonadotropins.

As follicles grow, the onset of expression of many genes occurs in a stage and cell-specific manner. McNatty et al. (2007) reviewed that a number of factors have been linked with early follicular activation and growth. Oocytes in primordial follicles express mRNAs and proteins for c-kit, estradiol receptor β and transforming growth factor β. Different growth factors such as growth differentiation factor 9 (GDF9), bone morphogenic protein 15 (BMP15) and BMP6 are also expressed by the oocyte of

developing follicles and affect early follicular growth. Granulosa cells of preantral follicles synthesize inhibin and follistatin, develop receptors to FSH and become responsive to steroids via androgen and estradiol receptors. The theca cells develop receptivity to LH, progestins, androgens, estradiol, activin, TGFβ and BMPs.

The mechanisms which initiate the growth of a primordial follicle to the primary stage of development are uncertain, but it may involve multiple factors both stimulatory and inhibitory. However, it is clear that primordial follicle growth is independent of gonadotropins, as there are no FSH receptors present on the granulosa cells until formation of primary follicles and LH receptors on theca cells are only evident when this cell-type differentiates in secondary follicles. Understanding the signals that initiate follicular activation and the factors necessary to sustain early growth stages, could be an important tool for manipulation of infertility, production of contraceptives and delay of menopause.

Follicular Dynamics

The use of ultrasound imaging to characterize ovarian follicular growth, first described by Pierson and Ginther (1984), was a major advancement in understanding follicular development in cattle. Several studies documented that follicular development progresses through stages of recruitment, selection and dominance, and is a recurring process in which one or two dominant anovulatory follicles develop before the ovulatory follicle (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989; Ginther et al., 1989a).

The majority of bovine estrous cycles are composed of either two or three follicular waves and emergence of the first wave occurs on the day of ovulation (day 0). The first follicular wave begins with recruitment of a cohort of follicles and is preceded by a

periovulatory FSH surge (Adams et al., 1992). At the end of the period of dominance (i.e., ovulation), circulating FSH concentrations begin to rise, increase over the next 48 hours, and peak approximately 12 to 24 hours before emergence of a new wave (i.e., recruitment of follicles). If a dominant follicle is removed (i.e., follicle ablation), FSH surge begins within 12 hours, which results in emergence of a new follicular wave within the next 24 hours (Adams et al., 2008). The cohort of recruited follicles increase in diameter and the variability in responses to FSH stimulation among follicles, likely determines which follicle continues to grow (selection) from the recruitment pool. Ginther et al., (1996) defined follicular deviation as the beginning of the greatest difference in growth rates (diameter changes between successive ultrasound examinations) between the largest follicle (i.e. dominant follicle) and the second largest follicle at or before the examination when the second largest follicle reached a maximum diameter. During the common growth phase, no follicle exerts dominance over its cohorts, even though the largest follicle becomes the dominant follicle in about 60% of cattle. At the time of deviation, the future dominant follicle averaged 8.5 mm in size, while the largest subordinate follicle had an average size of 7.2 mm (Ginther et al., 1996). The selected or dominant follicle becomes dependent on LH and produces high amounts of estradiol, which exerts an inhibitory action on FSH secretion and prevents recruitment of additional follicles. In addition, the selected follicle produces other factors such as IGFs, inhibins and follistatin to regulate FSH release and availability (Adams et al., 2008).

Continued growth of the first dominant follicle of the estrous cycle does not usually occur, due to the presence of a functional CL and high progesterone concentrations,

which exert a negative feedback to the pituitary, preventing ovulation and leading to atresia of the dominant follicle due to inadequate LH support. Initiation of a second follicular wave results in growth of a second dominant follicle on the ovary. Development and maturation of a second dominant follicle of the estrous cycle is associated normally with regression of the CL and subsequent ovulation. In contrast, the second wave may undergo atresia, and in this case, a third follicular wave is initiated. The third dominant follicle develops and becomes functional and capable to ovulate after luteolysis.

The end of selection and initiation of dominance occur during a period of declining FSH concentrations (Adams et al., 1992). Mihm et al. (1997) documented that during the decline in FSH serum concentrations, the increased growth and enhanced estradiol secretion of the dominant follicle were associated with increased intrafollicular concentrations of IGF-I and reduced concentrations of IGFBP3, IGFBP2 and total IGFBP. In addition, at the end of dominant follicle selection, concentration of total dimeric inhibins in intrafollicular fluid was reduced in the dominant follicle compared to pre-selection follicles. In contrast, no changes in dominant follicle intrafollicular fluid concentrations of progesterone, activin A, total α-inhibin immunoblot activity and 30 to 32 and 25kDa IGFBP were observed compared with pre-selection follicles. Amounts of the 35, 30 to 32 and 25kDa IGBPs were higher in subordinated follicles compared with the dominant follicle. These results indicated that the decline in FSH serum concentrations causes the diverse alterations in FSH dependent growth factors and hormones within the cohort of preselected follicles, leading to the selection of a dominant follicle. The increased secretions of estradiol and ovarian inhibin reach the pituitary and lead to a decreased FSH secretion. This FSH decline differentially induces

both continued growth and enhanced estradiol producing capacity of the dominant follicle and atresia of subordinate follicles.

Several studies support the idea that expression of LH receptors may be involved in the acquisition of deviation and ovulatory capacities. Using *in situ* hybridization, Xu et al (1995) showed an increase in LH receptor mRNA on granulosa cells from non detectable levels on day 2 (size averaged 6.7mm) to highly expressed levels on day 4 (size averaged 10.8mm). During the time of follicular selection, it was also observed an increase in LH receptor mRNA in thecal cells. Another study compared diameter deviation with granulosa cell LH receptor mRNA changes. The increased difference in LH receptor mRNA expression between the two largest follicles occurred before any increased difference in either follicle diameter or intrafollicular estradiol concentrations (Beg et al., 2001). Sartori et al. (2001) evaluated the temporal relationship between follicular diameter deviation with LH responsiveness of follicles in vivo, as assessed by follicle ovulatory capacity. Cows were challenged with purified LH at predetermined times after GnRH injections or at known follicular diameter, and varying doses of LH were used. Results showed that ovulatory capacity generally followed diameter deviation in response to a high dose of LH but not a low dose, showing that maximal growth of the dominant follicle required adequate concentrations of LH at about the time that the follicle developed ovulatory capacity. It also was shown that an increase in LH receptors and consequent LH responsiveness is an important component of continued growth of the dominant follicle and may be involved in selection.

Together, follicular recruitment and emergence of a follicular wave is regulated primarily by circulating FSH. The FSH concentrations are gradually inhibited and reach

a nadir at the time of deviation and selection of a dominant follicle. Intrafollicular factors and hormones, combined with the decline in FSH concentrations and an increase in granulosa cells LH receptors induce continue growth and development of the dominant follicle and enhance its estradiol producing capacity until it is sufficient to cause a LH surge and ovulation.

Corpus Luteum Development, Function and Luteolysis

Corpus Luteum Development and Function

The CL is a transient endocrine gland and its formation is initiated by a series of morphological and biochemical changes in both granulosa and theca cells of the preovulatory follicle (luteinization). Granulosa and theca cells differentiate into steroidogenic large and small luteal cells, respectively; however, the CL also consists of non-steroidogenic cells such as vascular endothelial cells, fibroblast and immune cells (Lei et al., 1991).

The main function of the CL is to synthesize and secrete progesterone, and adequate luteal function is crucial for regulation of the estrous cycle and maintenance of pregnancy in many species. Synthesis and action of progesterone is regulated by both luteotrophic and luteolytic factors.

In cows, development and function of the CL is supported primarily by luteinizing and growth factors. However, angiogenic factors, prostaglandins, noradrenaline and oxytocin should be considered as potent regulators of CL development as well as important factors regulating progesterone secretion (Skarzynski et al., 2001; Berisha and Schams, 2005). Moreover, progesterone also has autocrine and paracrine effects on function of the bovine CL in the early and mid stages of development. Skarzynski and Okuda (1999) reported that progesterone stimulated secretion of progesterone,

oxytocin and prostaglandins by the early CL. In contrast progesterone inhibited CL $PGF_{2\alpha}$ secretion at mid cycle. Therefore, progesterone affects bovine CL secretory function in a stage dependent fashion. In this same study, it also was reported that oxytocin and $PGF_{2\alpha}$ are potent stimulators of progesterone secretion in the early and mid luteal phases, suggesting that luteal oxytocin and $PGF_{2\alpha}$ are involved in mechanisms of CL development as autocrine/paracrine factors. In another study, Okuda et al. (2004) demonstrated that intra-luteal progesterone is an important factor supporting maintenance of the CL. Results of this study showed that blockage of the autocrine and/or paracrine action of progesterone by a specific antagonist reduced the viability of luteal cells by amplifying Fas L mediated apoptosis via an increase of *Fas* and *caspase*-3 expression and caspase-3 activity by bovine luteal cells. Kowalik and Kotwica (2008) investigated the progesterone receptor membrane component 1 (PGRMC1) mRNA expression in bovine CL measured on different days of the estrous cycle. The highest PGRMC1 mRNA expression was observed on days 6 to 10 of the estrous cycle and then declined. The lowest PGRMC1 expression was detected on days 1 to 5. Moreover, progesterone luteal content was the highest on days 6 to 10 and 11 to 16 and was positively correlated with the PGRMC1 mRNA expression ($r = 0.52$). Adrenergic factors are also known to be involved in development and secretory function of the bovine CL. In a review, Skarzynski et al. (2001) documented that noradrenaline strongly stimulated the synthesis and release of $PGF_{2\alpha}$ and PGE_2 in bovine luteal cells. Noradrenaline appears to play roles in development and maintenance of bovine CL through its action on progesterone secretion and stimulation of prostaglandins, especially in the early CL phase.

Angiogenesis and establishment of a functional blood supply are also important for CL growth and development. A series of promoters of angiogenesis, such as vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF) and insulin-like growth factors (IGF) have been identified as important factors in CL formation. Vascular endothelial growth factors (VEGF) have been found to be expressed in CL during development, function and regression. Berisha et al. (2000) documented high mRNA expression of VEGF and VEGFR-2 in CL tissue during early luteal phase followed by a decrease in mid and late luteal phases and a further decrease after CL regression. The mRNA expression of FGF-1 in bovine CL is increased during the mid luteal phase; in contrast, FGF-2 expression is highest during the early luteal phase (Schams and Berisha, 2002). The bovine CL had high expression of IGF-1, IGF-2 and IGFR-1 in the early luteal phase (days 1-4), followed by a decrease to a lower plateau in the cyclic CL (Schams et al., 2002). The IGF system may have indirect effects on angiogenesis in the early CL due to its proliferation and differentiation regulation of endothelial cells and also by affecting proliferation and differentiation of vascular tissues (Schams and Berisha, 2002).

Luteolysis

Luteolysis or regression of the CL is initiated when it loses capacity to synthesize and secrete progesterone, and this stage is designated as functional luteolysis. This is followed with a loss of the cellular structure of the CL, commonly known as structural luteolysis. In ruminants, luteal regression at the end of the estrous cycle is caused by episodic release of $PGF_{2\alpha}$ from the uterus, which reaches the CL through a counter current system between uterine vein and ovarian artery. In response to $PGF_{2\alpha}$, there is an initial intraluteal increase in blood flow, which promotes the release of different

vasoactive peptides that collectively cause a decrease in intraluteal blood flow, initiation of the luteolytic cascade, and subsequent decrease in progesterone secretion.

Many agents have been identified as mediators of both structural and functional luteolysis. Endothelin-1(ET-1), a potent vasoconstrictor, has a role in $PGF_{2\alpha}$ luteal regression in cows. Girsh et al (1996) reported that concentrations of ET-1 mRNA vary throughout the estrous cycle and the highest concentrations are present during luteal regression. Moreover, Milvae (2000) documented that ET-1 acts by binding to specific receptors located on large and small luteal cells to inhibit luteal progesterone synthesis while stimulating luteal $PGF_{2\alpha}$ production. The actions of $PGF_{2\alpha}$ and ET-1 result in a marked decrease in luteal blood flow and in combination with other luteal modulators induce functional and structural luteolysis. Besides ET-1, other vasoactive peptides, such as angiotensin II (Ang II) and atrial natriuretic peptide (ANP) are also considered important factors in mediating $PGF_{2\alpha}$ luteolytic actions (Schams and Berisha, 2004; Berisha and Schams, 2005).

Luteolysis also is mediated in part by a number of cytokines, including Fas L (Fas ligand), TNFα and IFNγ. The Fas antigen (Fas) is a member of the tumor necrosis family of cell surface receptors that triggers apoptosis when bound to the Fas ligand (Fas L). Taniguchi et al. (2002) demonstrated that Fas mRNA was highly expressed in bovine CL during the regressed luteal stage. Although Fas L alone did not induce cell death, bovine luteal cells became sensitive to Fas L in the presence of IFNγ or IFNγ in combination with TNFα. In addition, an in vitro study combined TNFα with IFNγ, which caused DNA fragmentation of luteal cells, supporting that luteal cells undergo apoptosis when treated with cytokines (Petroff et al., 2001). Furthermore, Benyo and Pate (1992)

documented that TNFα decrease steroidogenesis of luteal cells, inhibited luteal cell viability and stimulated prostaglandin synthesis. Another study outlined that in the bovine CL, TNFα interacted with $PGF_{2\alpha}$ and ET-1, and directly inhibited local release of progesterone. The low concentrations of progesterone combined with increased expression of TNFR type 1 facilitated TNFα apoptosis in endothelial cells leading to functional and structural regression of the CL (Friedman et al., 2000). Moreover, Benyo and Pate (1992) reported that exposure of luteal cell to TNFα or IFNγ increased the expression of class I MHC per cell as well as the overall percentage of cells expressing these molecules. In addition, when MHC molecules were examined on freshly isolated luteal cells collected on days 6, 10 and 18 of the estrous cycle, representing developing, fully functional and early regressing CL respectively, it was observed that class I MHC molecules expression did not vary significantly across the estrous cycle; however, expression of class II MHC molecules varied depending on the functional state of the CL. Class II MHC was expressed highly in most small and large luteal cells just before CL regression (d 18 of the estrous cycle) (Benyo et al., 1991). In this same study, expression of class II MHC was increased significantly when luteal regression was induced by $PGF_{2\alpha.}$ Furthermore, MHC molecule expression was lower in CL from pregnant animals compared with that in nonpregnant animals, suggesting that MHC molecules have a role in luteal regression. Pate and Keyes (2001) reviewed a series of studies on MHC molecules expression by the CL and reported that there is also evidence that CL expressed MHC molecules are functional immunologically. Luteal cells cultured with autologous T cells showed that luteal cells were effective stimulators of T cell proliferation and T cell proliferative response was greater in the presence of luteal

cells from regressing CL. These observations suggested that luteal cells could act as antigen-presenting cells, initiating a transient autoimmune response during luteolysis.

Collectively, intra-luteal progesterone is involved in a survival pathway in the CL by stimulating prostaglandins, oxytocin and its own production as well as by inhibiting apoptosis of the bovine CL. In addition, prostaglandins, oxytocin and noradrenaline as well as different angiogenic factors are potent regulators of adequate CL function and development. Furthermore, the actions of $PGF_{2\alpha}$ in combination with other intra ovarian factors such as ET-1, cytokines (including Fas L, TNFα and IFNγ) and MHC molecules induce functional and structural luteolysis.

Estrous Synchronization

Careful reproductive management of dairy cattle is essential to optimize fertility in current intensive production systems. Reasons for decline in reproductive performance of lactating dairy cows are multi factorial. Poor estrous expression and detection, low pregnancy per AI and increased embryo loss are exacerbated by intensive management, high milk production, occurrence of reproductive disorders, inadequate DMI, stressful environmental conditions and low BCS.

Development of synchronization systems to control and coordinate ovarian follicular dynamics and CL regression has provided producers with the ability to improve pregnancy per AI and embryo survival. These systems involve the use of pharmaceuticals that are comparable to the hormones regulating normal ovarian function in the cow. Exogenous $PGF_{2\alpha}$ promote shortening of the luteal phase and induce estrus in cows with a functional CL. In contrast, administration of progestins can lengthen the luteal phase and prevent ovulation during the treatment period. Moreover,

GnRH injections can be used in estrous synchronization protocols to turn over follicles, induce ovulation and subsequent CL formation.

Several studies documented the use of exogenous $PGF_{2\alpha}$ to promote CL regression and decline in progesterone concentrations during the bovine estrous cycle. However, early studies indicated that a limitation of $PGF_{2\alpha}$ administration was its inability to cause luteolysis in cattle when injected before day 5 of the estrous cycle (Lauderdale, 1972; Rowson et al., 1972). Lauderdale et al. (1974) demonstrated that the use of exogenous $PGF_{2\alpha}$ promoted CL regression and return to estrus without compromising fertility. In addition, Louis et al. (1974) described the sequence of changes in serum concentrations of different hormones after administration of $PGF_2\alpha$. Results of this study showed that administration of $PGF_{2\alpha}$ caused decrease in serum progesterone and subsequent increase in estradiol secretion, followed by an ovulatory surge of LH, onset of estrus and ovulation. Furthermore, Chenault et al. (1976) documented a decrease of progesterone concentrations to <0.5 ng/ml within 24 hours after administration of $PGF_{2\alpha}$.

Subsequent studies demonstrated that stage of estrous cycle at administration of $PGF_{2\alpha}$ had an effect on estrous response, interval from $PGF_{2\alpha}$ administration to the onset of estrus, and pregnancy per AI. Tanabe and Hann (1984) administered $PGF_{2\alpha}$ on days 7, 11 and 15 of the estrous cycle and observed estrus in 86, 90 and 98% of treated dairy heifers, respectively. They also observed significant differences in the interval to onset of estrus, where heifers that received $PGF_{2\alpha}$ at day 7, displayed estrus earlier (43.9h) than heifers treated on days 11 and 15. In another study, heifers were injected with $PGF_{2\alpha}$ after observed estrus at either early (days 5 to 7), middle (days 8 to

11) or late (days 12 to 15) diestrus. After $PGF_{2\alpha}$ injection, heifers were observed for estrus for five consecutive days and were inseminated at about 12 hours after estrus was first observed. Estrual response rates for early, middle and late diestrus were 43, 83.6 and 100 %, respectively. Heifers in the early diestrus group displayed estrus at an earlier time (average of 59h) compared to heifers in the middle (70h) and late (72h) diestrus groups. Pregnancy per AI was 56.8, 62.1 and 78.3% for early, middle and late diestrus groups respectively (Watts and Fuquay, 1985). In an attempt to ensure that a high proportion of cows would undergo CL regression and have a high rate of synchronization of estrus, early studies have used two injections of PGF2α at 11 or 14 days intervals to synchronize estrus (Roche, 1976; Stevenson et al., 1987; Morbeck et al., 1991). Although estrous synchronization was achieved, the variability in occurrence of estrus after the second $PGF_{2\alpha}$ injection was not sufficiently precise for the use of timed insemination without detection of estrus. Collectively, a number of studies have shown that synchronization of estrous with $PGF_{2\alpha}$ increased estrus detection rates and improved management for AI compared with daily estrous detection. However, estrus was not precisely synchronized and pregnancy per AI was consistently lower when timed insemination was used with $PGF_{2\alpha}$ compared with breeding after estrous detection (Lucy et al., 1986; Stevenson et al., 1987). Pregnancy per AIs following a single insemination at 80 hours (23%) or two inseminations at 72 and 96 hours (30%) after PGF2α were less ($P<0.05$) than those of cows inseminated 12 hours (54%) after detected estrus (Stevenson et al., 1987). Results of these studies indicated that administration of $PGF_2\alpha$ to promote CL regression at different times of the estrous cycle provides variable results and that a considerable variance exists as to when estrus will

occur after $PGF_{2\alpha}$ injection. This variation in responses is usually caused by the timing of $PGF_{2\alpha}$ injection relative to the stage of the follicular wave. Luteolysis induced by administration of $PGF_{2\alpha}$ in the presence of an active dominant follicle, results in an earlier occurrence of estrus than a PGF2α injection given at a time of the cycle where follicles might be in early stages of development and require additional time to develop and reach ovulatory capacity (Thatcher et al., 2001).

Extensive research on development of synchronization strategies has made clear that both follicular dynamics and CL life span should be controlled to precisely synchronize the estrous cycle in cattle and overcome problems and limitations associated with visual estrous detection.

Early studies indicated that the use of GnRH or an analogue caused stimulation of follicular growth and induction of follicular turnover or ovulation, according to the stage of follicular development at the time of treatment (Thatcher et al., 1989; Macmillan and Thatcher 1991). Moreover, Thatcher et al. (1989) demonstrated that treatment with GnRH followed 7 days later by $PGF_{2\alpha}$ injection increased the number of animals synchronized compared with administration of $PGF_{2\alpha}$ alone. By 7 days after GnRH injection, $PGF_{2\alpha}$ induces regression of the CL and allows final maturation of the synchronized dominant follicle.

Pursley et al. (1995) described a protocol for synchronizing the time of ovulation in cattle using a combination of GnRH and $PGF_{2\alpha}$. The protocol consisted of an injection of GnRH administered at a random stage of the estrous cycle to induce emergence of a new follicular wave and a dominant follicle. Seven days later, $PGF_{2\alpha}$ was injected intramuscularly to promote CL regression. A second injection of GnRH was given 2

days after $PGF_{2\alpha}$ injection. Ovulation was synchronized within an 8 hour period, 24 to 32 hours after the second injection of GnRH. The protocol allowed precise synchronization of ovulation because preovulatory follicles were at similar stages of growth and responsive to LH at the time of second GnRH injection. Schmitt et al. (1996) conducted two experiments to evaluate the efficacy of using TAI after an injection of GnRH agonist 24 or 48 h after $PGF_{2\alpha}$ injection in heifers. In the first experiment, heifers were randomly assigned to two treatments: in the TAI group (TAI), heifers were inseminated at a predetermined time; in the estrus group (AIE), heifers were inseminated after observation of standing estrus. Estrous cycle was synchronized by a GnRH injection on d 0 of the experimental sequence. On d 7, $PGF_{2\alpha}$ injection was administered and heifers in the TAI group received a GnRH injection 24 h later. TAI heifers were inseminated 15 hours after the last GnRH injection. In the second experiment, the same synchronization protocol was used; however, GnRH injection for the TAI group was given 48 h after the $PGF_{2\alpha}$ injection. Results of the first experiment showed a reduction in pregnancy and pregnancy per AIs for TAI compared with AIE (25.8 < 48.7% and 25.8 < 57.4 % respectively). In contrast, no differences in pregnancy per AI were observed in the second experiment (48% for AIE vs 45.5% for TAI). However, pregnancy per AI was reduced for TAI (45.5 < 61.2%). Moreover, Burke et al. (1996) compared conception and pregnancy per AI of lactating dairy cows assigned into two treatment groups. Both groups were synchronized by an injection of GnRH followed 7 days later with an injection of $PGF_{2\alpha}$. Cows in the timed AI group received a second injection of GnRH 48 hours after $PGF_{2\alpha}$ and were inseminated 16 hours later. Cow in the control group were inseminated 12 hours after detection of estrus. Pregnancy per AI was lower for the

timed insemination group (26.5 vs 41.5%). However, pregnancy per AI was 30.5% for the control cows and 29.0% for timed inseminated cows. Overall proportion pregnant by day 120 postpartum did not differ between the control and timed AI groups (58.8± 4.7% vs 56.2± 4.4%) respectively. A subsequent study evaluated pregnancy per AI using the Ovsynch, and a protocol consisting of $PGF_{2\alpha}$ injections and detection of estrus. Pregnancy per AI were similar among cows treated with Ovsynch protocol and bred between 16 and 20 hours after second GnRH compared with cows given two $PGF_{2\alpha}$ injections and bred 12 hours after detected estrus (37.8% vs 38.9%). Additionally, the use of Ovsynch protocol allowed timed AI of lactating cows without the need for estrous detection (Pursley et al., 1997). Furthermore, as a means of reproductive management, Pursley et al. (1997a) compared the Ovsynch protocol with a traditional $PGF_{2\alpha}$ method of synchronization followed by estrous detection. Cows were evaluated for pregnancy via ultrasonography 32 to 38 days after AI and non pregnant cows were treated again with the synchronization protocol used for that animal previously. Pregnancy per AIs for first service was similar (37% Ovsynch vs 39% PGF2α). However, in addition to eliminating the need for estrous detection, the use of Ovsynch protocol reduced days to first service and days open. Proportion pregnant at 60 and 100 days were higher for the Ovsynch group (53% >37, respectively). Variations on the intervals between $PGF_{2\alpha}$ and the second GnRH injection and between GnRH injections and insemination have been examined by numerous studies. Pursley et al. (1995) gave GnRH followed 7, 8 or 9 days later with a $PGF_{2\alpha}$ injection and a second injection of GnRH was administered at the same time of follicle development for all groups, that is, 48, 24 and 0 hours after PGF2α injection, respectively. Pregnancy per AIs were 54, 46 and 11% respectively.

Additionally, Stevenson et al. (1996) administered the second GnRH injection of the Ovsynch protocol 30 to 32 hours after PGF2α injection and AI 18 to 19 hours after GnRH and compared with a control group of cows given PGF2α and inseminated at estrus. If no estrus was observed, $PGF_{2\alpha}$ was reinjected 14 d later. Fertility status between treatment and control groups was compared after one fixed time insemination (treatment) and after insemination based on detected estrus after first or subsequent $PGF_{2\alpha}$ injection (control). Pregnancy per AI tended to be higher in the Ovsynch treated group. However, only 56% of cows in the control group were detected in estrus after $PGF_{2\alpha}$ injection (35.3% > 26.5%). In another study, Ovsynch, with insemination 16 h after the second GnRH injection, was compared with protocols using two injections of $PGF_{2\alpha}$ given in an interval of 14 days, and a single injection of $PGF_{2\alpha}$ with cows bred after the first observed spontaneous estrus. Ovsynch treated cows had higher pregnancy per AI and fewer days open than the other groups (i.e., 33% and 107 days open for Ovsynch vs 11% and 129 days for two injections of $PGF_{2\alpha}$, vs 12 % and 122 days for the single $PGF_{2\alpha}$ injection, and 6% and 116 days for untreated cows) (Momcilovic et al., 1998).

Collectively, the use of Ovsynch as a synchronization method results in acceptable pregnancy per AI following a fixed time insemination compared with other reproductive management strategies. In addition, Ovsynch allows insemination of 100% of cows enrolled in the program without the need of estrous detection.

Efficacy of estrous synchronization protocols using GnRH and $PGF_{2\alpha}$ depends on the ability of the first GnRH treatment to induce follicle turnover upon ovulation of a dominant follicle. In addition, stage of the estrous cycle at which a timed AI protocol is

initiated may affect subsequent pregnancy per AI. In an extensive study, Moreira et al. (2000) monitored follicle dynamics and CL development after initiation of an Ovsynch timed AI protocol at different stages of the estrous cycle. Ovsynch was initiated at days 2, 5, 10, 15 or 18. Results indicated that day of estrous cycle at which the Ovsynch was initiated affected the synchronization program and may influence subsequent pregnancy per AI in dairy heifers. Initiation of the TAI protocol at day 2, or the metestrus phase of the estrus cycle (i.e.,days 1 to 4), may lead to a failure of the first GnRH to synchronize a new follicular wave. Such failure may lead to development of an aged dominant follicle and GnRH induced ovulation of a poor quality oocyte that may compromise subsequent embryo development. Moreover, initiation of the Ovsynch at d 15 or late diestrus phase of the estrous cycle may lead to premature regression of the CL causing cows to ovulate before the second GnRH injection. This asynchrony causes a lower pregnancy per TAI to the synchronized service. Collectively, these observations showed that due to different reasons, Ovsynch initiated at day 2, day 15 or day 18 of the estrous cycle may result in an inappropriate conception to the TAI. Additionally, results suggest that the optimal stage to initiate the synchronization program corresponds to the early luteal phase between days 5 and 12 of the estrous cycle.

Based on the previous findings, a presynchronization strategy (Presynch) was developed in which two injections of $PGF_{2\alpha}$ administered 14 days apart preceded the initiation of Ovsynch 12 days after the second PGF2α injection (Moreira et al., 2001). The Presynch program will induce cows to be between days 5 and 11 of the estrous cycle at the time of the first GnRH injection of the Ovsynch; depending upon what day they expressed estrus after the second $PGF_{2\alpha}$ injection of presynchronization. Cows

enrolled on the Presynch-Ovsynch protocol had higher pregnancy per AI compared with cows that initiated the Ovsynch program at random stages of the estrous cycle. A modified Presynch protocol in which Ovsynch is initiated 14 days after the second $PGF_{2\alpha}$ injection has been shown to increase pregnancy per AI compared to Ovsynch protocol alone (49.6 vs 37.3%) (Navanukraw et al., 2004). Galvão et al. (2007) compared reproductive performance of lactating dairy cows subjected to 11 or 14 d intervals between presynchronization and initiation of TAI protocol. Reducing the interval between the presynchronization to the first GnRH injection of the TAI protocol resulted in increased pregnancy per AI evaluated on d 38 (40.5 vs 33.5%; P = 0.02) and 66 (36.4 vs 30.2%; P = 0.04) after TAI.

The use of an intravaginal device for the release of progesterone (CIDR, controlled internal drug-release, Eazi Breed™ CIDR® Pfizer Animal Health, New York, NY) has become available as an additional tool for synchronization of estrus. An extensive field trial investigated the efficacy of a CIDR insert containing 1.38 g of progesterone and $PGF_{2\alpha}$ injection in synchronizing estrus and shortening the interval to pregnancy in postpartum beef cows, beef heifers and dairy heifers (Lucy et al., 2001b). Animals were assigned to three groups and either received a CIDR implant for 7 days plus a $PGF_{2\alpha}$ injection on day 6 (CIDR+ $PGF_{2\alpha}$), received a single intramuscular injection of $PGF_{2\alpha}$ or were not treated (control group). Cows were diagnosed as anestrous, prepubertal or cyclic before the treatment period by analysis of blood progesterone concentrations. Estrous detection started at day 8, (1 day after CIDR removal or 2 days after $PGF_{2\alpha}$ injection) and continued for 31 days. Cattle were AI approximately 12 h after observed in estrus. Beef cows in the CIDR+ $PGF_{2\alpha}$ group

showed a greater incidence of estrus (P<0.001) during the first 3 days of the breeding period compared with cows treated with a single injection of $PGF_{2\alpha}$ or cows in the control group (59, 33 and 15%, respectively). Moreover, the improved estrus response led to an increase in pregnancy per AI during the 3 day period. In contrast, pregnancy per AI for the 31 day breeding period were similar for control (50%), $PGF_{2\alpha}$ (55%) and CIDR+ $PGF_{2\alpha}$ (58%) groups. Synchronization and pregnancy per AI also were improved in beef heifers that received the CIDR+ $PGF_{2\alpha}$ treatment. Dairy heifers treated with CIDR+ $PGF_{2\alpha}$ showed an improvement in the incidence of estrus during the first 3 days of the breeding period; however, pregnancy per AI during the first 3 days or the 31 days period were not improved for CIDR+ PGF2α compared to $PGF_{2\alpha}$ group. Anestrous cows and prepubertal heifers had lesser synchronization rates and pregnancy per AI during the 3 days and entire 31 days breeding period compared to cyclic animals. For anestrous cows, the proportion of cattle that were not observed in estrus declined rapidly in the CIDR+ $PGF_{2\alpha}$ treatment group. This effect of progesterone on increased ovarian/estrual activity involves increased LH secretion that stimulates follicular development leads to ovulation.

After first service, detection of non pregnant cows as early as possible to permit a subsequent resynchronization and timed AI are essential to maintain high reproductive performance of the herd. In addition to induced cyclicity in anestrous cows and heifers, a number of studies have showed that insertion of a CIDR after insemination can be used to synchronize the return to estrus (i.e., resynchronization) of non pregnant cows. In a review, Macmillan and Peterson (1993) summarized results of a series of trials using a CIDR-B insert to synchronize return to estrus of previously inseminated lactating

dairy cows. In one trial, cows received a new CIDR-B device starting from 14 to 17 days after first insemination and removed 21 days after insemination. Pregnancy per AI to first service was 64 and 63.6 % for the CIDR-B treated and control groups respectively. The CIDR B treatment was an effective tool to increase the percentage of second inseminations made 23 or 24 days after first inseminations. However pregnancy per AI to second inseminations were similar between CIDR-B treated cows compared with control cows (65.5% vs 70.9%). Treatment with CIDR-B device has proven to be effective in situations of low estrous detection rates, which contribute to long return to service intervals. Van Cleef et al. (1996) showed that in heifers, the use of used CIDR devices from days 17 to 22 after insemination was effective in producing a synchronous return to estrus in most nonpregnant heifers, and the CIDR insert did not harm fertility to the initial insemination. Pregnancy per AI for untreated heifers and heifers that received a CIDR implant was 43.5% and 50% respectively. In contrast, another study reported reduced pregnancy per AI of heifers that received used CIDR inserts between days 16 and 21 after timed AI compared to untreated heifers; however, the method used was effective in synchronizing returns to estrus (Xu and Burton, 1999). Moreover, Chenault et al. (2003) documented that administration of CIDR inserts to lactating dairy cows on day 14 after insemination with removal 7 days later was an effective method of synchronizing return to estrus, However, treatment with a CIDR insert was associated with reduced pregnancy per AI to the pretreatment AI. Pregnancy per AI for control cows and CIDR treated cows were 36.7% and 32.7% respectively. Furthermore, El-Zarkouny and Stevenson (2004) reported that the use of a CIDR insert from 13 to 20 days after AI increased the synchrony of return to estrus, but failed to increase the

reinsemination rate of nonpregnant cows during the 6 days after removal of the insert. Use of a CIDR insert increased embryonic survival between days 29 and 57 of the prior TAI compared with the control group (65.5% vs 44.3%). In another study, Chebel et al (2006) evaluated the effects of CIDR insert during a presynchronization protocol on initiation of cyclicity, reproductive responses and also the efficacy of the CIDR to synchronize return to estrus after insemination. Holstein cows were assigned randomly into one of three presynchronization treatments. During presynchronization, all cows received two injections of $PGF_{2\alpha}$ on study d 35 and 49. Cows enrolled in the control (CON) treatment were inseminated on detection of estrus from study d 49 to 62. Cows enrolled in the CIDR estrus detection (CED) treatment received a CIDR insert from study d 42 to 49 and were inseminated on detection of estrus from d 49 to d 62, and cows enrolled in the CIDR TAI (CTAI) treatment received the same treatment as CED but cows were not inseminated following removal of the CIDR. On study d 62 all cows from the CTAI treatment and cows from the CON and CED treatments not yet observed in estrus were enrolled in an Ovsynch protocol. After AI cows were blocked by presynchronization treatments and within each block, were randomly assigned to either received a CIDR insert from day 14 to 21 (RCIDR) or to not receive a resynchronization treatment (RCON). No differences in pregnancy per AI at 31 and 60 days after AI were observed between CON and CED groups, indicating that the use of a CIDR insert during presynchronization did not improve fertility. However, the use of a CIDR during the presynchronization program improved cyclicity by increasing the proportion of anestrous cows that initiated cyclicity by study d 62 (i.e., within 13 days after the end of the voluntary waiting period). Cows cycling by study d 49 had higher pregnancy per AI

on d 31 and 60 after insemination compared with anestrous cows. In addition, an interaction between presynchronization and resynchronization treatments were observed for pregnancy per AI at day 31 after first AI. The RCIDR cows had a higher pregnancy per AI when they were presynchronized as CED or CTAI compared with cows presynchronized as CON. Regardless of the presynchronization protocol, cows that received CIDR insert after AI had reduced pregnancy losses from d 31 to 60 of gestation . Bartolome et al., (2009; in press) compared pregnancy per AI for first service in control cows and cows receiving a CIDR insert during the Presynch/Ovsynch protocol. Moreover, second service pregnancy per AI was evaluated in cows resynchronized with or without a CIDR insert between d 14 and 23 after first AI. No difference in overall pregnancy per AI for first service at 30 and 55 d was observed for CIDR (36.1% and 33.6%) and control (34.1% and 28.8%) groups. However, pregnancy loss between 30 and 55 d was lower for cows in the CIDR compared to cows in the control group (7.0% vs 15.6%). Furthermore the use of a CIDR insert between d 14 and 23 after AI did not affect pregnancy outcome to the prior insemination and did not improve second service pregnancy per AI. In addition, no differences in pregnancy loss between 30 and 55 d for second service was detected between cows receiving a CIDR insert for resynchronization compared with control cows.

The administration of a CIDR might be an alternative reproductive management tool as part of Presynch/Ovsynch synchronization programs. Moreover, administration of CIDR after insemination might be considered for resynchronization protocols. However, the benefits of using a CIDR insert are not consistent. Thus, additional studies

are needed to evaluate its effectiveness in induction of cyclicity, synchronization of follicular development and ovulation, pregnancy outcome and embryonic survival.

Pregnancy Associated Glycoproteins (PAGs)

Placentation in ruminants is relatively noninvasive and is classified as synepitheliochorial cotyledonary. Cotyledons, or villous trophectoderm, introduce themselves into the crypts of the maternal caruncles and form interdigitated regions of partially fused fetal cotyledonary and maternal caruncles, known as placentomes. Cells of the trophectoderm are specialized and subdivided into two morphologically and functionally distinct cell types referred as mononucleated and binucleated trophoblast cells. The binucleated cells migrate and fuse with maternal uterine epithelial cells and deliver secretory products directly into the maternal system. Among the secretory products are the pregnancy associated glycoproteins (PAGs), also known as pregnancy specific protein B (PSPB) (Butler et al., 1982) and pregnancy serum protein of M_r 60 kDa (PSP60) (Mialon et al., 1994).

Bovine PAGs (boPAGs) were first discovered during attempts to develop an early pregnancy test in cattle. In Butler et al. (1982) injected rabbits with extracts of placental cotyledons with the aim of raising antisera with placental specificity. Antibodies not directed against placental antigens were removed by absorption with tissue extracts from nonpregnant animals. Pregnancy specific protein B (PSPB) was isolated and partially purified from bovine placental tissue. Moreover, Sasser et al. (1986) detected PSPB in pregnant cow serum as early as 15 days after conception. In addition, Zoli et al. (1991) isolated bovine pregnancy associated glycoprotein (bPAG) from fetal cotyledons, and subsequently developed a radioimmunoassay test able to detect bPAG in peripheral blood of pregnant cows as early as day 22 of pregnancy (Zoli et al., 1992).

Although bovine PSPB and bPAG were detectable in the plasma of some pregnant cows as early as 15 to 20 days after AI, more accurate and reliable results were achieved by 28 to 30 days after insemination (Humblot et al., 1988; Zoli et al., 1992). Xie et al. (1991) used an antiserum directed against purified boPAGs to screen cDNA libraries from late placental tissue and found that the cDNAs shared 86% nucleotide sequence identities with each other and 60% sequence identity to pepsinogens. However, the boPAGs mutations in and around their active sites identified them as inactive members of the aspartic proteinase family.

Collectively, early studies demonstrated that although the detection of PAGs could be used as the basis of a pregnancy test in cattle, positive pregnancy diagnosis in the first 30 days of pregnancy was uncertain because antigen concentrations in the maternal blood were low and variable (Sasser et al., 1986; Zoli et al., 1992; Mialon et al., 1993). Moreover, concentrations of PAGs rose markedly close to term, and due to its long circulating half-life, PAGs could still be detected in blood 80 to 100 days postpartum, compromising pregnancy diagnosis in cows bred within this early postpartum period (Mialon et al., 1993; Zoli et al., 1992; Kiracofe et al., 1993). In addition, several PAGs have been cloned from bovine and ovine placenta (Xie et al., 1997).). While some of these proteins were localized to binucleated cells (Xie et al., 1991; Xie et al., 1997a), other PAGs were expressed throughout the trophectoderm (Xie et l., 1994). Analysis of the bovine genome resulted in the identification of 18 distinct PAG genes and 14 pseudogenes (Telugu et al., 2009).

In an attempt to identify candidate PAGs that were suitable antigens for an improved pregnancy test in cattle, Green et al. (2000) studied the relationship among

the various PAG family members in terms of their structures and phylogenetic origins and determined whether these features were correlated with expression patterns during pregnancy. The cloning work described by this study confirmed that numerous PAG genes were expressed in the placenta in cattle and also identified novel PAG genes. Moreover, *in situ* hybridization showed the distribution of PAGs into two groups, those expressed predominantly in binucleated cells and those PAGs expressed throughout the trophectoderm. Phylogenetic analysis trees demonstrated that the two kinds of PAGs were represented by two distinct groupings comprising ancient and modern PAGs, based on the relative times at which they arose during evolution. PAGs found to be expressed throughout the trophectoderm were more ancient members of the family and were represented by relatively fewer genes compared with PAGs expressed predominantly in binucleated cells. Many of the PAG*s* in the modern group have amino acid substitutions at conserved positions within the catalytic center that may preclude these PAG*s* from functioning as enzymes. In contrast, ancient PAGs, possess all the hallmarks of typical aspartic peptidases and are predicted to be active enzymes (Telugu et al., 2009). Additionally, RNase protection assays showed that different PAGs were temporally expressed throughout pregnancy and identified PAG family genes expressed predominantly by binucleated cells in early pregnancy, which could be good candidates for a pregnancy test. Observations from this study led to purification of a mixture of PAGs expressed by binucleated cells and development of a mononucleated based enzyme linked immunosorbent assay (ELISA) that specifically detected PAG molecules predominantly expressed in early pregnancy by binucleate cells. Furthermore, the efficiency of this assay was not compromised by persistence of PAGs in the circulation

during the post partum period (Green et al., 2005). A recent study used the ELISA test described by Green et al. (2005) with slight modifications to evaluate the accuracy of a PAG ELISA test in identifying pregnancy status 27 days after timed AI. Pregnancy diagnosis by transrectal ultrasonography (TU) was performed and pregnancy outcomes by TU served as a standard to test the accuracy of the PAG ELISA test. The ability of the PAG ELISA to identify pregnant cows (sensitivity) 27 days after AI varied from 93.5 to 96.3% and was similar to the accuracy of the TU method (93.7 to 97.8%). Specificity of the PAG ELISA test ranged from 91.7 to 96.8% (Silva et al., 2007).

The measurement of PAGs in maternal blood is an alternative reproductive management tool that can be used for early pregnancy diagnosis in cattle and may be an indicator of embryo/fetal well being and pregnancy loss. In cows, losses of pregnancy prior to day 24 after AI characterize early embryonic death and losses between days 24 and 42 to 50 after AI indicate late embryonic losses. Fetal death is distinguished by pregnancy losses after day 50 of pregnancy (Santos et al., 2004).

Early studies showed that peripheral concentrations of PSPB (Patel et al., 1995) or bPAG (Patel et al., 1997) were correlated to the stage of gestation, number of fetuses and fetal viability. Szenci et al. (1998) performed pregnancy diagnosis in lactating dairy cows between 27 and 59 days after AI by transrectal ultrasonography while concurrently measuring plasma bPAG1 concentrations. High concentrations of bPAG1 and progesterone confirmed that four cows, initially checked pregnant by transrectal ultrasonography where indeed pregnant at the time of the initial examination but experienced embryonic/fetal mortality afterwards. Fetal death, determined by ultrasonography was preceded by a decrease in plasma concentrations of bPAG1. One

study measured bPAG1 concentrations in plasma before and after induction of late embryonic mortality by either trans-cervical inoculation with *Arcanobacterium pyogenes* or prostaglandin treatment in heifers on days 30 to 38 of pregnancy. Inoculation with *Arcanobacterium pyogenes* caused late embryonic mortality in 4 of 5 heifers and concentrations of bPAG1 decline to the pregnancy cut-off value within 4 to 8 days post inoculation. In heifers treated with $PGF_{2\alpha}$, plasma bPAG1 concentrations reached the pregnancy/open cut-off value 5 to 8 days after treatment. It was observed that bPAG1 concentrations started to decrease steadily when it was accompanied by the subsequent death of embryo, suggesting that measurement of bPAG1 plasma concentrations may be used as an alternative method for determining late embryonic mortality or viability of the fetus in cattle (Szenci et al., 2003). Another study examined whether bPAG1 measurements during the early fetal period could be associated with early fetal loss. Lactating dairy cows had blood collected and transrectal ultrasonography done on days 35, 42, 49, 53 and 63 of pregnancy or until pregnancy loss. Of the 98 cows investigated, 18.4% suffered early fetal loss. bPAG 1 and progesterone concentrations were not significantly different between the cows that suffered fetal loss and cows that maintained pregnancy. However, based on the bPAG1 concentrations on day 35 of gestation, the odds of fetal loss was 10 and 6.8 times more likely in cows with low (< 2.5 ng/ml) and high (> 4.0 ng/ml) bPAG1 concentrations compared with medium bPAG1 concentrations (2.5- 4.0ng/ml). Therefore, bPAG1 concentrations on day 35 of gestation might be used as a predictive tool for fetal loss (López-Gatius et al., 2007). Increasing levels of PAGs during gestation and further increase within 10 days prior to calving might be indicatives of the involvement of PAGs

in the parturition process. Studies evaluated changes in PAGs responses during late pregnancy by inducing parturition with $PGF_{2\alpha}$. Animals with a normal parturition showed increasing and high levels of PAGs. The patterns of plasma PAGs were altered in the cases of calving difficulty and stillbirth (Kindahl et al., 2002; Kornamatitsuk et al., 2002). Chavatte-Palmer et al. (2006) evaluated fetal and placental development by repeated ultrasonography observations and measurement of PAG plasma concentrations in groups of pregnancies resulting from somatic cell nuclear transfer (SCNT) compared with pregnancies that did not result from SCNT. Two different radioimmunoassays were used to measure plasma concentrations of PAGs and results indicated that cows carrying clone pregnancies that went to term had significantly higher concentrations of PAGs compared with control cows. Moreover, PAGs concentrations from days 34 to 50 of pregnancy were lower in clone recipient cows suffering early pregnancy loss than those which maintained pregnancy. Measurement of PAGs may be a practical noninvasive tool to follow recipient cows carrying a cloned fetal placental unity.

Although measurement of PAGs in plasma is useful to detect pregnancy and monitor fetal development in ruminants, the functional role of these molecules is still unclear. A proposed role in which PAGs act in an immunodulatory capacity has been reported by different studies. Dosogne et al. (1999) investigated immunossupression in periparturient cows by PAGs. From 3 weeks before until 5 weeks after parturition, the phagocytosis and oxidative burst activity of polymorphonuclear neutrophil (PMN) leukocytes isolated from blood and PAG concentrations in plasma were evaluated in dairy cows. A significant decrease in oxidative burst activity of PMN was observed between 1 and 3 weeks after calving. In addition, it was documented a peak in plasma

bPAG concentration immediately prior to the decline in PMN activity. It was hypothesized that there could be an association between high PAG concentrations around parturition and lowered PMN activities. Moreover, Hoeben et al. (1999) documented that high concentrations of bPAG around parturition significantly inhibited the proliferation of bone marrow progenitor cells cultured in vitro, suggesting a possible involvement of PAGs in post partum immunosuppression. In addition, day 14 uterine endometrial explants from non pregnant cows were cultured with PSPB, which induced release of the alpha chemokine granulocyte chemotactic protein-2 (GCP-2). Induction of GPC-2 was blocked by addition of antiserum against PSPB (Austin et al., 1999). Furthermore, different possible functions may be related to the diverse localization of the ancient and modern PAG groups. Ancient PAGs, mainly expressed at the microvillar junctions may be involved in binding the surfaces together or in establishing an immunological barrier. In contrast, the expression of modern PAGs mainly in the developing maternal villi of the placentomes ideally positions them to manipulate the maternal immune system (Wooding et al., 2005). Additionally to possibly identify roles of PAGs in immune regulation, a series of in vitro studies reported that PAGs may be involved directly and indirectly in luteotrophic activity. Administration of PAG1/PSPB to cultured bovine luteal cells promoted the release of PGE_2 (Del Vecchio et al., 1995 and 1996; Weems et al., 1998) and increased progesterone (Del Vecchio et al., 1996; Weems et al., 1998a). In contrast, Del Vecchio et al. (1995) reported no increase in progesterone in luteal cells treated with PSPB. Administration of PSPB to bovine cultured endometrial explants of day 16 of the estrous cycle increased secretion of PGE_2 and $PGF_{2\alpha}$ (Del Vecchio et al., 1990). Ninety days pregnant ewes were

ovariectomized and blood samples were collected at day 0 (laparotomy) and at162 hours. Results showed that plasma PSPB and estradiol 17β concentrations were increased in ewes ovariectomized at 90 days of pregnancy, compared with intact ewes. Increased concentrations of PSBP were preceded by an increase in estradiol. In vitro studies with placenta slices of the same ovarioectomized ewes collected 7 days after laparotomy revealed an increase in progesterone, PGE_2, estradiol 17β and PSPB secretions compared with secretion by placenta of intact ewes. Conclusions were that estradiol may regulate PSPB secretion, which may influence placental PGE_2 secretion, which affects placental secretion of progesterone in sheep (Weems et al., 1999). Moreover, secretion of PGE_2 by cultured ovine endometrium was increased by PSPB treatment on days 60 and 90 post breeding. Thus it was concluded that secretion of PGE_2 by caruncular/placental tissues after 50 days post breeding might be regulated by PSPB (Weems et al., 2003).

In an attempt to gain some preliminary insight into PAG function in vivo, Egen et al. (2009) examined whether these circulating placental antigens were important in pregnancy by auto-immunization of ewes against a mixture of purified native ovine PAGs. Ewes were immunized against PAGs or kept as controls. Blood samples for analyses of PAG concentration were collected at the time of estrus (day 0) and on days 10, 14 to 28 and then weekly throughout the remainder of pregnancy. Pregnancy was confirmed by ultrasound 30 days after breeding. Of the 22 animals immunized against PAGs, seven developed no or little reactivity toward PAGs. Control animals had detectable PAGs around day 21 of pregnancy. Ewes that expressed low immunoreactivity had measurable PAGs by day 22 of pregnancy and their PAGs serum

concentration throughout pregnancy did not differ from the controls. In contrast, ewes exhibiting moderate to high anti-PAG immunoreactivity had significantly lower PAG concentrations than controls; in these ewes, circulating PAGs did not become detectable until around day 48 of pregnancy. Although the ability to coat PAGs with antibodies was successful, the decrease in circulating PAGs in the immunized ewes did not affect pregnancy per AI, lamb number or lamb birth weight, suggesting that systemic role of PAGs may be secondary to their local role at the placenta-uterine interface.

The function (s) of PAG family genes may be complex and combined with their spatial and temporal expression throughout pregnancy. Some PAG family members may be involved in adhesion, implantation and maternal recognition of pregnancy, while others may have a role in immune regulation and remodeling of the fetal-maternal unit during placenta development. However, in view of the magnitude of their production and their survival through years of evolution, it seems unlikely that the PAGs lack a role during pregnancy.

Hypothesis and Objectives

Early embryo mortality accounts for major reproductive wastage in dairy farms. In the past few decades, the shift toward more productive cows has been associated with a decline in fertility. Moreover, the extended NEB experienced in early lactation associated with changes in BCS, occurrence of health disorders and high milk production have significant carry over effects on reproductive efficiency. In addition, lower concentrations of steroid hormones in lactating cows compared with nonlactating cows might exert negative effects on reproductive performance. Moreover, understanding the molecular regulation of embryo development and endometrium remodeling during the early gestation period is important for identification of genes

regulating implantation, placentogenesis and maintenance of pregnancy. The hypothesis of the experiment described in Chapter 3 was that lactation would cause a differential expression of endometrial and conceptus expression of genes at day 17 after AI in both cyclic and pregnant cows. Therefore objectives of this study were to characterize postpartum metabolic and hormonal differences between nonlactating and lactating dairy cows, to evaluate lactation and pregnancy effects on endometrium and conceptus gene expression, and to characterize associations between conceptus and endometrial expression of genes in early pregnancy (d 17).

Several factors participate in the complex transition period such as reestablishment of postpartum reproductive performance and subsequent establishment and maintenance of pregnancy. Experiments described in the present thesis have the overall objective of understanding mechanisms involved in early pregnancy, developing platforms to improve fertility in dairy cattle and to test an alternative reproductive management tool for early pregnancy diagnosis.

Careful reproductive management of dairy cattle is essential to optimize fertility in current intensive production systems. Development of synchronization systems to control and coordinate ovarian follicular dynamics, CL regression and induction of ovulation has provided producers with the ability to improve pregnancy per AI and embryo survival. Moreover, an alternative reproductive management tool such as measurement of PAGs in maternal blood is used for early pregnancy diagnosis in cattle and may be an indicator of embryo/fetal well being and pregnancy loss. The hypothesis of the experiment described in Chapter 4 was that early resynchronization of cows diagnosed nonpregnant using a CIDR insert would reduce days open, improve

pregnancy per AI and reduce embryo loss. Objectives of this study were to characterize pregnancy per AI to AI for first and second services in cows receiving a Presynch/Ovsynch and a CIDR/Resynchronization program (Resynch) versus a Presynch/Ovsynch and a Control resynchronization program (Control), and to compare concentrations of progesterone and PAGs after first AI between d 18 and d 60 for pregnant cows and between d 18 and d 30 for nonpregnant cows of the Resynch and Control programs.

CHAPTER 3
EFFECTS OF LACTATION AND PREGNANCY ON METABOLIC AND HORMONAL RESPONSES AND CONCEPTUS AND ENDOMETRIAL GENE EXPRESSION OF HOLSTEIN DAIRY CATTLE

Objectives were to characterize postpartum metabolic and hormonal differences between nonlactating and lactating dairy cows, evaluate lactation and pregnancy effects on endometrium and conceptus gene expression, and characterize associations between conceptus and endometrial expression of genes in early pregnancy (day 17). Pregnant heifers (n=33) were assigned randomly after calving to a lactating group (L, n=17) and a nonlactating group (NL, n=16). L cows were fed a TMR (1.65Mcal NEL/kg, 16.5% CP) ad libitum. NL cows were fed a maintenance ration (1.45 Mcal NEL/kg, 12.2% CP) once per day. Rectal temperatures and blood, for analyses of progesterone and metabolites, were collected thrice weekly. Ovarian ultrasonography and BW measurements were performed weekly. BCS was measured every 14 days. All cows were pre-synchronized and enrolled in a TAI protocol (Presynch/5 days CIDRSynch); 10 cows in the L and 12 in the NL were TAI. On day 17 after GnRH/TAI, all cows were slaughtered and endometrial, conceptus, oviductal and ovarian tissues collected. The Bovine Affymetrix GeneChip was used to assess conceptus and endometrial gene expression. Standard and partial correlation analyses were used to evaluate associations of conceptus and endometrium expression of PAGs with other genes of interest. Temporal changes in BCS and BW did not differ between L and NL cows. L cows had higher body temperature than NL cows (38.4 > 38.2ºC). NL cows cycled earlier than L (26.3 < 34.7days postpartum; $P < 0.04$). Mean plasma concentrations of NEFA postpartum did not differ between NL and L (247.2 vs 207.5 mEq/L). Cows in L group had greater ($P < 0.01$) plasma concentrations of BHBA (4.90 > 2.97 mg/dL) and

BUN (11.64 > 6.5 mg/dL) and lower (P < 0.01) plasma glucose concentrations (74 < 79.9 mg/dL) than NL cows. Plasma progesterone concentrations from GnRH or TAI (d 0) until d 17 were lower for L cows than NL cows (P < 0.01). All conceptuses (n=13) expressed PAG2, PAG8, PAG11 and PAG12.The same PAG family genes were observed in the endometrium of some pregnant cows. Simple and standard partial correlation analyses detected associations of conceptus PAG11 with prostaglandin regulatory genes. Moreover, endometrium PAG11 was associated with endometrial prostaglandin regulatory genes, trophoblast cell specific genes, genes involved in invasion and implantation, and progesterone regulatory genes. In conclusion, lactation\diet altered metabolic status even though BW and BCS were the same between L and NL. Lactation delayed initiation of cyclicity and lowered concentrations of progesterone in pregnant cows during a programmed period following an induced ovulation. Moreover, early expressions of PAG genes within the endometrium of pregnant cows and in the conceptus with specific candidate genes may lead to the elucidation of the role (s) of PAG genes during early pregnancy.

Introduction

Adequate synchronization of embryonic development and remodelling of the endometrium are crucial to support fetal-placental development throughout gestation. Hence, early alterations of maternal or embryo environments may affect the embryo-uterine dialogue and lead to pregnancy failure. Establishment of the placenta involves implantation, when fetal derived placental cells (trophoblasts) fuse to the endometrium and deliver secretory products into the maternal system. Numerous molecules, including proteins, cytokines, hormones, and growth factors coordinate the fetal-maternal interface and systemically moderate maternal anatomy, endocrinology,

immunology and physiology to create an appropriate environment for fetal development and survival.

In ruminants, among the molecules recognized as being synthesized and secreted by trophoblast cells are the PAGs, also known as Pregnancy Specific Protein B (PSPB) (Butler et al., 1982) and Pregnancy Serum Protein of M_r 60 kDa (PSP60) (Mialon et al., 1994). In cattle, it has been identified 18 distinct PAG genes and 14 pseudogenes (Telugu et al., 2009). PAG genes are temporally expressed throughout pregnancy and spatially distributed into two distinct groups: those PAG genes expressed predominantly in trophoblast binucleated cells (i.e., modern PAG genes) and those expressed throughout the trophectoderm (i.e., ancient PAG genes) (Green et al., 2000). The measurement of PAGs in maternal blood is an alternative reproductive management tool that can be used for early pregnancy diagnosis in cattle and may be an indicator of embryo/fetal well being and pregnancy loss (Sasser et al., 1986; Patel et al., 1995, Kindahl et al, 2002; Green et al, 2005). However, the functional role (s) of these molecules is still unclear. The function (s) of PAG family genes may be combined with their spatial and temporal expression throughout pregnancy and may be involved in adhesion, implantation and remodeling of the fetal-maternal unit during placenta development (Wooding et al., 2005), immune regulation (Dosogne et al., 1999; Hoeben et al., 1999; Austin et al., 1999) and prostaglandin synthesis and regulation (Del Vecchio et al., 1990; Weems et al., 1998 and 2003).

Early embryo mortality accounts for major reproductive wastage in dairy farms. Up to 40% of total embryonic losses are estimated to occur between days 8 and 17 of pregnancy (Thatcher et al., 1994). An important event on d 16 to 17 after estrus is the

maintenance of the CL. This process is established by the ability of the conceptus to secrete sufficient amounts of IFN-τ, which regulates secretion of $PGF_{2\alpha}$ in the uterine endometrium (Thatcher et al., 2001). Changes involved in the process from a cyclic to a pregnant state not only depend on adequate production of antiluteolytic signals from the conceptus, but also the response of the endometrium to those signals. Inadequate communication between the conceptus-maternal unit leads to early embryonic mortality. To reduce these early losses and improve reproductive efficiency of cattle, a precise understanding of the dialogue between fetal-maternal-placental unit in the establishment and maintenance of pregnancy is needed. However, little is known about the complex molecular regulation of embryo development and endometrial remodeling during early gestation in cattle. Thus, elucidating the mechanisms that control embryo and endometrial development in early gestation is important for identification of genes regulating implantation, placentogenesis and maintenance of pregnancy. The development of microarray analysis has provided an opportunity to study early gestation expression of several thousand genes simultaneously.

In the past few decades, the shift toward more productive cows has been associated with a decline in fertility. Despite progress in synchronizing estrus and artificial insemination, the reproductive performance of dairy cows has not improved substantially (López-Gatius, 2003). Although pregnancy per AI in large commercial dairy herds is only 35-40% for mature lactating cows, dairy heifers have pregnancy per AIs above 65% (Butler, 2005). Sartori et al. (2004) compared fertilization and early embryo development between lactating and nonlactating dairy cattle, and observed a reduction in fertilization rates and poorer embryonic development in lactating dairy cows.

Moreover, studies comparing ovarian structures and circulating steroids of heifers, nonlactating and lactating cows showed that in general lactating dairy cows ovulated larger follicles and formed larger CL despite having lower serum steroid concentrations. This inverse relationship is probably due to greater steroid metabolism in lactating cows. We hypothesized that lactation would cause a differential expression of endometrial and conceptus expression of genes at day 17 after AI in both cyclic and pregnant cows. Therefore objectives of this study were to: 1) characterize postpartum metabolic and hormonal differences between nonlactating and lactating dairy cows, 2) evaluate lactation and pregnancy effects on endometrium and conceptus gene expression, and 3) characterize associations between conceptus and endometrial expression of genes in early pregnancy (d 17).

Material and Methods

Study Population

The study was conducted from August 2007 to April 2008 at the University of Florida Dairy Unit. A total of 39 pregnant dairy heifers purchased in New York were enrolled in the study. Heifers were evaluated for pregnancy and vaccinated at the time of arrival. All first calf heifers were housed in sod-based pens at around 3 weeks before expected calving and fed a close up ration of 1.51Mcal net energy of lactation (NE_L)/kg, 15.2% crude protein (CP), and a cation-anion difference (DCAD) of -4.5mEq/100g. After calving cows were randomly assigned into two treatment groups (Nonlactating [NL] and Lactating [L]) and moved to a free-stall facility. NL heifers were dried off immediately (i.e., not milked) after calving. The L cows were milked twice daily. Samples for measurement of milk protein and fat were collected once a month and analyzed by Southeast Milk laboratory (Belleview, FL) using a Bentley 2000 NIR analyzer. NL cows

were fed a far off dry cow ration consisting of 80% sorghum silage, 8.8% citrus pulp, 5% cottonseed meal, 5% molasses and 1.2% mineral/vitamin premix (1.45 Mcal NE_L/kg, 12.2% CP) once per day to meet maintenance requirements. L cows were fed a total mixed ration (TMR) consisting of 38% corn silage, 18% corn meal, 8% alfalfa hay, 6.9% corn gluten feed, 5.4% wet brewer grains, 5.2% soybean meal, 4.5% soyplus, 3.7% molasses, 3.3% citrus pulp, 3.3% whole cottonseed and 3.7% of a mineral/vitamin premix (1.65Mcal NE_L/kg and 16.5% CP) with ad libitum access to feed and water. Complete dietary and chemical composition of the diets is depicted in Table 3-1.Of the 39 pregnant heifers enrolled, six were excluded from the experiment because of death or failed to complete the study due to occurrence of reproductive disorders. Thus, a total of 33 cows completed the study.

Study Design

Lactating (n=17) and nonlactating (n=16) groups of cows were managed in separate pens. All cows were placed on a Presynch/CIDRSynch protocol. However, only 10 cows in the L and 12 in the NL were TAI and, the non inseminated cows were considered as cyclic (L, n=7; NL, n=4). Presynch was initiated with an i.m. injection of GnRH (100µg ; Cystorelin, Merial Ltd., Duluth, GA) at 44 ± 3 d after parturition, followed 7 days later by a injection of $PGF_{2\alpha}$ (25 mg ; Lutalyse, Pfizer Animal Health, New York, NY). At 12 days after $PGF_{2\alpha}$, CIDRSynch was initiated by administering 100µg of GnRH and inserting a intravaginal progesterone insert (CIDR, Pfizer Animal Health, New York, NY) for 5 days. Cows received an injection of $PGF_{2\alpha.}$ (25 mg) at the time of CIDR removal and another $PGF_{2\alpha.}$ (25 mg) injection 12 h later. GnRH (100µg) was injected 72 hours after the first $PGF_{2\alpha}$, and cows were TAI twice (i.e., a.m. and p.m.). On day 17

after GnRH/TAI, inseminated and noninseminated (i.e., cyclic) cows were slaughtered (Figure 3-1).

Rectal Temperature, Body Weight and Body Condition Score

Rectal temperatures were collected thrice weekly using a digital thermometer. Body weight measurements were performed weekly and body condition score was measured every 14 days.

Ovarian Ultrasonography

Ovarian ultrasound (linear array 7.5 MHz transrectal transducer, Aloka SSD-500, Aloka Co. Ltd, Wallingford, CT, USA) examinations were performed weekly starting at 14 ± 3 days after parturition until the day before slaughter. Presence, number and size of CL and follicles were recorded.

Blood Metabolites

Blood samples used for measurement of blood metabolites and hormones were collected from all cows from both treatments thrice weekly from the day of parturition until the day of CIDR insertion. The samples were obtained from the coccygeal vessels into evacuated tubes containing EDTA (10 mL Vacutainer®; BD, Franklin Lakes, NJ, USA) and placed on ice immediately after collection. Samples were centrifuged for 30 min, and plasma was stored at -20 ^{o}C. Plasma concentrations of non esterified fatty acids (NEFA) (NEFA-HR; Wako Chemical USA, Inc.) and β-hydroxy butyric acid (BHBA) (Autokit 3-HB; Wako Chemical USA, Inc.) were determined by using an enzymatic colorimetric method/assay. A Technicon Autoanalyzer (Technicon Instruments Corp., Chauncey, NY) was used to determine concentrations of blood urea nitrogen (BUN) (a modification of Coulombe and Favreau, 1963 and Marsh et al., 1965) and plasma glucose (a modification of Gochman and Schmitz, 1972).

Plasma Progesterone Concentrations

Blood samples used for measurement of progesterone concentrations were collected from all cows from both treatments thrice weekly from the day of GnRH/TAI until the day of slaughter. The samples were obtained from the coccygeal vessels into evacuated tubes containing EDTA (10 mL Vacutainer®; BD, Franklin Lakes, NJ, USA) and placed on ice immediately after collection. Samples were centrifuged for 30 min, and plasma was stored at -20 ^{o}C. Plasma concentrations of progesterone were determined by a solid-phase, no extraction radioimmunoassay (Coat-A-Count Progesterone, DPC® Diagnostic Products Corporation, Los Angeles, CA, USA). The standard curve dilution consisted of duplicated plain tubes used for total counts and non-specific binding. A 100µL of increasing concentrations of calibrators (0.1, 0.25, 0.5, 1, 2, 5, 10 and 20 ng/mL) were added to the tubes. The sensitivity of the assay was 0.1 ng/mL. The intra-assay coefficients of variation for d 4 (1.25 ng/mL ± 0.04) and d 8 (3.65 ng/mL ± 0.16) of cycle were 10% and 12.7% respectively.

Tissue Collection

All cows were slaughtered in the abattoir of the Department of Animal Sciences at the University of Florida. Reproductive tracts were collected within 10 minutes of slaughter. The uterine horn ipsilateral to the CL was cut along the anti-mesometrial border and the entire uterine horn was exposed. If the cow was inseminated then the uterine horn was examined for presence of a conceptus. If present, the conceptus was collected, measured, weighed and frozen in liquid nitrogen for subsequent RNA analyses. Intercaruncular tissue samples for *in situ* hybridization were collected from the ipsilateral horn. Tissue was washed twice in Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen Corporation, Carlsbad, CA, USA) and placed in formaldehyde

solution. Subsequently, tissue was stored at 4^{o}C for 4 to 6 h, then rinsed twice in DPBS and placed in 5 to 10 mL (i.e., depending on tissue size) 10% sucrose solution at 4^{o}C. After 24 to 26 hours, sucrose saturated tissues were rinsed briefly in DPBS, embedded in Optimal Cutting Temperature Compound (Tissue-Tek O.C.T. compound, Sakura Finetek Europe B.V., The Netherlands) and frozen. Caruncular and intercaruncular tissues were collected separately for RNA analyses. Tissue sections were cut (i.e, endometrium through to the serosa) at different locations along the ipsilateral horn adjacent to the location of the conceptus. Same procedure was followed for cyclic cows that were sampled in the same relative areas. Endometrial tissue was dissected from the myometrium and different sections of caruncular and intercaruncular tissues were frozen in liquid nitrogen. Ovarian and oviduct tissues were also collected and frozen for subsequent RNA analyses. Each ovary (i.e. CL and non CL bearing ovaries) was dissected from the uterus and weighed. CL were removed from the ovary, weighed and frozen in liquid nitrogen. Contralateral and ipsilateral oviducts were removed and isthmus and ampulla were dissected, weighed and frozen in liquid nitrogen.

RNA Extraction

RNA from conceptus and intercaruncular endometrial tissues was extracted using TRIzol® reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to instructions provided by the manufacturer. Samples were purified (PureLink® Micro-to-Midi kit; Invitrogen Corporation, Carlsbad, CA, USA) and RNA concentrations and purity were determined (Agilent 2100 Bioanalyzer, Agilent Technologies, Inc., Santa Clara, CA, USA). All samples were further processed for amplification and labeling and had a RNA integrity number > 7.5, which is related to the ratio of 18S and 28S ribosomal subunits. Samples were placed in aliquots and stored at -80^{o}C. Amplification and biotin labeling

were performed with an initial 200 ng of RNA by using the MessageAmp™ III (Applied Biosystems, Inc., Foster City, CA, USA) according to manufacturer's guidelines. Samples were then tested in the bioanalyzer for quality determination and subsequently submitted for fragmentation and hybridization in the bovine microarray. (Affymetrix® GeneChip Bovine Genome Array, Affymetrix®, Inc., Santa Clara, CA, USA).

Conceptus and Endometrial Gene Expression

The Bovine Affymetrix® GeneChip (Affymetrix®, Inc., Santa Clara, CA, USA), used for conceptus and endometrial gene expression, contains approximately 24,000 probe sets that represent over 23,000 bovine transcripts and includes approximately 19,000 UniGene clusters. The Affymetrix GeneChip uses 25-mer oligonucleotides to measure the abundance of mRNA transcripts and each probe set contains 11 probe pair replicates. Each probe is designed to hybridize to a specific sequence, and there is a mismatch probe that has one mismatch nucleotide, which allows for accurate quantitation and subtraction of background fluorescence and hybridization of non specific sequences. The specific probe and its mismatch constitute a probe pair and the probe set on the chip is comprised of 11 probe pairs for that bovine transcript. Hybridization controls are used to assess both, chip quality and/or hybridization efficiency and are also used to standardize signal intensity for the chip. The hybridization controls used in the genechip are bioB, bioC, bioD (E.coli) e cre (P1 B. subtilis). The housekeeping or control genes include actin, GAPDH, efla, 5.8S rRNA, 12S rRNA, 18S rRNA, cyclophilin B, GSH transferase, lactophorin, and translation initiation factor elF-4E.

Genes of Interest

Correlations between PAG family gene members, which are expressed exclusively by trophoblast cells, and a set of early pregnancy conceptus and endometrium expressed genes were evaluated. Genes of interest included genes expressed exclusively by trophoblast cells (i.e., interferon tau and trophoblast kunitz domain proteins) and genes expressed in both pregnant and cyclic animals, such as prostaglandin regulatory genes (i.e., prostaglandin synthases and prostaglandin receptor genes), genes involved in invasion and implantation (i.e. dickkopf homolog and pentraxin-related gene), immune system related genes (i.e., serpins, interferons, interleukins, tumor necrosis factors), growth factors genes (i.e., fibroblast growth factors, transforming growth factors), cholesterol and steroid metabolism genes (i.e., low density lipoprotein genes, cytochrome P450 genes) and progesterone regulatory genes (i.e., progesterone receptor genes, progesterone immnunodulatory binding factor) (Table 3-2).

Statistical Analysis

Temperature, weight, body condition score, metabolic and progesterone responses between treatments were analyzed using repeated measures analyses of the mixed model procedure (PROC MIXED) of SAS (SAS/STAT, ver. 9.1, SAS Institute Inc). This procedure applies methods based on the mixed model with special parametric structure on the covariance matrices. The data were tested to identify the covariance structure that provided the best fit for the data (Akaike's information criterion). Covariance structures tested included compound symmetry, autoregressive order 1, and unstructured. The covariance structure used was autoregressive order 1. The model included the effects of treatment, day of measurement, interaction of treatment ×

day of measurement, with cow nested within treatment as the random effect. Survival analysis using Cox Proportional Hazards Regression (PROC PHREG) of SAS was performed to evaluate cyclicity of the two treatment groups. Homogeneity of Regression analyses for progesterone concentrations of nonlactating and lactating cyclic and pregnant cows were conducted using the PROC GLM procedure of SAS. Regression analyses were performed to determine the best-fit
curves among treatments in relation to day of measurement. Linear, quadratic and cubic curves were tested. All variables were analyzed with the main effects of treatment, day of measurement, cow within treatment and the interaction of treatment × day of measurement. Cow nested within treatment was considered to be random.

Gene expression statistical tests were performed primarily using Bioconductor statistical software (http://www.bioconductor.org/), which is an open source and development software packet for analysis of microarray through put data based primarily on the R programming language (Gentleman et al. 2004). All array data were first preprocessed and normalized using the Affy package (Bolstad et al. 2003). Raw data was normalized by the Robust Multichip Analysis (RMA) approach (Bolstad et al. 2003),then the probeset's detection call was estimated by using the Wilcoxon signed rank-based algorithm. Probesets that were absent in all the study samples were removed from further analyses. For each gene represented on the microarray, a two-way ANOVA was conducted to identify genes that were regulated differentially for lactation (NL and L), reproductive status (cyclic and pregnant) and the interaction of lactation and reproductive status. A linear modeling approach and the empirical Bayes statistics as implemented in the Limma package (Smyth 2004) of the R software were

employed for differential expression analysis. Differentially expressed genes were than ranked by p-values, and genes with p-value less than 0.01 were considered as differentially expressed at a statistically significant level. Hierarchical clustering of the data was computed on log-transformed and normalized data using complete linkage and Pearson correlation distances. Computations and visualization were done using R packages. In order to understand more about cellular mechanisms among the physiological states, functional enrichment analyses (i.e., application of pathway and Gene Ontology programs) for selected statistical genes also were performed. The biological significance of all the annotated significant genes was determined using the web tool DAVID (http://david.abcc.ncifcrf.gov/home.jsp) and Pathway Studio software from Ariadne Genomics Inc., (http://www.ariadnegenomics.com). The Fisher's exact probability test was used to identify those statistically over-represented gene ontology (GO) terms or pathways. Conceptus and endometrium expression of PAG genes were associated with other expressed genes by correlation analyses. PROC CORR of SAS was used to determine simple Pearson correlation coefficients. Standard partial correlation analyses were performed by multivariate analysis of variance (MANOVA) using PROC GLM of SAS. The MANOVA analyses was used to analyze the relationship of more than one dependent variable at a time and allowed for testing the effect of one independent variable on more than one dependent variable. The MANOVA analysis detects differences in correlations among dependent variables when holding an independent variable constant.

Results

Breeding and Fertility

Distribution of experimental animals is depicted in Table 3-3. The experiment had a total of 33 cows. Six out of the 12 (i.e., 50%) cows inseminated in the NL and 8 out of 10 (i.e., 80%) in the L group had a conceptus recovered at day 17 after AI. Moreover, NL and L groups had a total of 4 and 7 noninseminated (i.e., cyclic) cows respectively.

Rectal Temperature, Body Weight, Body Condition Score and Milk Production

Temporal changes in body weight and body condition score did not differ between L and NL. L cows had higher mean rectal temperatures than NL cows (38.4 ± 0.04 > 38.2 ± 0.04^{o}C; P < 0.01) (Figure 3-2). Body weight averaged 509.7 ± 13.2 kg for cows in the L group and 516.6 ± 13.6 kg for cows in the NL group (P < 0.79) (Figure 3-3). Cows in the L and NL had a mean body condition scores of 3.08 ± 0.07 and 3.18± 0.07 respectively (P < 0.35) (Figure 3-4).

L cows had an average milk production of 24.56 ± 0.84 kg (Figure 3-5). Average milk production of L cyclic cows ranged from 18.9 ± 0.40 to 32.0 ± 0.43kg. Overall average milk production of L cyclic cows was 25.0 ± 0.23kg. Cows in the L pregnant group had average milk production ranging from 17.2 ± 0.37 to 27.1 ± 052 kg. Lactating pregnant cows overall milk production was 23.7 ± 0.20 kg. In addition, average milk fat and protein percentages were 3.44 ± 0.24 % and 2.98 ± 0.04 %, respectively.

Ovarian Ultrasonography

Survival analysis of the occurrence of first CL formation in both L and NL is depicted in Figure 3-6. Cows in the NL cycled earlier than cows in the L (26.4 ± 2.01< 34.7 ± 3.09 days postpartum; P < 0.04; hazard ratio = 0.464). Moreover, 22.2% (n=

4/18) cows of the L group did not cycle by day 55 after parturition. Daily rate of resumption of cyclicity was 53.6% lower for L than NL cows.

Blood Metabolites

Mean plasma concentrations of NEFA did not differ between treatments ($P < 0.25$). Mean NEFA concentrations for the L and NL groups were 207.5 ± 23.91 mEQ/L and 247.2 ± 24.75 mEQ/L (Figure 3-7). Cows in the L had greater mean plasma concentrations of BHBA (4.90 ± 0.23 > 2.97 ± 0.23 mg/dL; $P < 0.01$, Figure 3-8) and BUN (11.64 ± 0.32 > 6.5 ± 0.33 mg/dL; $P < 0.01$; Figure 3-9) throughout the postpartum period than NL cows. Concentrations of glucose in plasma were lower significantly for L compared to NL cows (74.0 ± 1.12 < 79.9 ± 1.17 mg/dL; $P < 0.01$; Figure 3-10) throughout the postpartum period.

Progesterone

Results of regression analyses conducted to examine homogeneity of regression for plasma progesterone curves from day 0 (GnRH injection or TAI) until day 17 (day of slaughter) indicated a lactation effect ($P < 0.01$). Plasma progesterone concentrations of cows in the L group only rose to approximately 5.5 ng/mL by day 17, while plasma progesterone concentrations of cows in the NL group reached around 7 ng/mL by the day of slaughter. Moreover, the increase in plasma progesterone concentrations was less for L pregnant cows compared to L cyclic cows ($P < 0.01$). In contrast there was no difference in the progesterone curves between cyclic and pregnant cows of the NL group (Figure 3-11). An additional analysis was undertaken to examine the linear increases in plasma progesterone from day 0 to day 8 after GnRH to induce ovulation. Results indicated a lactation effect ($P < 0.01$), a pregnancy effect ($P < 0.01$) and an interaction effect ($P < 0.01$) on the linear increase in plasma progesterone

concentrations (0.44 ± 0.04ng/mL/day; Figure 3-12). The rate of rise in plasma progesterone concentrations per day for NL cyclic and pregnant cows from days 0 to 8 were 0.48 ± 0.05 ng/mL and 0.52 ± 0.04 ng/mL. In contrast, L cyclic and pregnant cows had a daily plasma progesterone rise of 0.45 ± 0.04 ng/mL and 0.30 ± 0.03 ng/mL from days 0 to 8. The L pregnant cows had a lower rate of plasma progesterone rise than L cyclic cows ($P<0.01$). Mean plasma progesterone from GnRH injection or TAI (d 0) until d 17 was lower for L cows compared with NL cows (3.04 ± 0.10 ng/mL < 3.67 ± 0.12 ng/mL ; $P < 0.002$). Plasma progesterone concentrations of cyclic and pregnant cows as a main effect did not differ. Cyclic and pregnant cows of the NL and L groups had mean plasma progesterone concentrations of 3.54 ± 0.12 ng/mL and 3.07 ± 0.11 ng/mL respectively.

Furthermore, microarray analyses showed a lactation by pregnancy interaction effect ($P < 0.04$) on the expression of endometrial CYP39A1 (cytochrome P450, family 39, subfamily A, polypeptide 1), a gene involved in cholesterol and steroid synthesis. The CYP39A1 participates in cholesterol catabolism and is involved in the conversion of cholesterol into bile acids. CYP39A1 oxysterol 7 α hydroxylase preferentially catalyzes the 7 α hydroxylation of 24 hydroxycholesterol for subsequent cholesterol clearance .Expression level of CYP39A1 was highest in the L pregnant group (6,680 ± 388.6) compared with cows in the L cyclic (4,836 ± 644.8) ,NL cyclic (5,780 ± 734.0) and NL pregnant (5,290 ± 220.7) groups (lactation by pregnancy interaction; $P < 0.04$).

Slaughter Data

Mean CL weights were greater ($P < 0.10$) for L than NL cows (6.39 ± 0.37 g > 5.42 ± 0.44 g, respectively). In contrast mean CL weights did not differ ($P < 0.27$) between cyclic and pregnant cows as a main effect (5.65 ± 0.41 g and 6.26 ± 0.41 g,

respectively) and the interaction between lactation and pregnancy status was not significant($P < 0.72$) (Table 3-4). Conceptus length ($P < 0.57$) and weights ($P < 0.24$) did not differ between pregnant cows of the NL and L groups (Table 3-4).

Pregnancy Associated Glycoproteins

All conceptuses (n=13) expressed the PAG genes 2, 8, 11 and 12 (Table 3-5). The gene PAG11 was the PAG family gene member most abundantly expressed by d 17 conceptuses (i.e., expression level = 53,831 ± 2,620). Additional PAG family genes were expressed by some of the conceptuses: PAG 1 (n=1), PAG 7 (n=3), PAG 9 (n=1), PAG 10 (n=1), PAG 17 (n=2), PAG 18 (n=1), PAG 20 (n=1) and PAG 21 (n=5). Table 3-6 shows the conceptus and endometrial expression levels of genes interrelated at d 17 of pregnancy. Simple correlation (**r**) analyses detected associations of PAG 11 with the following genes expressed in conceptuses: PGH2 synthase (prostaglandin H 2 synthase [Cox-2]; $r = 0.87$; $P < 0.01$), PTGES (prostaglandin E synthase; $r = 0.76$; $P < 0.01$), PTGES3 (prostaglandin E synthase 3 [cytosol]; $r = 0.69$; $P < 0.02$) and $PGF_{2\alpha}R$ (prostaglandin $F_{2\alpha}$ receptor; $r = -0.73$; $P < 0.01$). Standard partial correlation (pr) analyses holding PGH2 synthase as a constant showed a decrease in the correlations of PAG 11 with PTGES ($pr = 0.50$; $P < 0.09$), PTGES3 ($pr = 0.17$; $P < 0.59$) and $PGF_{2\alpha}R$ ($pr = -0.47$; $P < 0.11$). Moreover, PAG11 and PGH2 synthase were still highly correlated when standard partial correlation analyses were run holding PGES ($pr = 0.76$; $P < 0.01$), PGES3 ($pr = 0.74$; $P < 0.01$) or $PGF_{2\alpha}R$ ($pr = 0.78$; $P < 0.01$) constant (Figure 3-13). Prostaglandin related expression of genes in the conceptus was not affected by lactation.

PAG genes, which are expressed exclusively by trophoblast cells (i.e., ancient PAGs), were observed in the endometrium of pregnant cows at d 17 of pregnancy.

Among the PAG genes expressed in the endometrial tissue were PAG 2 (n=4), PAG 8 (n=1), PAG 11 (n=7) and PAG 12 (n=3) (Table 3-5). PAG 11 was expressed in a greater number of cows and at a higher level than the other PAG genes observed in the endometrial tissue (i.e., expression level = 84.15 ± 39). Simple correlation analyses (r) detected associations of PAG 11 with expressed genes of trophoblast cells such as TKDP1 (trophoblast Kunitz domain protein 1; r = 0.86; P < 0.01) and TP1 (trophoblast protein 1; r = 0.67; P < 0.01). PAG 11 also was correlated with genes expressed in the endometrium of both cyclic and pregnant cows, such as genes involved in conceptus invasion and implantation, PTX3 (pentraxin-related gene; r = 0.50; P < 0.01) and DKK1 (dickkopf homolog 1; r = 0.43; P < 0.02); prostaglandin regulatory genes such as HPGD (hydroxyprostaglandin dehydrogenanse 15; r = 0.56; P < 0.01), and a gene related to progesterone regulation, PGRMC2 (progesterone receptor membrane component 2; r = 0.35; P < 0.07). A series of pr analyses revealed a direct correlation of PAG11 with TKDP1 (pr =0.72; P< 0.01) when holding TP1 constant (Figure 3-14). Moreover, when standard partial correlations were run holding PAG 11 as a constant, TKDP1 was correlated with TP1 (pr = 0.52; P < 0.01). However, no correlation was observed between PAG11 and TP1 when TKDP1 was hold as a constant (pr = 0.008; P < 0.96). In addition, associations between TP1 and PTX3 were observed when TKDP1 was held constant (pr = 0.52; P < 0.01). Moreover, having TP1 as a constant, showed that PTX3 was highly correlated with DKK1 (pr = 0.61; P < 0.01). A negative association between PTX3 and PTGDS (prostaglandin D2 synthase; pr = -0.60; P < 0.01) was observed when PIBF1 (progesterone induced blocking factor 1) was held constant. Furthermore, PIBF1 was negatively correlated with PTGDS holding PGRMC2 constant (pr = -0.70; P

< 0.01). PIBF1 also had a positive correlation with PGRMC2 (pr = 0.74; P < 0.01) when PTGDS was held constant. In addition, results of standard partial correlations showed that HPGD was correlated negatively with PTGDS (pr = -0.42; P < 0.04) and positively correlated with PGRMC2 (pr = 0.50; P < 0.01) (Figure 3-14).

Endometrial expression of HPGD and PGRMC2 was not affected by pregnancy or lactation. Pregnant cows had lower expression of PTGDS compared with cyclic cows. Moreover, pregnancy enhanced the expression of PIBF1, DKK1 and PTX3. In addition, DKK1 expression was inhibited by lactation. Presence of genes expressed exclusively in trophoblast cells of the endometrium, such as TP1, TKDP1 and PAG11, were observed only in pregnant cows and their expression did not differ between L and NL cows.

Discussion

Various metabolomic responses such as plasma concentrations of NEFA, BHBA, BUN and glucose are indicative that L cows undergo marked metabolic changes associated with homeoretic processes in response to lactogenesis and galactopoeisis. The alterations in metabolites reflect mobilization of lipids and proteins during a period of negative energy balance (NEB) postpartum. The period of NEB begins about one week before calving and undergoes a further decline over the next 2-3 weeks, reaching its nadir about 2 weeks postpartum (Butler and Smith, 1989; Bell, 1995). In the present study, metabolic changes related to lactational status were observed even though there was no differences in body weight and body condition score between L and NL cows. Lactational status did not affect overall postpartum NEFA concentrations (P < 0.25). NEFA released from lipid stores are taken up by the liver, where they may be oxidized to provide energy or partially oxidized to produce ketone bodies that are transported for

utilization elsewhere in the body. NEFA may also be esterified to triacylglycerols, which accumulate in the liver and compromise liver function (i.e., fatty liver). Beta-hydroxybutyrate (BHBA), the predominant form of ketone bodies in blood is an index of fatty acid oxidation. It has been documented that at least 50% of all dairy cows probably go through a temporary period of subclinical ketosis during the first weeks of lactation (Wathes et al., 2007). Moreover, blood urea concentrations are variable during the NEB period. Degradation of rumem degradable protein contributes to a rise in circulating ammonia concentrations, especially during NEB, which also affects urea production by the liver. This impaired liver function commonly associated with the postpartum period is involved in a reduction of the metabolic clearance of urea (Wathes et al., 2007). L cows had higher plasma concentrations of BHBA compared with NL cows, reflecting the higher energy demand associated with lactation. In addition, lactation impacted BUN plasma concentrations. L cows had higher BUN concentrations compared to NL cows, which may be explained by the different amounts of protein in the diets offered to the two treatment groups throughout the study period (Table 3-1). However, no health problems were detected that were related to altered metabolic status of the L cows. Furthermore L cows had lower plasma concentrations of glucose due to utilization by the mammary gland compared to NL cows. The availability of glucose for L cows results from the increase in hepatic gluconeogenesis and the decrease in oxidation of glucose by peripheral tissues to direct glucose to the mammary gland for lactose synthesis (Overton and Waldron, 2004). The high glucose utilization rate of the mammary gland led to the lower steady state concentrations of glucose in plasma.

The extended NEB experienced in early lactation has significant carry-over effects on reproductive efficiency. Postpartum ovulation is dependent on the re-establishment of pulsatile LH secretion and estradiol production by the dominant follicle. Low energy availability suppresses LH secretion and reduces ovarian responsiveness to LH stimulation, increasing the interval to the first ovulation (Butler, 2003). Fernandes et al. (1978) evaluated LH time responses to GnRH starting at 3 days postpartum in dairy cows. Results indicated that postpartum pituitary responsiveness to GnRH stimulation was not restored until 10 days or more after parturition. Moreover, reductions in concentrations of metabolic hormones like insulin and IGF-I also influence follicular development and peripartum ovulation. Cows that experience an early postpartum rise in IGF-1 concentrations in plasma have enhanced ovarian follicular development coupled with increased estradiol secretion (Beam and Butler, 1997 and 1999) and earlier occurrence of ovulations (Thatcher et al., 1996). Our results showed that lactation delayed resumption of postpartum ovulation. NL cows cycled earlier than L cows (26.4 ± 2.01< 34.7 ± 3.09 days). Sartori et al. (2004) described differences in ovarian dynamics and reproductive hormones during the estrous cycle between lactating cows and heifers. Maximal volume of luteal tissue was larger for cows than heifers; even though maximal serum progesterone concentration was lower for cows compared with heifers (5.6 ± 0.5 vs 7.3 ± 0.4 ng/mL). Another study documented greater progesterone concentrations in heifers compared with lactating cows, even though, lactating cows had greater amounts of luteal tissue (Sartori et al., 2002). In accordance with previous studies, L cows of the present study had lower plasma concentrations of progesterone and greater CL weight compared with NL cows.

Lactation, pregnancy and their interaction was detected in plasma progesterone concentrations from day 0 (GnRH/TAI) to day 8 during the period of early CL development after GnRH injection prior to slaughter. NL cows had a greater daily increase in plasma progesterone concentrations compared to L cows. Differences in sizes of ovarian structures and lower serum estradiol and progesterone concentrations in lactating cows versus heifers or lactating cows versus nonlactating cows may be related to a greater steroid metabolism observed in lactating dairy cows (Sangsritavong et al., 2002). Interestingly, in the present study, L pregnant cows had a lower increase in plasma progesterone concentrations compared with L cyclic cows. In contrast there was no difference in the progesterone curves between cyclic and pregnant cows of the NL group. Bilby et al. (2004) have documented lower plasma progesterone concentrations in nonlactating pregnant compared with nonlactating cyclic cows from d 0 to d 17 of a synchronized estrous cycle In the present study, microarray analyses detected a lactation by pregnancy interaction on day 17 endometrial expression of CYP39A1, a gene involved in metabolism of cholesterol and steroids. Pregnant L cows had higher expression of CYP39A1 compared with cyclic L cows and NL cyclic and pregnant cows. A preferential difference of greater CYP39A1 expression in pregnant L cows than cyclic L cows may contribute to the lower plasma progesterone concentrations observed in L pregnant cows. If greater cholesterol is cleared by the endometrium and liver in lactating dairy cows than less cholesterol would be available for uptake by the ovary for the synthesis of progesterone by the CL in lactating dairy cows.

The dialogue between trophoblast cells of the conceptus and epithelial cells of the

endometrium is critical to CL maintenance and embryo survival. Identification of genes involved in the control of early embryo and endometrial development is important to elucidate the molecular mechanisms that regulate recognition and maintenance of pregnancy. In the present study, an array of genes known to be important during early pregnancy was identified (Table 3-2). The following classes of genes were identified in both conceptuses and endometrial tissue that were deemed important for early embryo development and endometrial programming to sustain a pregnancy: prostaglandin regulatory genes (i.e., prostaglandin synthases and prostaglandin receptor genes), trophoblast cell specific genes (i.e., interferon tau and trophoblast kunitz domain proteins), genes involved in invasion and implantation (i.e. dickkopf homolog and pentraxin-related gene), immune system related genes (i.e., serpins, interferons, interleukins, tumor necrosis factors), growth factor genes (i.e., fibroblast growth factors, transforming growth factors), genes involved in cholesterol and steroid metabolism (i.e., low density lipoprotein genes, cytochrome P450, family 39, subfamily A, polypeptide 1) and progesterone regulatory genes (i.e., progesterone receptor genes, progesterone immnunodulatory binding factor). In the present study, prostaglandin related genes expressed in the conceptus were not affected by lactation. Moreover, no effect of pregnancy or lactation was observed in endometrial expression of HPGD and PGRMC2, However, lower endometrial expression of PTGDS was observed in pregnant cows. In contrast, pregnancy enhanced endometrial expression of PIBF1, DKK1 and PTX3. In addition, DKK1 expression was inhibited by lactation. Genes expressed exclusively by trophoblast cells, such as TP1, TKDP1 and PAG11, were present in the

endometrium of pregnant cows, and their expression did not differ between L and NL cows.

Trophoblast cells of the ruminant placenta are specialized and subdivided into two morphologically and functionally distinct cell types referred to as mononucleated and binucleated trophoblast cells. These cells deliver different secretory products directly into the maternal system. Maintenance of the CL to sustain pregnancy and embryo survival are established through the secretion of IFN-*τ* by trophectoderm cells (Thatcher et al., 2001). IFN-*τ* inhibits CL regression through the suppression of pulsatile release of $PGF_{2\alpha}$ in the uterine endometrium due to suppression in oxytocin receptors. In addition, IFN-*τ* affects synthesis of cytokines that contribute to the immune modulation of the conceptus-maternal interface. In the present study, we were able to associate conceptus IFN- *τ* expression with other trophectoderm expressed genes such as PAGs. PAG genes are expressed in trophoblast mononucleated and binucleated cells. Although several studies have documented the measurement of plasma PAG concentrations as an alternative early pregnancy diagnosis tool (Humblot et al., 1988; Zoli et al., 1992; Green et al, 2005; Silva et al., 2007), the functional role of these molecules are still to be determined. In the present study, expression of PAG genes was examined specifically at d 17 of pregnancy. Different PAG family genes were identified in both conceptuses and endometrial tissues at d 17 of pregnancy. To date it has been documented that PAG genes are expressed exclusively by the placenta. However, the present study detected expression of PAG genes in endometrial tissue at d 17 of pregnancy. All PAG genes observed in the endometrium were members of the ancient PAG group (i.e., expressed throughout the trophectoderm). Presence of PAG

genes and other genes expressed exclusively by trophoblast cells (i.e., TP1, TKDP1) in endometrial tissue at d 17 of pregnancy may reflect the presence of trophoblast cells in the endometrium tissue due to the intimate relationship between the trophectoderm and the endometrium at this time of pregnancy. Mononucleated trophoblast cells have apical surface membranes that are modified to form microvillar processes that interdigitate with maternal uterine epithelial cells forming the fetal-maternal contact zone (Igwebuike, 2006).

Based on studies that reported possible functional roles for PAG genes, a series of both simple and partial correlation analyses between PAG genes expressed by the conceptus and endometrial tissues at d 17 of pregnancy and various candidate genes of interest, including genes expressed exclusively by trophoblast cells and genes expressed in the endometrium of both pregnant and cyclic animals, was undertaken. Correlation analyses were conducted with the intent of identifying associations that might be important functionally for pregnancy and to provide a basis for future studies to identify the possible function (s) of PAG genes. In the present study, all conceptuses (n=13) expressed PAGs 2, 8, 11 and 12, which are all members of the ancient PAG group indicating that these genes are expressed by both trophectoderm mononucleated and binucleated cells. Moreover, other ancient and modern (i.e., expressed in binucleate cells) PAG family genes also were expressed by some of the conceptuses. Endometrial tissue of pregnant cows at day 17 expressed ancient PAG family genes 2, 8, 11 and 12. These are likely to be associated with trophectoderm cells and consistent with expression being detected only in endometrial tissue of pregnant cows. PAG11

was the PAG gene most highly expressed in both conceptus and endometrial tissues at d 17 of pregnancy, which focused our correlation analyses on this PAG family gene.

A proposed role in which PAG genes act in an immunodulatory capacity has been reported by different studies. Dosogne et al. (1999) investigated immuno-supression in periparturient cows by PAGs. From 3 weeks before until 5 weeks after parturition, the phagocytosis and oxidative burst activity of polymorphonuclear neutrophil (PMN) leukocytes isolated from blood and PAG concentrations in plasma were evaluated in dairy cows. A significant decrease in oxidative burst activity of PMN was observed between 1 and 3 weeks after calving. In addition, it was documented that a peak in plasma bPAG concentrations occurred immediately prior to the decline in PMN activity. Moreover, Hoeben et al. (1999) documented that high concentrations of bPAG around parturition significantly inhibited the proliferation of bone marrow progenitor cells cultured in vitro, suggesting a possible involvement of PAGs in postpartum immunosuppression. In addition, day 14 uterine endometrial explants from nonpregnant cows were cultured with PSPB, which induced release of the alpha chemokine granulocyte chemotactic protein-2 (GCP-2). Induction of GPC-2 was blocked by addition of antiserum against PSPB (Austin et al., 1999). Furthermore, different possible functions of PAG genes may be related to the diverse localization of the ancient and modern PAG groups. Ancient PAG genes, mainly expressed at the microvillar junctions of the fetal-maternal interface may be involved in binding together the fetal maternal surfaces or establishing an immunological barrier. In contrast, the expression of modern PAG genes (i.e., exclusively expressed in trophoblast binucleated cells) occurs mainly in the developing maternal villi of the placentomes which ideally positions them to

manipulate the maternal immune system (Wooding et al., 2005). In the present study, no correlations between conceptus expressed PAG11 and genes involved in immune control were observed at d 17 of pregnancy. However, simple correlation analyses showed that endometrial PAG11 was correlated with PTX3, a gene that is produced by various tissues in response to proinflammatory signals. In humans, both trophoblast conditioned medium and trophoblast explants increased PTX3 mRNA expression in endometrial stromal cells (Popovici et al., 2008). In addition, Tranguch et al. (2007) documented that PTX3 null mice had compromised implantation and decidualization processes. Moreover, endometrial PAG11 of the present study was correlated with DKK1, a gene that antagonizes Wnt/β-catenin signaling, by inhibition of the Wnt coreceptors Lrp5 and Lrp6. In addition, DKK1 has high affinity for the transmembrane proteins Kremen1 and Kremen2, which are also involved in the modulation of Wnt signaling. In addition, DKK1 is involved in early development of head structures anterior to the midbrain and it has been documented to function as a suppressor of tumor growth in colon cancer cells in humans (Niehrs, 2006; Aguilera et al., 2006). Furthermore, it has been documented that DKK1 promotes trophoblast cell invasion in mice (Peng et al. 2008). The associations between PAG11 and PTX3, and PAG11 and DKK1 in the endometrium identify these genes as possible candidates for future studies involving the role of PAGs during embryo development, trophoblast cell invasion and implantation. Furthermore, PTX3 was correlated positively with DKK1, suggesting that PTX3 might be acting in combination with DKK1 in the establishment of implantation. In addition, PTX3 was correlated negatively with prostaglandin D2 synthase gene (PTGDS) (Figure 3-14). It has been documented that prostaglandin D 2 is converted to

a biologically active $PGF_{2\alpha}$ stereoisomer (9α, 11β $PGF_{2\alpha}$) through the action of an enzyme called 11-ketoreductase (Theodore et al., 1985). The 9α, 11β $PGF_{2\alpha}$ has vasoconstrictive and smooth muscle contractile properties. The negative correlation between PTX3 and PTGDS might suggest an alternative way to attenuate $PGF_{2\alpha}$ like effects at this stage of pregnancy. Moreover, results of standard partial correlation analyses showed a negative association between PTGDS and HPGD. Hydroxyprostaglandin dehydrogenanse is known to be involved in the degradation of prostaglandins such that a sequential reduction in PTGDS would enhance HPGD to increase $PGF_{2\alpha}$ metabolism in early pregnancy. Parent et al. (2006) have documented HPGD expression in bovine endometrium during the estrus cycle and early pregnancy. Furthermore, Knickerbocker et al. (1986) demonstrated that estradiol induced both $PGF_{2\alpha}$ and 13- 14 dihydro 15-keto $PGF_{2\alpha}$ secretion from the uterus of cyclic cows. Endometrial expression of HPGD is possibly associated with the tight regulation of prostaglandin activity (i.e., production and degradation) in the endometrial tissue. The negative correlation between PTGDS and HPGD genes at d 17 of pregnancy might be a link between formation and destruction of $PGF_{2\alpha}$ in the endometrium. This tight regulation possibly allows an adequate functionality of reproductive events such as luteolysis, maintenance of the CL in pregnancy, implantation and parturition. In addition, HPGD was correlated positively with expression of the PGRMC2 gene (Figure 3-14), which encodes a protein that binds progesterone. Thus, this correlation indicates that endometrial progesterone binding might be associated with prostaglandins degradation in early pregnancy. Interestingly, simple correlation analyses showed that endometrial PAG 11 was positively correlated with HPGD and PGRMC2. These associations may

be important in the elucidation of functions for PAG genes. PAG genes might be involved in the prostaglandin and progesterone regulation of early pregnancy.

Standard partial correlations indicated a positive association between endometrial PGRMC2 and PIBF1. Following recognition of fetal derived antigens, maternal lymphocytes develop an increased sensitivity to progesterone by an activation-induced appearance of progesterone binding sites. Szkeres-Bartho et al. (2001) documented that PIBF1 inhibition of natural killer (NK) cell activity is demonstrated by an altered cytokine production both in vitro and in vivo. Moreover, PIBF1 inhibits arachidonic acid release by exerting a direct effect on inhibiting the phospholipase A2 enzyme. Thus through this mechanism, PIBF1 possibly attenuates prostaglandin synthesis and reduces IL-12 production, which decreases NK activity and favors normal pregnancy development. Furthermore, women that had a normal pregnancy outcome had greater PIBF-positive lymphocytes compared with women showing clinical signs of threatened preterm pregnancy termination (Szkeres-Bartho et al., 2001). Thus, the mechanisms above describe PIBF1 as an anti-abortive agent. The positive correlation between gene expressions of PGRMC2 and PIBF1 in the endometrium may implicate such cellular regulatory events are involved in normal pregnancy outcomes in the cow. In addition, PTGDS, which is antagonizes the effects of $PGF_{2\alpha}$, was negatively correlated with PIBF1 at d 17 of pregnancy (Figure 3-14). This negative correlation would actually enhance PIBF1 since PTGDS was decreased due to the negative association with the gene expression of PTX3. Thus the regulation of arachidonic acid availability and its metabolism to eicosonids and subsequent metabolism (i.e., PGF) by the network of coordinated expression of genes may be affecting maintenance of pregnancy.

Simple correlation analyses showed that endometrial PAG 11 was correlated highly with trophoblast expressed genes such as TKDP1 and TP1 (i.e., interferon tau). Moreover, standard partial correlations holding TP1 as constant resulted in a high association between TKDP1 and PAG 11 (Figure 3-14). TKDP1 belongs to a placenta specific multigene family restricted to ruminant ungulates. Even though TKDPs function is not yet established, most TKDPs are expressed during early pregnancy and may be important regarding the interactions of the conceptus-maternal unit (Maclean et al., 2003). Association of PAG11 with trophoblast cell specific genes at d 17 of pregnancy is important in elucidating the role that these molecules are playing in the ruminant placenta during early pregnancy.

Additionally, in an attempt to identify possible roles of PAGs, a series of in vitro studies were conducted to identify direct or indirect involvement of PAGs on luteotrophic regulation of CL function. Administration of PAG1/PSPB to cultured bovine luteal cells promoted the release of PGE_2 (Del Vecchio et al., 1995 and 1996; Weems et al., 1998a) and increased progesterone secretion (Del Vecchio et al., 1996; Weems et al., 1998b). In contrast, Del Vecchio et al., (1995) reported no increase in progesterone from luteal cells treated with PSPB. Administration of PSPB to bovine cultured endometrial explants of day 16 of the estrous cycle increased secretion of PGE_2 and $PGF_{2\alpha}$ (Del Vecchio et al., 1990). In another study, 90 day pregnant ewes were ovariectomized, and blood samples were collected at day 0 (laparotomy) at 12:00 a.m. and every 6 h through 6:00 a.m. of day 7 (i.e., 162 hours later) for analyses of progesterone, estradiol-17β, PGE_2 , $PGF_{2\alpha}$, and PSPB. Plasma PSPB and estradiol 17β concentrations were increased in ovariectomized ewes compared with intact ewes. PSBP increased

concentrations were preceded by an increase in estradiol. In vitro studies with placenta slices of the same ovariectomised ewes collected 7 days after laparotomy revealed an increase in progesterone, PGE_2, estradiol 17β and PSPB secretions compared with secretion by placenta of intact ewes. Conclusions were that estradiol may regulate PSPB secretion to stimulate placental PGE_2 secretion that in turn sustains placental secretion of progesterone in sheep (Weems et al., 1999). The dynamics of progesterone secretion during pregnancy in cattle is largely sustained by the CL not by the placenta.

Conceptus expression of PAG11 was correlated positively with PGH2 synthase, PTGES, and PTGES3, and negatively correlated with $PGF_{2\alpha}R$ genes of the conceptus (Figure 3-13). However, standard partial correlation analyses holding PGH2 synthase as a constant showed a decrease in the correlations between PAG11 and PTGES, PTGES3 and $PGF_{2\alpha}R$, suggesting that PAG11 may primarily affect upstream expression of PGH2 synthase e that than leads to downstream regulation of PGES, PGES3 and $PGF_{2\alpha}R$ expression. These correlations between PAG11 and genes involved in the prostaglandin cascade in early pregnancy within the conceptus may implement a role of PAG11 with mechanisms involved in maintenance of pregnancy, invasion of the endometrium, maintenance of the CL through the endometrium, immunoregulation within the endometrial environment and placentation.

Conclusion

In conclusion, lactation\diet altered metabolic status even though BW and BCS were the same between L and NL. Lactation delayed initiation of cyclicity and lowered concentrations of progesterone in pregnant L cows during a programmed period following an induced ovulation. Early expressions of PAG genes within the conceptus

and endometrium of pregnant cows and its association with other genes infer a possible role of PAGs in pregnancy maintenance and implantation by regulation of prostaglandin metabolism.

Table 3-1. Dietary ingredients and chemical composition of diets

	Treatments		
Item	Prepartum	Nonlactating	Lactating
Ingredient, (% of dietary DM)			
Corn silage	42.0	—	38.0
Sorghum silage	—	80.0	—
Oat silage	20.0	—	—
Corn meal	10.6	—	18.0
Alfafa hay	—	—	8.0
Corn gluten feed	—	—	6.9
Wet brewers grains	—	—	5.4
Soybean meal	13.0		5.2
Cottonseed meal	—	5.0	—
SoyPlus	—	—	4.5
Molasses	3.0	5.0	3.7
Citrus pulp	5.0	8.8	3.3
Whole cottonseed	—	—	3.3
Mineral/Vitamin premix	6.4[a]	1.2[b]	3.7[b]
Chemical composition, % DM	42.0		
DM	34.2	39.2	45.2
Crude protein	15.2	11.6	16.5
RDP	10.3	8.0	11.2
NDF	34.2	48.4	33.1
ADF	21.5	33.5	19.3
Crude fat	3.2	2.9	4.3
TDN	66.0	63	70
$NE_{L,}$ Mcal/kg	1.54	1.44	1.63
Ca	1.70	0.69	0.80
P	0.30	0.27	0.41
Mg	0.37	0.32	0.32
K	1.66	1.70	1.26
Na	0.17	0.20	0.36
S	0.32	0.22	0.25
Cl	1.03	0.68	0.35
DCAD, mEq/100g	-4.5	20	23

[a]Mineral and vitamin premix contained 22.8% CP, 22.9% Ca, 0.20% P, 0.2% K, 2.8% Mg, 0.7% Na, 2.4% S, 8% Cl, 147 mg/kg of Mn, 27 mg/kg of Fe, 112 mg/kg of Cu, 95 mg/kg of Zn, 7 mg/kg of Se, 8 mg/kg of I, 11 mg/kg of Co, 268,130 IU of vitamin A/kg, 40,000 IU of vitamin D/kg, and 1129 IU of vitamin E/kg (DM basis).
[b]Mineral and vitamin premix contained 26.4% CP, 10.2% Ca, 0.90% P, 3.1% Mg, 1.5 % S, 5.1% K, , 8.6 % Na, 1500 mg/kg of Zn, 512 mg/kg of Cu, 339 mg/kg of Fe, 2231 mg/kg of Mn, 31 mg/kg of Co, 26 mg/kg of I, 7.9 mg/kg of Se, 147,756 IU of vitamin A/kg, and 787 IU of vitamin E/kg (DM basis).

Table 3-2. Genes of interest expressed at d 17 of pregnancy

Gene symbol	Gene name
Endometrium	
DKK1	Dickkopf homolog 1[a]
HPGD	Hydroxyprostaglandin dehydrogenase
IGFBP	Insulin-like growth factor binding proteins[b]
ILR	Interleukin receptors[b]
IL	Interleukin genes[b]
LDLR	Low density lipoprotein receptor
LRP	Low density receptor-related proteins[b]
LHB	Luteinizing hormone beta polypeptide
OXTR	Oxytocin receptor[b]
PAG	Pregnancy associated glycoprotein[b]
PGR	Progesterone receptor
PGRMC2	Progesterone receptor membrane component[b]
PIBF1	Progesterone Immunomodulatory binding factor 1[b]
PTGDS	Prostaglandin D2 synthase[b]
PTGER	Prostaglandin E receptor[b]
PTGES	Prostaglandin E synthase[b]
$PTGF_{2\alpha}R$	Prostaglandin $_{2\alpha}$ F receptor
PGH2	Prostaglandin endoperoxidase synthase 2
PTX3	Pentraxin Related Gene[b]
TGIF	TGF beta induced factor[2]
TGFB	Transforming growth factors, beta[b]
TKDP1 (1,2,3,4,5)	Trophoblast Kunitz domain protein (1,2,3,4,5)[b]
TNFSF	Tumor necrosis factor (ligand) superfamily[b]
TNFRSF	Tumor necrosis factor receptor superfamily[b]
TP1	Trophoblast protein 1[b]
CYP39A1	Cytochrome P450, family 39, subfamily A, polypeptide 1[a]
Conceptus	
FGF	Fibroblast growth factors
FGFR	Fibroblast growth factor receptors
IL	Interleukin genes
IRF	Interferon regulatory factors
PAG	Pregnancy associated glycoprotein
PTGDS	Prostaglandin D2 synthase
PTGER	Prostaglandin E receptor
PTGES	Prostaglandin E synthase
$PTGF_{2\alpha}R$	Prostaglandin $_{2\alpha}$ F receptor
PGH2	Prostaglandin endoperoxidase synthase 2
TGFB	Transforming growth factors, beta
TKDP1 (1,2,3,4,5)	Trophoblast Kunitz domain protein (1,2,3,4,5)
TNFSF	Tumor necrosis factor (ligand) superfamily
TP1	Trophoblast protein 1

[a] Genes affected by lactation and pregnancy.
[b] Genes affected by pregnancy.

Table 3-3. Distribution of experimental animals

	Cyclic	Pregnant	Nonpregnant	Total Cows
Nonlactating (no.)	4	6	6	16
Lactating (no.)	7	8	2	17

Table 3-4. CL weight, conceptus length and conceptus weight from nonlactating and lactating cows during day 17 of estrus cycle and pregnancy

	Nonlactating		Lactating	
	Cyclic (4)[a]	Pregnant (6)	Cyclic (7)	Pregnant (8)
CL Weight (g) [b]	4.95 ± 0.66	5.81 ± 0.60	6.15 ± 0.56	6.60 ± 0.52
Conceptus length (cm)		20.0 ± 7.17		25.1 ± 5.07
Conceptus Weight (mg)		34.75 ± 16.04		60.0 ± 11.91

[a]Number of cows in parenthesis.
[b]Nonlactating vs Lactating: $P < 0.10$.

Table 3-5. Endometrial and conceptus PAG genes expression at d 17 of pregnancy

PAG Genes	Gene Expression Level		
	Conceptus (13) [a]	Endometrium Cyclic Cows	Endometrium Pregnant Cows
2	28,818.95 ± 1,435	4.22	52.78 ± 36 (4) [a]
8	10,352.84 ± 1,892	4.22	8.87 (1)
11	53,831.91 ± 2,620	4.23	84.15 ± 39 (7)
12	9,187.08 ± 1,170	4.22	11.73 ± 5.9 (3)

[a] Number of animals in parenthesis.

Table 3-6. Endometrial and conceptus expression levels of genes interrelated at d 17 of pregnancy

Genes	Expression Level				Lactation p-value	Pregnancy p-value	Lactation x Pregnancy p-value
	Lactating Cyclic	Lactating Pregnant	Nonlactating Cyclic	Nonlactating Pregnant			
Endometrium							
DKK1	1,291 ± 335.6	7,087 ± 701.3	2,998 ± 789.8	8,523 ± 641.7	< 0.01	< 0.001	0.06
HPGD	7,830 ± 741.2	8,313 ± 644.9	7,672 ± 990.9	8,328 ±506.6	0.91	0.43	0.90
PGRMC2	1,343 ± 66.6	1,320 ± 77.3	1,321 ± 98.2	1,192 ± 31.9	0.29	0.28	0.44
PIBF1	41.4 ± 4.4	65.4 ± 7.3	45.8 ± 7.5	48.1± 2.9	0.37	0.03	0.07
PTGDS	2,933 ± 156.7	1,672 ± 78.1	2,911 ± 368.9	1,848 ± 162.8	0.50	< 0.001	0.43
PTX3	67.7 ± 15.3	538.6 ± 83.0	61.8 ± 21.7	537.5 ± 106.7	0.82	< 0.001	0.83
TKDP1	4.3 ± 0.0	27.3 ± 197.6	4.3 ± 0.0	50.0 ± 180.9	0.69	< 0.01	0.68
TP1	4.2 ± 0.0	33.2 ± 432.8	4.2 ± 0.0	69.2 ± 111.6	0.62	< 0.01	0.62
Conceptus							
PGES	___	1,210 ± 420.5	___	1,409 ± 662.9	0.55	___	___
PGES3	___	8,716 ± 292.2	___	8,835 ± 420.6	0.84	___	___
$PTGF_{2\alpha}R$	___	22.3 ± 4.8	___	21.5 ± 7.6	0.78	___	___
PGH2	___	13,641 ± 379.4	___	12,285 ± 1,526.7	0.25	___	___

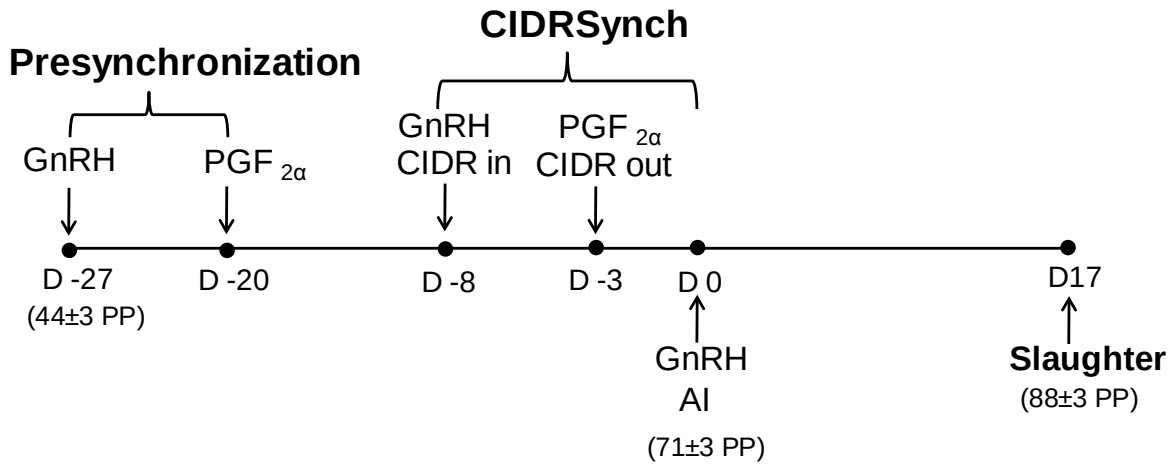

Figure 3-1. Experimental design; AI= artificial insemination; PP= days post partum; CIDR= controlled internal drug releasing containing progesterone; GnRH= injection of gonadotropin releasing hormone; $PGF_{2\alpha}$= prostaglandin $F_{2\alpha}$ injection; CIDRSynch= protocol for synchronization of ovulation.

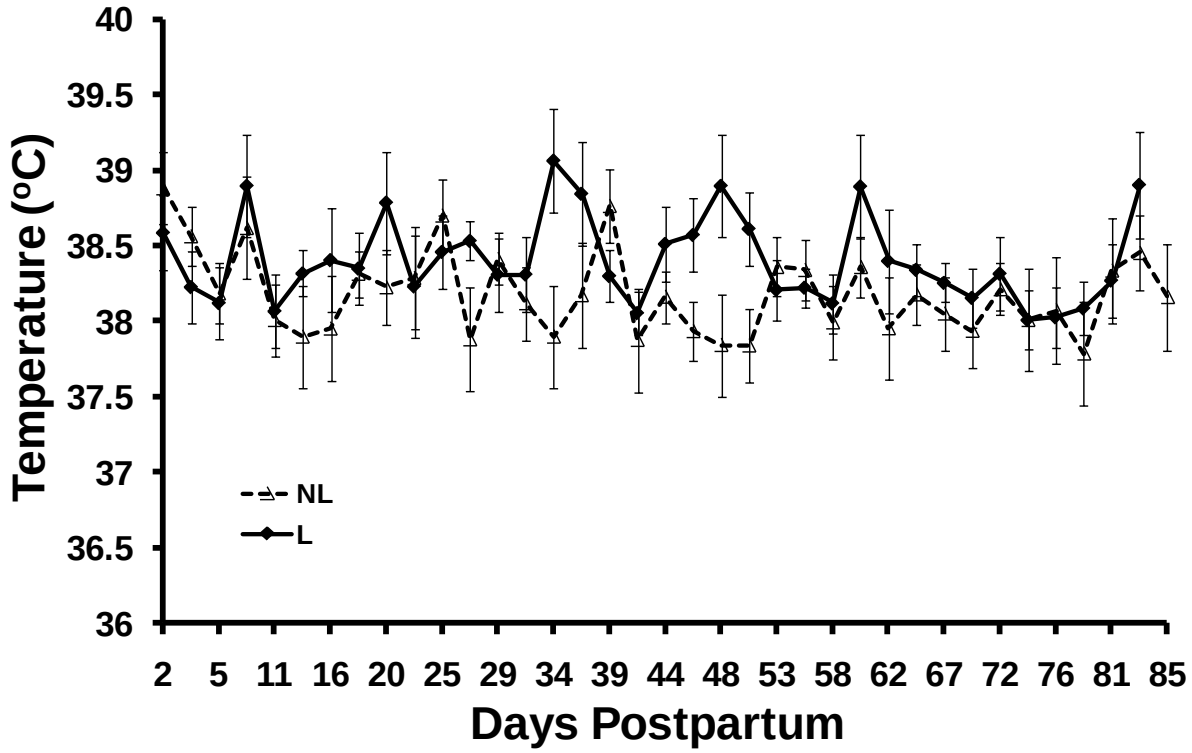

Figure 3-2. Rectal temperature (oC) of L and NL cows throughout the study period. The LSM ± S.E.M. for rectal temperatures for L and NL were 38.4 ± 0.04 oC and 38.2 ± 0.04 oC respectively.

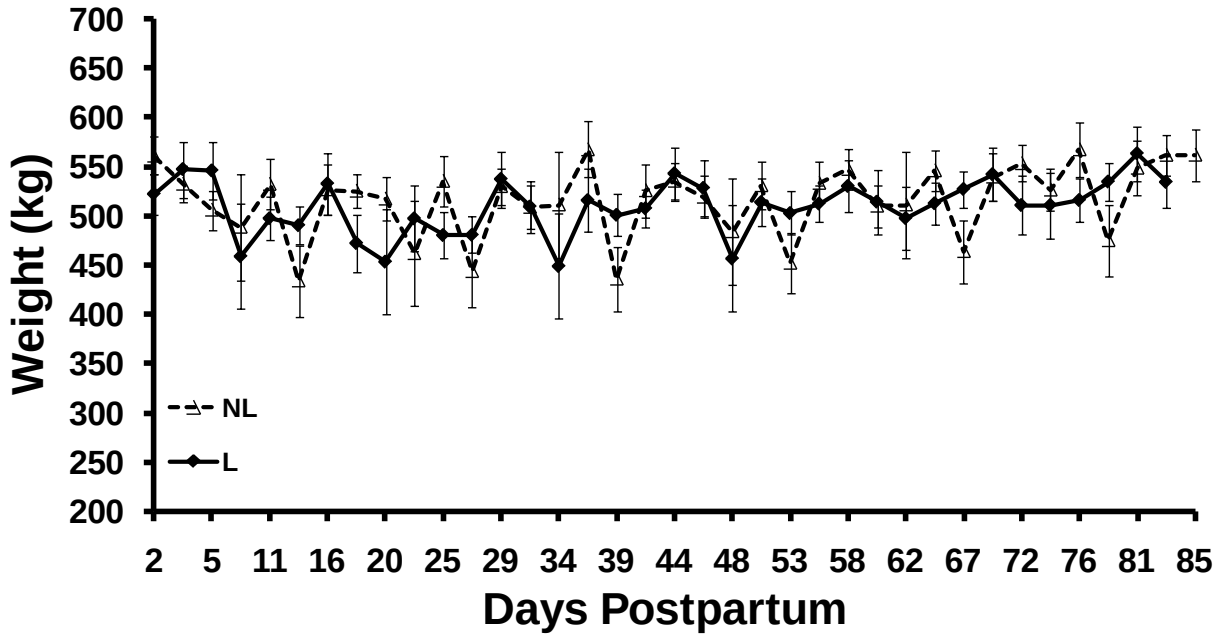

Figure 3-3. Body weight (kg) of L and NL cows throughout the study period. The LSM ± S.E.M. for body weight for L and NL were 509.7 ± 13.2 kg and 516.6 ± 13.6 kg respectively.

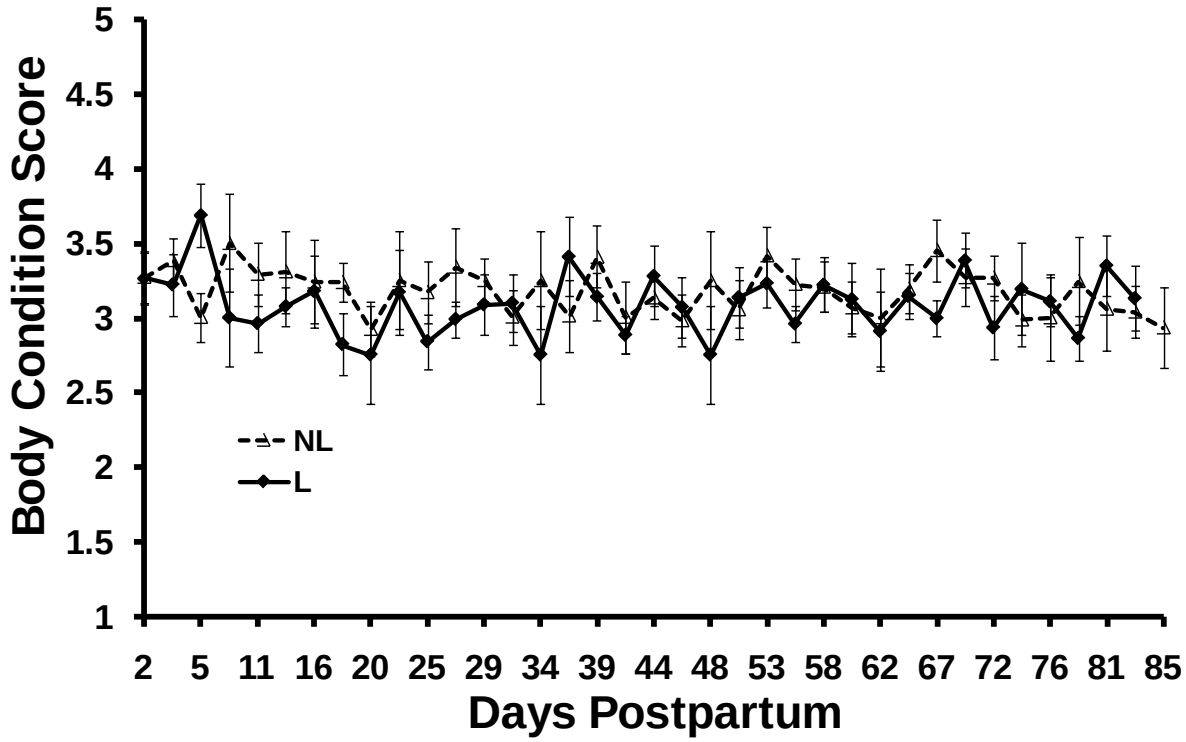

Figure 3-4. Body condition score of L and NL cows throughout the study period. The LSM ± S.E.M. for body condition score for L and NL were 3.08 ± 0.07 and 3.18± 0.07 respectively.

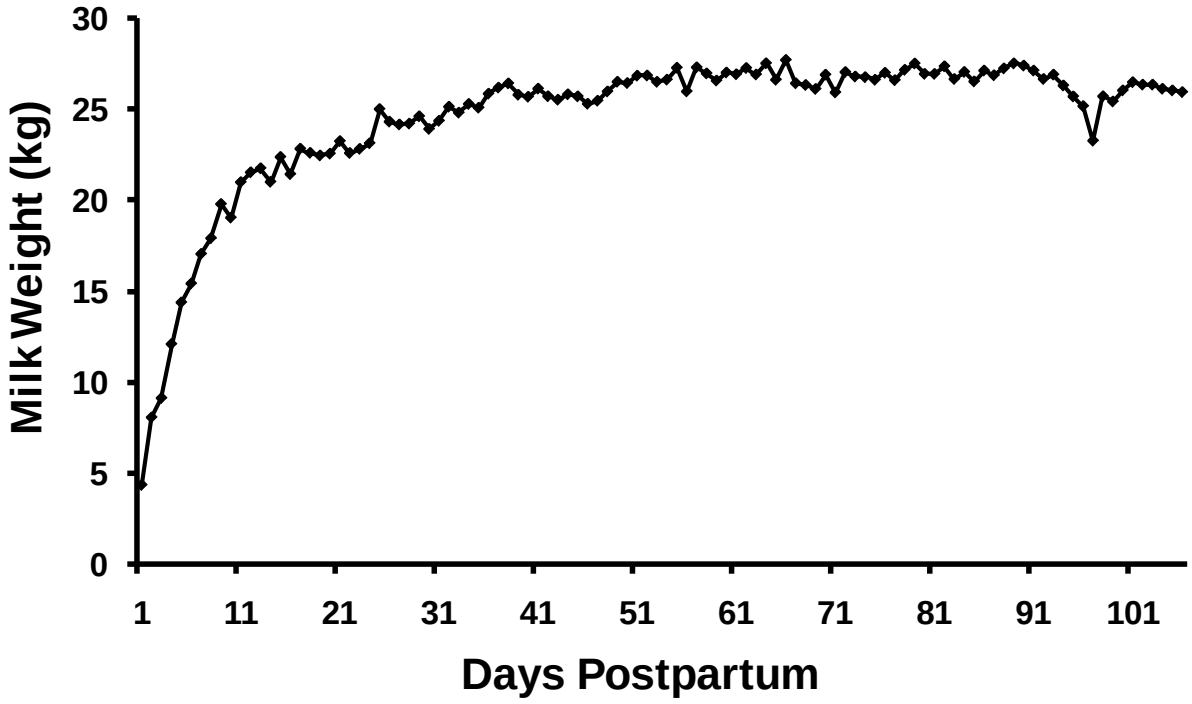

Figure 3-5. Milk weight throughout the study period. The LSM ± S.E.M. for milk weight was 24.56 ± 0.84 kg.

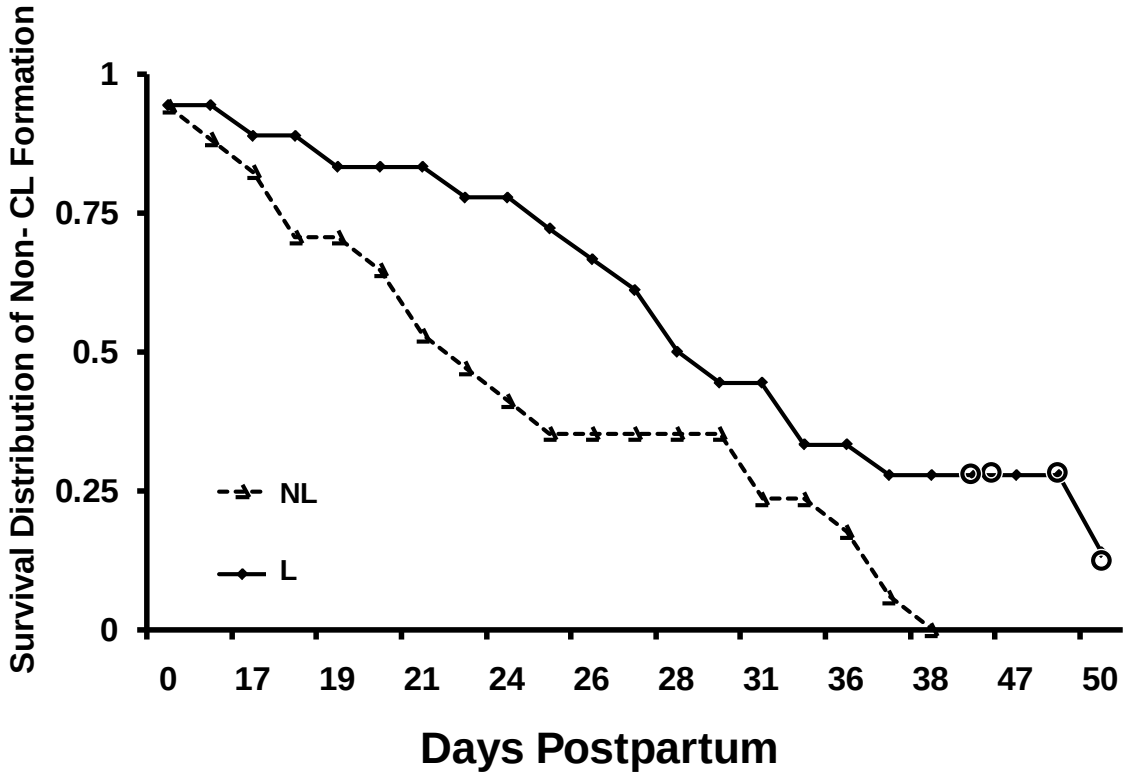

Figure 3-6. NL and L survival curve of non CL formation. Cows in NL cycled at d 26.3 ± 2.01, while cows in the L cycled at d 34.7 ± 3.09 (P<0.04). Hazard ratio = 0.464. L cows (n= 4) that had not cycled by day 55 after parturition are represented by circles on days 39, 47 49 and 55.

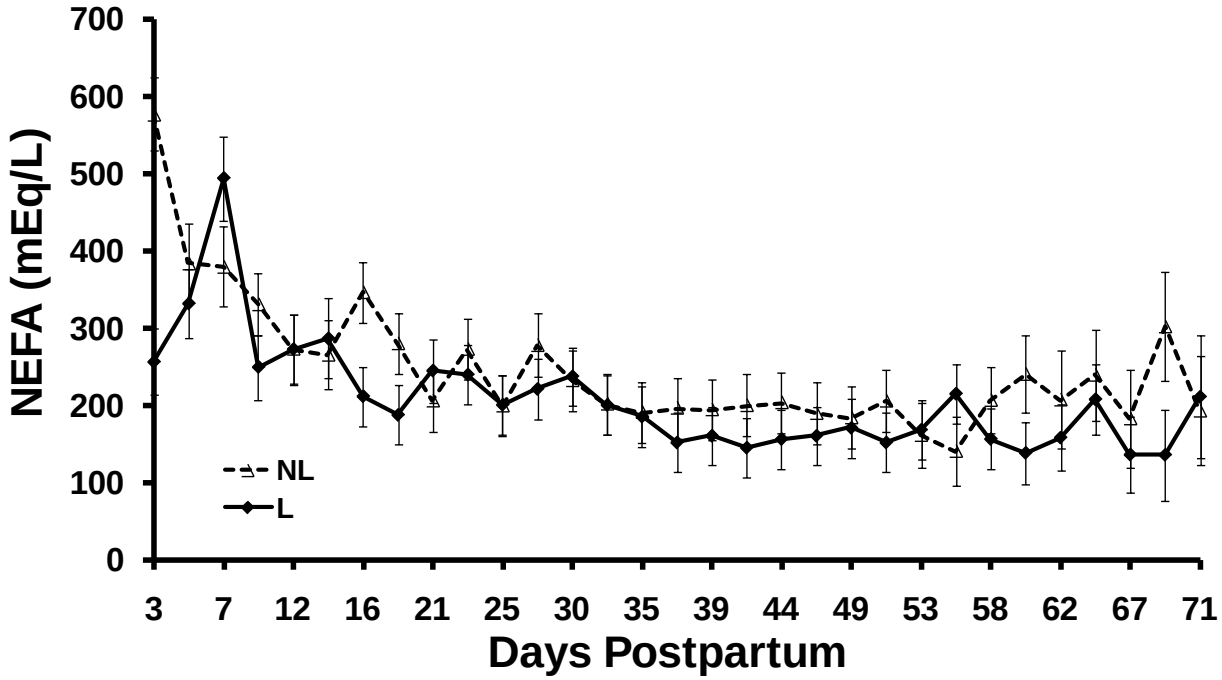

Figure 3-7. NEFA plasma concentrations (mEQ/L) of L and NL cows.The LSM ± S.E.M. for NEFA plasma concentrations for L and NL cows were 207.5 ± 23.91mEQ/L and 247.2 ± 24.75mEQ/L respectively.

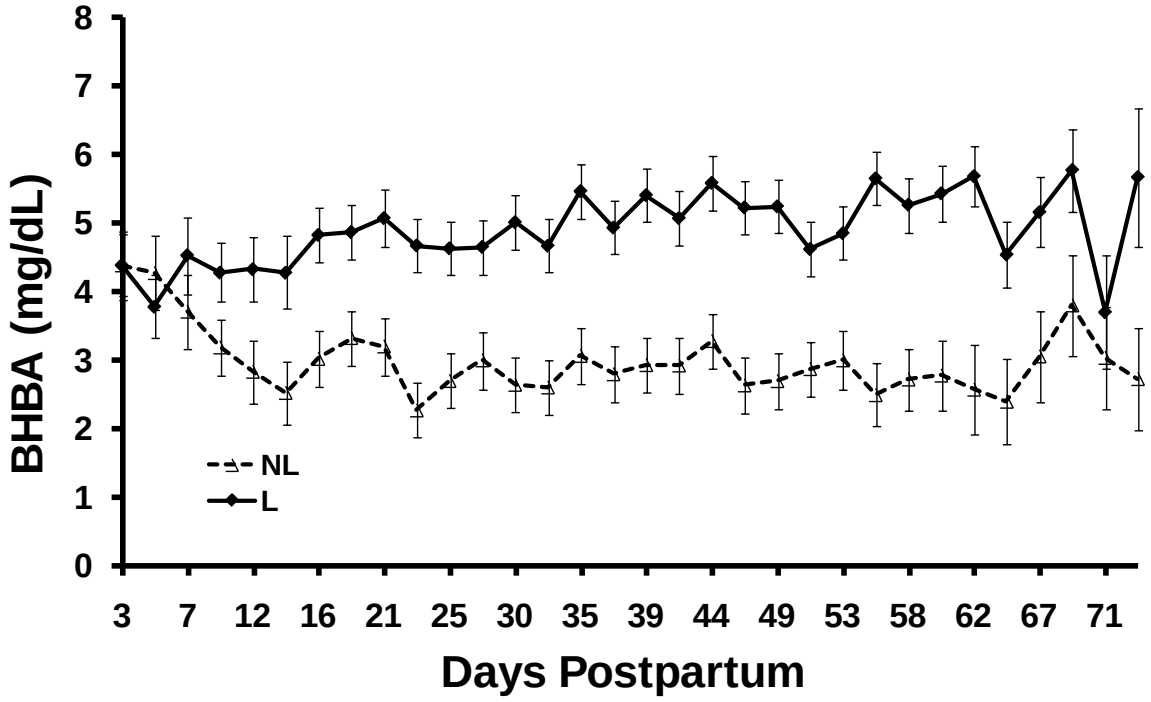

Figure 3-8. BHBA plasma concentrations (µg/mL) of L and NL cows. The LSM ± S.E.M. for BHBA plasma concentrations for L and NL cows were 4.90 ± 0.23 > 2.97 ± 0.23 µg/mL respectively ($P < 0.01$).

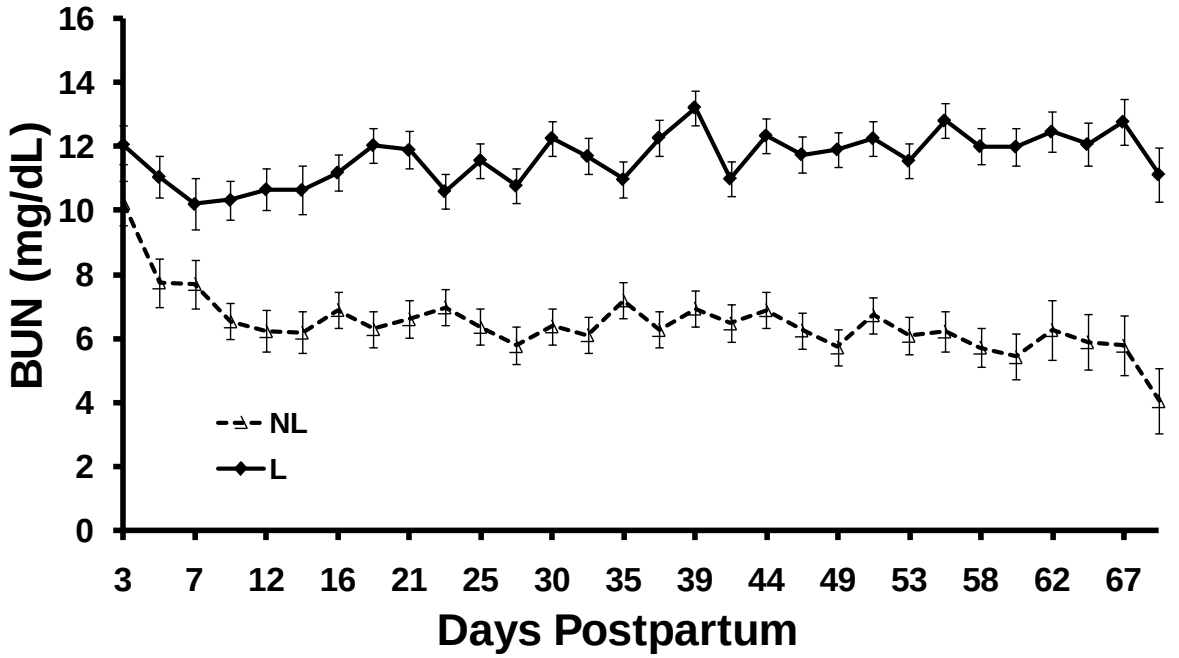

Figure 3-9. BUN plasma concentrations (mg/dL) of L and NL cows. The LSM ± S.E.M. for BUN plasma concentrations for L and NL cows were 11.64 ± 0.32 > 6.5 ± 0.33 mg/dL respectively ($P < 0.01$).

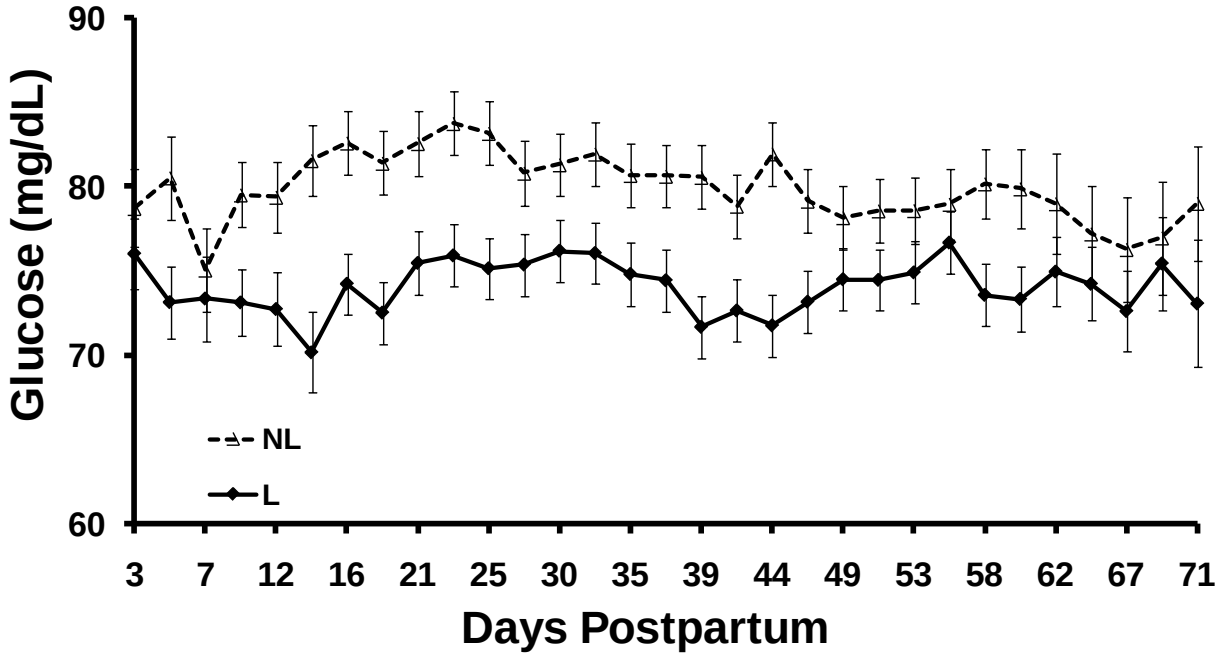

Figure 3-10. Glucose plasma concentrations (mg/dL) of L and NL cows. The LSM ± S.E.M. for glucose plasma concentrations for L and NL cows were 74.0 ± 1.12 < 79.9 ± 1.17 mg/dL respectively ($P < 0.01$).

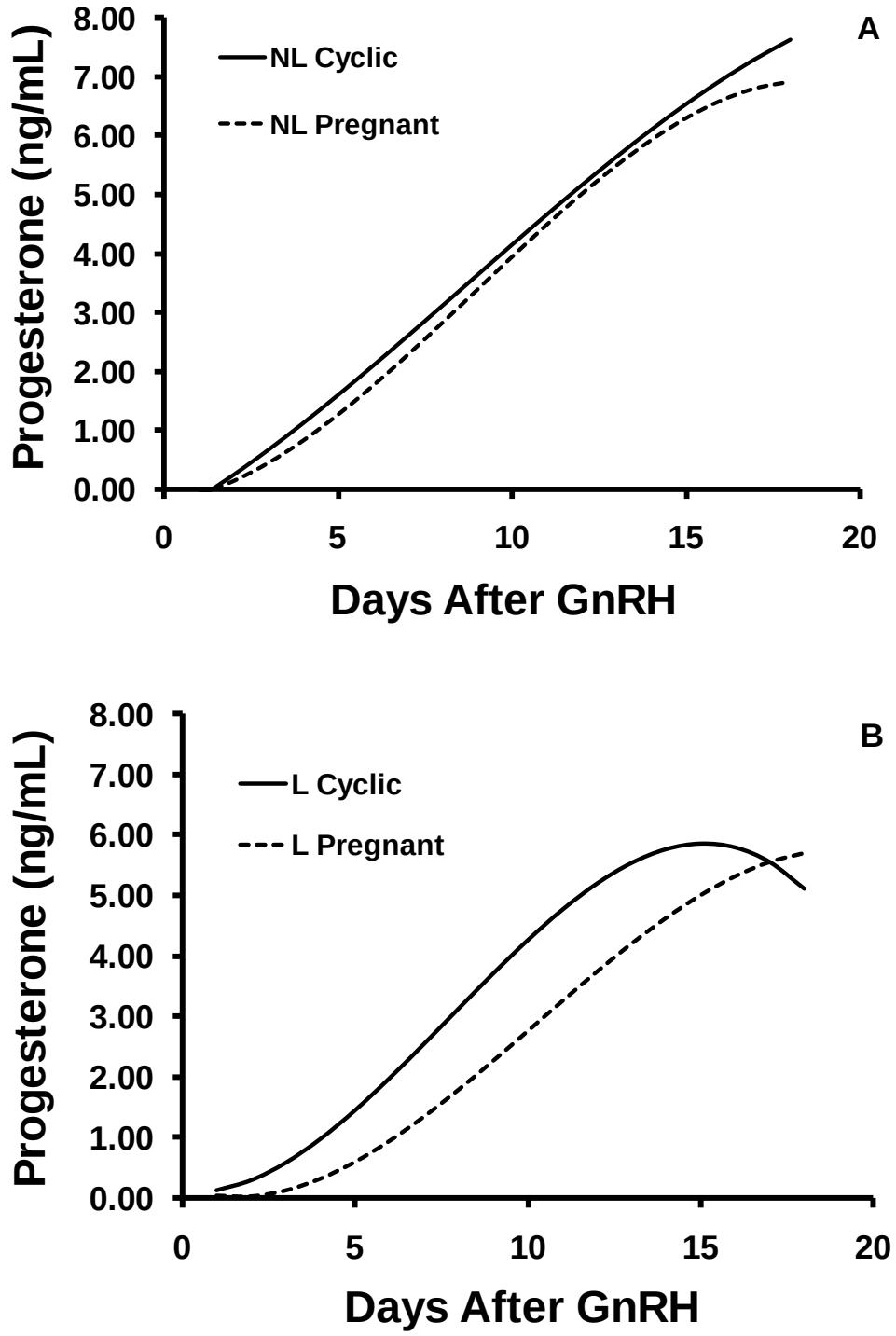

Figure 3-11. Third order regression analyses of progesterone plasma concentrations (ng/ml) of NL (A) and L (B) cyclic and pregnant cows from d 0 (GnRH/TAI) to d 17 (day of slaughter). The overall LSM ± S.E.M. for progesterone plasma concentrations for NL and L cyclic and pregnant cows were 3.67 ± 0.12 ng/mL and 3.04 ± 0.10 ng/mL ($P < 0.002$).

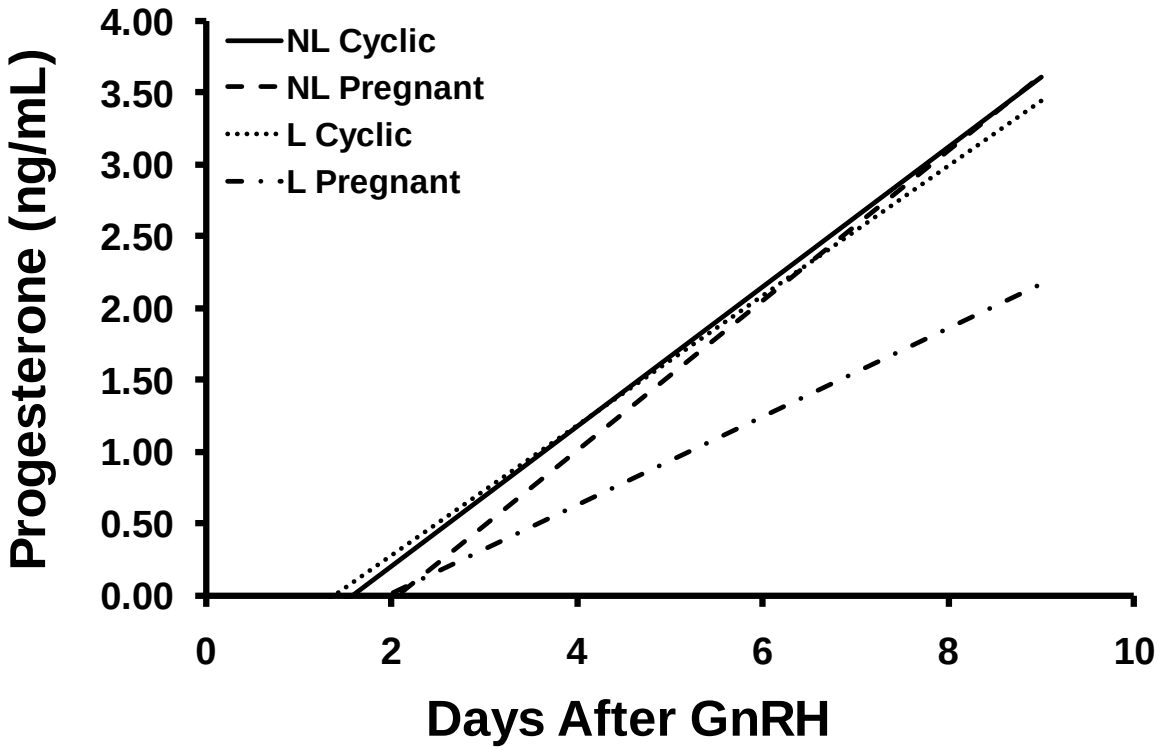

Figure 3-12. Linear regression analyses of progesterone plasma concentrations (ng/ml) of NL and L cyclic and pregnant cows from d 0 (GnRH/TAI) to d 8 before slaughter. The rate of rise in plasma progesterone concentrations per day for NL cyclic and pregnant cows from days 0 to 8 were 0.48 ng/mL and 0.52 ng/mL. L cyclic and pregnant cows had a daily plasma progesterone rise of 0.45 ng/mL and 0.30 ng/mL from days 0 to 8.

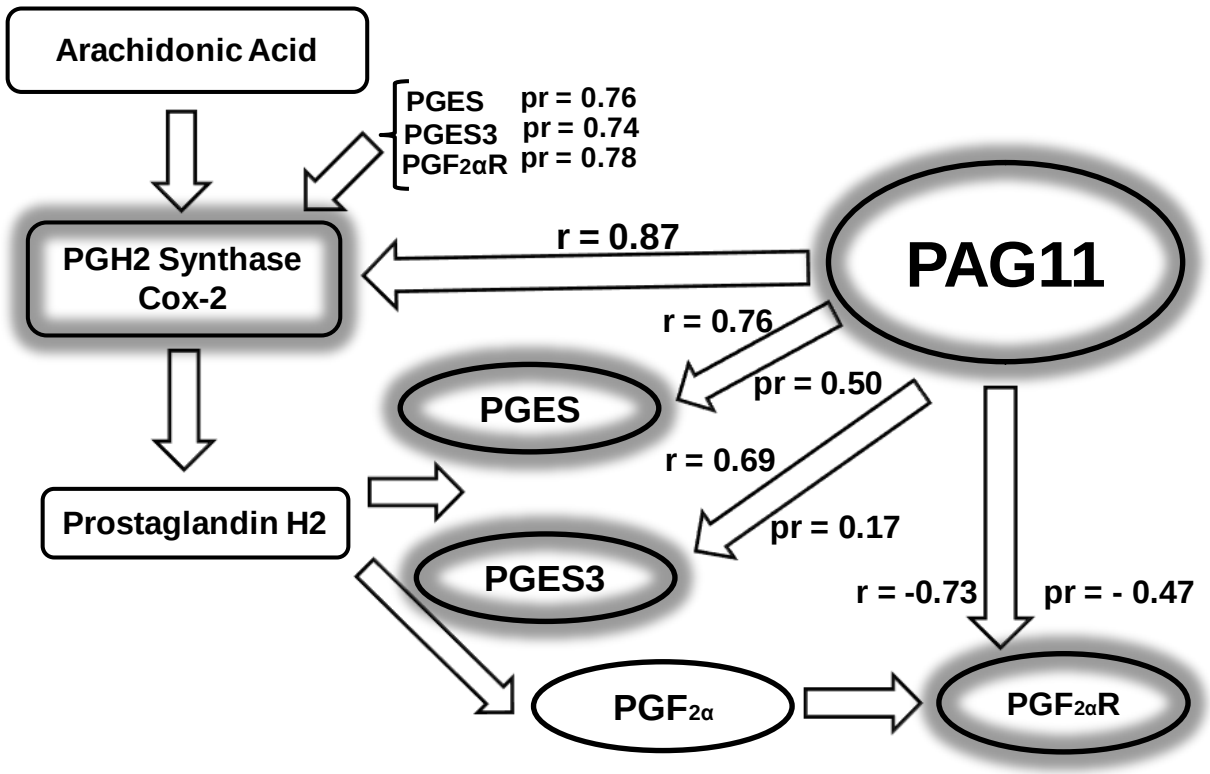

Figure 3-13. Conceptus gene expression; simple (r) and standard partial (pr) correlations associations of PAG11 with prostaglandin regulatory genes. PGH2 synthase (prostaglandin H 2 synthase [Cox-2]); PTGES (prostaglandin E synthase; PTGES3 (prostaglandin E synthase 3 [cytosol]); and PGF2αR (prostaglandin F2α receptor). Grey shadow represents genes that were correlated with PAG 11 in both simple and partial correlation analyses.

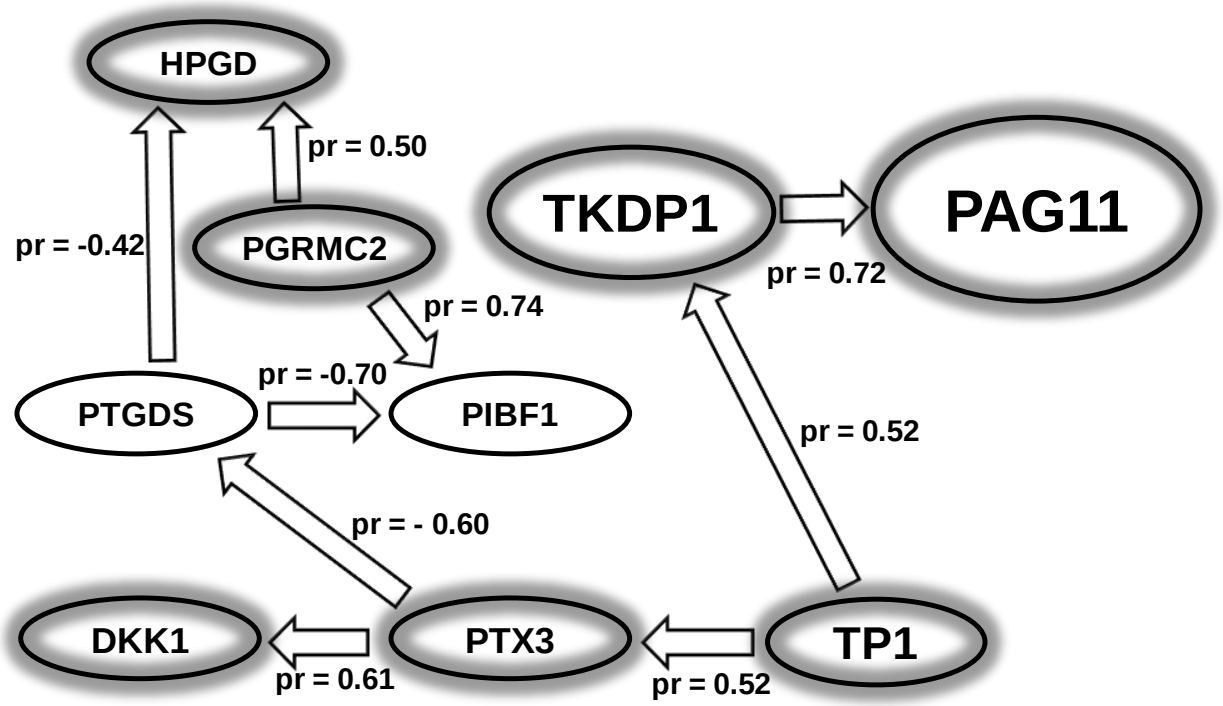

Figure 3-14. Endometrium gene expression; standard partial correlations (pr) associations of PAG 11 with other functional genes. TKDP1 (trophoblast Kunitz domain protein 1); TP1 (trophoblast protein 1); PTX3 (Pentraxin-Related Gene); DKK1 (Dickkopf Homolog 1); HPGD (Hydroxyprostaglandin dehydrogenanse 15); PGRMC2 (progesterone receptor membrane component 2); PTGDS (prostaglandin D2 synthase) and PIBF1 (progesterone induced blocking factor 1). Grey shadow represents the genes that were correlated with PAG11 in simple correlation analyses.

CHAPTER 4
EFFECTS OF RESYNCHRONIZATION PROGRAMS ON PREGNANCY PER AI, PROGESTERONE AND PREGNANCY ASSOCIATED GLYCOPROTEINS (PAGS) IN PLASMA OF LACTATING DAIRY COWS

Objectives were to develop a timed AI resynchronization program to improve pregnancy per AI and to evaluate responses of circulating progesterone and PAGs in lactating cows. Cows (n=1578) were presynchronized with two injections of $PGF_{2\alpha}$ given 14 d apart starting at 42±3 d postpartum followed by Ovsynch (GnRH, 7 d $PGF_{2\alpha}$, 56 h GnRH, 16 h TAI [d 0]). The Resynch group received a controlled internal drug releasing (CIDR) insert containing progesterone from d 18 to 25, GnRH at d 25, pregnancy diagnosis at d 32, and nonpregnant cows received $PGF_{2\alpha}$., GnRH 56h after and TAI 16 h later (d 35). The Control group was diagnosed for pregnancy at d 32 and nonpregnant cows received GnRH, d 39 $PGF_{2\alpha}$, GnRH 56 h after, and TAI 16 h later (d 42). Pregnancy was re-confirmed at d 60 after AI. Ovarian structures were examined in a subset of cows at the time of GnRH and $PGF_{2\alpha}$ injections. Blood samples for analyses of progesterone and PAGs were taken every 2 d from d 18-30 in 100 cows and continued weekly to d 60 of pregnancy (n=43). Pregnancy per TAI at d 32 for pre-enrolment AI (45.8%, n=814; 45.9%; n=764) and pregnancy losses at d 60 (6.7% and 4.0%) did not differ for Resynch and Control, respectively. Resynchronized service pregnancy per AI (36%, n=441 and 39.5% n=412) and pregnancy losses (6.3% and 6.7%) did not differ for Resynch and Control. Days open for pregnant cows after two TAI were less for the Resynch group (96.2 vs 99.5 d). Cows in the Resynch had more large follicles at the time of GnRH. The number of CL did not differ between groups at the time of $PGF_{2\alpha}$. Plasma progesterone was greater for pregnant cows in the Resynch group (18-60 d; 6.6 > 5.3 ng/ml), and plasma concentrations of progesterone at d18

were greater for pregnant than nonpregnant cows (5.3 > 4.3 ng/ml). Plasma PAGs in pregnancy were lower for cows in the Resynch group on d 39 (2.8 < 4.1 ng/ml) and 46 (1.34 < 3 ng/ml). Cows pregnant at d 32 that lost pregnancy by d 60 (n=7) had lower (P < 0.05) plasma concentrations of PAGs at d 30 than cows that maintained pregnancy (n=36) (2.9 < 5 ng/ml). PAGs at d 30 (> 0.33ng/ml) were predictive of a positive d 32 pregnancy diagnosis (Sensitivity 100%, Specificity 90.6%). In conclusion, Resynch and Control protocols had comparable pregnancy per AI for first and second TAI services, but pregnancy occurred 4 d earlier in the Resynch group. CIDR and/or GnRH increased progesterone during pregnancy. Dynamics of PAGs were indicative of pregnancy status and pregnancy loss.

Introduction

Accurate and early detection of nonpregnant cows combined with a program for resynchronization of ovulation will improve reproductive efficiency of dairy herds. Acceptable pregnancy per AI has been achieved with the use of ovulation synchronization programs for timed AI (TAI) to maximize submission rates for insemination at the designated voluntary waiting period (Chebel et al, 2006).

A presynchronization protocol with two injections of $PGF_{2\alpha}$ administered 14 d apart and initiation of the Ovsynch protocol (Pursley et al., 1997) 12 d later (Moreira et al., 2001) increased pregnancy per AI to first service in lactating dairy cows. Cows diagnosed nonpregnant after a Presynch+Ovsynch and TAI should be resynchronized as early as possible when diagnosed nonpregnant. Protocols for resynchronization of ovulation (Resynch) can increase the effective AI service rate and reduce the interval between AI services (Fricke. 2002). Optimization of the stage for re-synchronization

after the initial TAI is a physiological approach to reduce the interval between TAI services (Bartolome et al., 2005a; Bartolome et al 2005b; Bartolome et al., 2005c).

The measurement of PAGs as an indicator of non-pregnancy or, alternatively, early pregnancy in cattle can serve as a tool to help optimize reproductive management (Sasser et al., 1986; Zoli et al., 1992; Green et al, 2005; Silva et al., 2007). Furthermore, monitoring the circulating concentration of PAGs can also assist in identification of embryo/fetal well being and pregnancy loss (Patel et al., 1995, Patel et al., 1997; Kindahl et al, 2002; Szenci et al, 2003; Gábor et al, 2007; Kornmatitsuk et al, 2002).

Objectives of this study were to: 1) characterize pregnancy per AI for first and second services in cows receiving a Presynch/Ovsynch and a CIDR/Resynchronization program (Resynch) versus a Presynch/Ovsynch and a Control resynchronization program (Control), and 2) compare concentrations of progesterone and PAGs after first insemination between d 18 and d 60 for pregnant cows and between d 18 and d 30 for nonpregnant cows of the Resynch and Control programs.

Material and Methods

Study Population

The study was conducted from October 2007 to July 2008 in a large commercial dairy of north central Florida with 3900 milking cows. A total of 1772 primiparous and multiparous lactating dairy cows were enrolled. Cows were housed in free stall barns and fed a total mixed ration (TMR) with ad libitum access to feed and water. Diets were formulated to meet NRC (2001) nutrient requirements for net energy of lactation (NE_L), crude protein (CP), fiber, mineral and vitamins for postpartum cows. Cows were milked three times a day and the mean 305 d mature equivalent milk production for primiparous and multiparous cows was 11,878 kg and 11,922 kg, respectively.

Study Design

Primiparous and multiparous cows were managed in separate pens and assigned to two treatment groups after calving (Resynch and Control) based upon odd and even last numbers of the ear tags. Multiparous cows were scheduled to receive initial hormonal injections on Mondays and were TAI on Thursdays. Primiparous cows received hormonal injections on Tuesdays and were scheduled to receive TAI on Fridays. All cows were submitted to a Presynch+Ovsynch protocol. Presynch was initiated with an i.m. injection of $PGF_{2\alpha}$ (25 mg; Lutalyse, Pfizer Animal Health, New York, NY) at 45 ± 3 d after parturition. Fourteen days later, Ovsynch was initiated by administering of GnRH (100µg; Cystorelin, Merial Ltd., Duluth, GA) on d 0, 25 mg of $PGF_{2\alpha}$ on d 7, 100µg of GnRH 56 h later and TAI 16 h after the GnRH injection. Of the 1772 cows enrolled, 194 (10.9%) were excluded because they were sold, died or failed to complete the hormonal injection sequences for the Presynch + Ovsynch or Resynch protocols. Thus, a total of 1578 cows completed the study (Figure 4-1).

Resynch Treatment

Cows of the Resynch treatment group (n=441) received an intravaginal progesterone insert (CIDR®, Pfizer Animal Health, New York, NY) for 7 d starting at d 18 after TAI. Cows received a 100 ug i.m. injection of GnRH at the time that CIDR insert was removed at d 25 after TAI. Pregnancy status was assessed 7 d later at 32 d after TAI by ultrasonography (Easi-Scan, BCF Technology; Rochester MN, USA). The presence of an embryo with a heartbeat was the criterion used to determine pregnancy. Cows diagnosed pregnant were re-examined by palpation per rectum of the uterus and its contents at d 60 after TAI to reconfirm pregnancy status and quantify pregnancy loss. Nonpregnant cows continued Resynch by receiving an i.m. injection of 25 mg $PGF_{2\alpha}$ at

d 32 after TAI followed by a GnRH injection (100 ug) 56 h after the $PGF_{2\alpha}$ injection. All nonpregnant cows were TAI 16 hours after the GnRH injection. Pregnancy diagnosis by ultrasonography was conducted 32 d after the second TAI and pregnancy re-confirmed at 60 d (Figure 4-1).

Control

Cows assigned to the Control group (n=413) underwent ultrasonography at d 32 after TAI to determine pregnancy status. Pregnant cows had pregnancy reconfirmed at d 60 after TAI. All nonpregnant cows received an injection of GnRH followed by an i.m. injection of $PGF_{2\alpha}$ (25 mg) at d 39 after TAI, a GnRH injection (100 ug) 56 h later and TAI at 72 h after $PGF_{2\alpha}$. The second service pregnancy diagnosis by ultrasound was conducted 32 d after TAI and pregnancy re-confirmed at 60 d (Figure 4-1).

Ovarian Ultrasonography

Ovarian ultrasound examinations were performed in a subgroup of cows (n = 149) of both resynchronization programs at the time of GnRH and $PGF_{2\alpha}$ injections. Presence of corpus luteum (**CL**) and follicle sizes were recorded. Follicles were classified according to their diameter as small (<10mm), medium (between 10 and 15mm) and large (>15mm). Following first service pregnancy diagnosis (d 32), all cows enrolled in the experiment (n = 1578) had their ovaries checked by ultrasound for the presence or absence of CL, regardless of whether the cows were pregnant or nonpregnant.

Blood Collection for Pregnancy Associated Glycoproteins ELISA Assay

Blood samples for measurement of PAGs were collected from a subgroup of cows (n = 100) from both resynchronization programs. Samples were collected between d 18 and 60 after first insemination for pregnant cows and between d 18 and 30 after first

insemination for nonpregnant cows. Sampling was every other day from d 18 to d 30 and once a week from d 32 to d 60 after TAI. Additional blood samples for measurement of PAGs were collected from a subgroup of cows (n = 103) on the day of parturition. Samples were collected from the coccygeal vessels into evacuated tubes containing EDTA (10 mLVacutainer®; BD, Franklin Lakes, NJ, USA) and placed on ice immediately after collection. Samples were centrifuged for 30 min, and plasma stored at -20^{o}C until assayed for PAGs.

Blood samples were analyzed for concentrations of PAGs using a 'sandwich' ELISA assay performed as described previously (Green et al., 2005), with some modifications. Specifically, a different detection rabbit polyclonal antibody (Ab20) was employed. This antibody was raised against a mixture of PAGs that had been isolated based on their affinity toward the aspartic peptidase inhibitor, pepstatin A. Late pregnant (3rd trimester) cotyledons from a twin pregnancy were homogenized, and PAGs were obtained after binding to pepstatin-agarose under neutral (pH 7) and acidic (pH 5) pH conditions; elution of PAGs from the matrix was performed by increasing the osmolarity and pH. Detailed methods describing PAG purification in this manner have been reported elsewhere (Green and Roberts, 2006; Green et al., 2005; Wooding et al., 2005). The eluted PAGs were further enriched by binding and eluting them from an anion exchange matrix (MonoQ; BioRad). Binding was at pH 7.5 in 20 mM Tris buffer; elution of PAGs was between 0.1 and 0.2 M NaCl.

The Ab20 immunoglobulins were isolated by Protein A affinity chromatography (Green and Roberts, 2006; Green et al., 2005) and further purified by binding and elution from a PAG matrix. Briefly, 1 mg of the PAG mixture was biotinylated by

conjugation to NHS LC-LC biotin (Pierce; cat. No 21343). The final molar ratio of biotin to PAG was 20:1. The conjugation reaction was allowed to proceed for 3 hr at room temperature on a rotator/mixer. The PAG-biotin conjugate was then dialyzed overnight (50,000 MWKO) against Tris-buffered saline (10mM Tris, pH 8.0; 150 mM NaCl). The biotinylated PAG was added to 1mL of an avidin-agarose slurry (Vector; cat. No A2010) and agitated for 2 hr on a rotator/mixer. The matrix was washed several times with PBS and then equilibrated in TBS. Purified Ab20 immunoglobulin was applied to the PAG-avidin matrix, washed with TBS (30 column volumes), and then eluted with 0.1M glycine, pH 2.0 followed by a 0.5M glycine, pH 2.0 elution. The eluted fractions were neutralized with 1M Tris pH9.5 and dialyzed against PBS. The anti-PAG immunoglobulin was concentrated by using an Amicon Ultra device (Millipore UFC903024) and quantified by BCA assay with rabbit IgG as standard. The purified Ab20 was used as the detection antibody in the ELISA at 0.1 µg per well, which differed from the previous assay format that used 1 µg per well (Green et al., 2005).

Procedure description. Each well of a 96-well plate (Costar) was coated with 100 µl of 10 µg/ml sheep anti-mouse (Fc specific, Jackson Labs prod #515-005-071) antibody. This was diluted in 0.1M sodium bicarbonate, pH 9.5 and stored at 4^{o}C overnight. On the next day, the orientating trap antibody was shaken out of the plate(s) and tapped against paper towels to remove residual. Blocking solution (300 µl; 0.1M Sodium Bicarbonate pH 9.5, 2% ovine serum albumin, 1% nonfat dry milk, 0.02% (w/v) sodium azide) was placed into the wells. After 1 hour of incubation, blocking solution was removed and 100 µl of monoclonal mixture (50 ng each of A6, J2, L4/100 µl) diluted in TBST (with blocking proteins) was added into each well. Plates were

incubated at room temperature for 1 hour. Plates were then washed five times on an ELISA plate washer (BioTek). TBST (50 μl) with blocking proteins (e.g. 1/20 dilution of blocking solution) was added to each well to keep them moist. 100μl of each experimental sample was added to adjacent wells, with the duplicates oriented horizontally. Also duplicate dilutions of the PAG standard curve were run. The standard was diluted in bull serum or nonpregnant heifer serum (NPHS). The blanks were wells of bull serum or NPHS only. Plates were incubated at 4C overnight. The next morning, plasma was shaken out of the wells and plates were washed 10 times on an ELISA plate washer. Plates were turned 180 degrees between the wash cycles. A 100 μl aliquot of 15 μg/ml polyclonal Ab20 (antibody to native PAGs) was added to each well, diluted in TBST+2% NPHS followed by incubation for 1 hour at room temperature. After the 1 h incubation, the fluid was shaken out and wells were washed. A 100 μl aliquot of AP-conjugated goat anti-rabbit IgG (Jackson Labs prod #111-055-144) diluted 1:2000 in TBST or AP buffer was added to the wells and the plates were incubated again for 3 minutes at room temperature. Plates were washed and 100 μl of 1 mg/ml para-nitrophenyl phosphate PNPP (Sigma, St. Louis, MO, USA) was added to each well. Plates were read by a plate reading at 405nm wavelength after 15 to 30 minutes. Pooled error of variance of the duplicates of 331 samples gave a coefficient of variation of 22.3%. The inter- and intra-assay coefficients of variation for replicates run in three assays for d 28 of pregnancy were 11.8% and 14.6% respectively and for d 41 were 9.3% and 16.3% respectively.

Plasma Progesterone Concentrations

The measurement of plasma progesterone concentrations was performed from the same blood samples used for the measurement of PAGs. The samples were obtained from the coccygeal vessels into evacuated tubes containing EDTA (10 mL Vacutainer®; BD, Franklin Lakes, NJ, USA) and placed on ice immediately after collection. Samples were centrifuged for 30 min, and plasma was stored at -20°C. Plasma concentrations of progesterone were determined by a solid-phase, no extraction radioimmunoassay (Coat-A-Count Progesterone, DPC® Diagnostic Products Corporation, Los Angeles, CA, USA). The standard curve dilution consisted of duplicated plain tubes used for total counts and non-specific binding. A 100µL of increasing progesterone concentrations of calibrators (0.1, 0.25, 0.5, 1, 2, 5, 10 and 20 ng/mL) were added to the tubes. The sensitivity of the assay was 0.1 ng/mL. The intra-assay and inter-assay coefficients of variation for the replicates ran in each assay for plasma at d 4 of cycle (1.27 ± 0.04 ng/mL) were 8.1% and 10.2% respectively and for d 8 of the cycle (4.14 ± 0.05 ng/mL) were 2.8% and 5.3% respectively.

Statistical Analysis

The proportions of pregnant cows (pregnancies per TAI) to first and second services were analyzed by logistic regression using PROC LOGISTIC of SAS (SAS/STAT, ver. 9.1, SAS Institute Inc). The model for pregnancies per TAI for the first and second services included the effects of two different reproductive management programs (Control vs Resynch), parity (primiparous vs multiparous), sires, inseminators, occurrence of different clinical disorders, and milk weight averaged for 3 months (quartiles). Original modeling was performed using backward stepwise selection with the significance level for retention in the model set at $\alpha \leq 0.10$. A subsequent logistic

regression model was run including only variables that had a P $\leq$ 0.10 0 from the backward stepwise logistic regression analysis. The model fit statistics were performed by comparison of the difference in the deviances by likelihood-ratio statistic test. Differences in frequency and proportion of ovarian structures (presence or absence of CL, number of CL and small, medium and large follicles) between treatments were analyzed by a multinomial logistic regression model using GLogit from SAS. In the model, one category of the dependent variable is chosen as the comparison category. The least squares means for number of CL for each treatment were obtained using the GLIMMIX procedure for generalized linear mixed models from SAS. The progesterone and PAGs responses between resynchronization treatments were analyzed using repeated measures analyses of the mixed model procedure (PROC MIXED) of SAS. Days open were calculated using general linear model (PROC GLM) procedure of SAS. The ability of the PAG ELISA assay to identify pregnant cows (sensitivity) and the ability to identify open cows (specificity) were tested by running a ROC (Receiver Operating Characteristic) curve for concentrations of PAGs on d 28 and 30 of pregnancy.

Results

Breeding and Fertility

Reproductive outcomes for the pre-enrolment and resynchronized TAI are summarized in Table 4-1. There was no difference in pregnancy per AI at the pre-enrolment between the Resynch and the Control treatment groups on d 32 (P < 0.90) and d 60 (P < 0.54) of pregnancy. The overall pre-enrolment pregnancies per AI were 45.9% (n = 724) on d 32, and 43.4% (n = 685) on d 60.

The resyncrhonization pregnancies per AI of 37.7% (n = 322) on d 32 and 35.3% (n = 301) on d 60 after resynchronized TAI did not differ between the two treatment

groups (Table 4-1). In addition, the overall pregnancy losses were 5.4% and 6.5% for pre-enrolment and resynchronized TAI. Pregnant cows in the Resynch treatment group had fewer days open (i.e., days between calving and conception) compared with cows in the Control group (96.2 vs 99.5 d; $P < 0.01$).

The occurrence of different health disorders had an impact on pregnancy per AI at d 32 after pre-enrolment TAI. Cows that developed digestive problems (13.9%; n = 219) had a decrease in pregnancy per AI from 47.2% (healthy animals) to 37.9% ($P < 0.01$). The frequency of mastitits of the study population was 24.1% (n = 380) with a decrease in pregnancy per AI from 47.4% to 41.3% ($P < 0.06$). Occurrence of dystocia (3.4%; n = 55) and retained fetal membranes (5.5%; n = 88) also impacted pregnancies per AI from 46.9% to 27.2% ($P < 0.005$) and from 46.5% to 34% ($P < 0.06$), respectively.

Ovarian Ultrasonography

Ovarian responses at the time of GnRH and $PGF_{2\alpha}$ injections for a subgroup of cows of both treatment groups are depicted in Tables 4-2, 4-3 and 4-4. The average number of CL for nonpregnant cows in the Resynch treatment group was lower than nonpregnant cows of the Control group at the time of GnRH injection (0.61 vs 0.92; $P < 0.01$; Table 4-2). There was also a significant difference in occurrence of cows with no CL versus 1 CL between treatments ($P < 0.01$). The odds of cows to have no CL in the Resynch group compared to 1 CL were 3.73 times greater than cows of the Control group (Table 4-2). The average number of medium and large follicles per cow of the Resynch group was greater compared to the Control group (1.16 > 0.94; $P < 0.04$). In the Resynch group a greater proportion of cows had a large follicle (54 vs 28; $P < 0.01$) at the time of GnRH injection than the Control group (Table 4-3). The mean number of CL at the time of $PGF_{2\alpha}$ injection was not different between the Control and Resynch

groups (0.88 vs 0.82; P < 0.32). However, the occurrence of a CL with a large follicle was less for the Resynch group compared to the Control (P < 0.01) at the time of $PGF_{2\alpha}$ injection (11.4% < 40.5%) (Table 4-4). The presence or absence of a CL was recorded for all nonpregnant cows following pre-enrolment in the experiment (n = 854; Table 4-5). Indeed cows in the Resynch group had a slightly higher occurrence of a CL (80.0 % > 73.3%: P < 0.02).

Pregnancy Associated Glycoproteins

The overall concentrations of PAGs in plasma did not differ (P < 0.30) between the two treatment groups for pregnant cows (Resynch group [n = 20] 1.68 ± 0.27 ng/mL and Control [n = 23] 2.07 ± 0.25 ng/mL). However, concentrations of PAGs (Figure 4-2) were lower in the Resynch group compared to Control on d 39 (2.80 ± 0.46 vs 4.13 ± 0.44 ng/mL; P < 0.04) and d 46 (1.34 ± 0.46 vs 3.03 ± 0.44 ng/mL; P < 0.01). The profiles of PAGs concentrations in the plasma of 43 pregnant cows (Figure 4-3) revealed that pregnant cows (n = 36) maintaining pregnancy had higher (P < 0.05) plasma concentrations of PAGs at d 30 after AI compared to cows undergoing late embryo mortality (LEM) between d 32 and 60 (n = 7) of pregnancy (4.98 ± 0.42 > 2.91 ± 0.96 ng/mL). The sensitivity for diagnosis of pregnancy at d 28 was 95.3% with a specificity of 88.3%, when concentrations of PAGs were > 0.41ng/mL for a diagnosis of pregnancy. On d 30 of pregnancy, sensitivity and specificity were 100% and 90.6%, respectively when cows with plasma concentrations > 0.33 ng/mL were considered pregnant.

Plasma concentrations of PAGs on the day of parturition did not differ between the two treatment groups and were not associated with parity, calving difficulty and occurrence of peripartum health disorders. However, cows that delivered female calves

had significantly lower plasma concentrations of PAGs on the day of parturition compared with cows that delivered male calves (327.8 ± 26.54 ng/mL < 395.2 ± 21.64 ng/mL; $P < 0.05$).

Progesterone

Pregnant cows of the Resynch group maintained higher ($P < 0.003$) progesterone concentrations than the Control group even after removal of the CIDR insert (Figure 4-4). The mean concentrations of progesterone for the Resynch and Control groups were 6.59 ± 0.30 ng/mL and 5.30 ± 0.28 ng/mL, respectively. All cows diagnosed pregnant at d 32 (n = 36) had higher concentrations of plasma progesterone ($P < 0.02$) compared to nonpregnant cows with a CL (n = 35) when sampled at d 18 (5.26 ± 0.30 > 4.31 ± 0.30 ng/mL). In nonpregnant cows (n = 57), there were no main effects of treatment and day on plasma concentrations of progesterone. However, an interaction of treatment by day was detected ($P < 0.03$; Figure 4-5). Nonpregnant cows of the Resynch group had a timely synchronized decrease in progesterone between 24 to 26 d after TAI. In contrast, CL regression, formation of a new CL, and subsequent increases in progesterone occurred at different times between 18 and 30 days for the Control group such that there was no clear synchronized pattern of ovulation compared to the Resynch group.

Discussion

Supplemental progesterone via a CIDR insert between d 18 and 25 after the pre-enrolment TAI and injection of GnRH at removal of the CIDR insert for resynchronization did not affect pregnancy per TAI for the pre-enrolment or pregnancy loss between d 32 and 60 of gestation compared to the Control group. Overall pregnancy per AI to the Presynch/Ovsynch 56 h protocol (45.9%) and subsequent pregnancy losses to pre-enrolment (5.4%) were considered to be good for a large

commercial dairy herd. Pregnancy per AI in US herds average 34% (Santos et al., 2004).

The effect of progesterone supplementation with a CIDR insert on pregnancy per AI was evaluated in a series of studies. Treatment using a CIDR insert from d 14 to 21 after insemination was associated with a 4% reduction in pregnancy per AI to the pre-enrolment AI (Chenault et al., 2003). However, CIDR inserts improved synchrony of return to estrus and did not affect pregnancy per AI during the resynchronization period. In contrast, Galvão et al. (2007) reported that the insertion of a CIDR for resynchronization between d 14 and 21 post-insemination with or without estradiol injection at the time of CIDR removal did not affect pregnancy rate, embryonic or fetal survival. Moreover, Bartolome et al. (accepted) documented that the use of a CIDR insert between d 14 and 23 after AI did not affect pregnancy outcome to the pre-enrolment AI and did not improve second service pregnancy per AI. In addition, no differences in pregnancy loss between 30 and 55 d for second service was detected between cows receiving a CIDR insert for resynchronization compared with control cows. In a meta analysis, Mann and Lamming (1999) observed that supplementation of progesterone before d 6 after AI in lactating cows, significantly increased pregnancy per AIs. In addition, a reduction in pregnancy loss between d 31 and 60 of gestation was attributed to insertion of a CIDR insert between d 14 and 21 after first AI (Chebel et al., 2006). Similarly, El-Zarkouny and Stevenson (2004) observed an improvement in embryo survival between d 29 and 57 of gestation in cows that received a CIDR insert from d 13 to 20 after AI. Although various studies are conflicting, the present results agree with Galvão et al. (2007) and Bartolome et al. (accepted) indicating that insertion

of a CIDR in early pregnancy between 14 to 18 d for a 7 d period did not improve pregnancy per AI or reduce pregnancy loss in the pre-enrolment and resynchronized AI.

Reproductive performance is linked to health in the weeks immediately before and after calving, and timely achievement of subsequent pregnancy in turn has a substantial impact on profitability (De Vries, 2006). Chebel et al (2004) have associated reproductive success with periparturient diseases, and several studies reveal reduction in fertility for cows affected by disorders of the reproductive tract, lameness, mastitis and metabolic disorders (LeBlanc, 2008; Garbarino et al., 2004; Schrick et al., 2001; Opsomer et al., 2000). In accordance with previous studies, our results indicate that the occurrence of digestive problems, mastitis, dystocia and retained fetal membranes decreased pregnancy per AI.

Use of exogenous progesterone promotes induction of cyclicity and better synchronization of estrus in lactating cows (Rhodes et al., 2003). Examination of ovarian structures of a subsample of cows from the Resynch and Control groups at the time of GnRH and $PGF_{2\alpha}$ injections showed clear differences in ovarian structures between the two groups. Cows in the Resynch group had a lower occurrence of CL at the time of GnRH injection compared to the Control group. Likewise, cows with the CIDR insert had a higher percentage of large follicles. During the period of CL regression between 18 and 25 d, presence of a CIDR insert likely sustained follicle growth and blocked ovulation such that at the time the CIDR insert was removed and GnRH was injected, a sustained large dominant preovulatory follicle was present. The active dominance of this follicle accounted for the lower number of medium and small follicles in the Resynch group. In contrast nonpregnant cows of the Control group likely

had undergone spontaneous CL regression and a normal preovulatory follicle developed and ovulated. Consequently a developing first wave follicle would be present at the time GnRH was injected on d 32. This likely accounted for a greater frequency of CL and medium size follicles (i.e., 10 to 15 mm) in nonpregnant cows of the Control group on d 32.

Maintenance of a large follicle in the presence of a progestin has been well documented (Sirois and Fortune, 1990; Savio et al., 1993a; Savio et al., 1993b). Clearly, the two treatment groups had distinct differences in ovarian CL and follicle dynamics that was further verified at the time of $PGF_{2\alpha}$ injection. The GnRH injection at d 25 for the Resynch group appeared to induce a greater synchronization of large follicles than the control group. This is suggested based on equal frequency of CL at $PGF_{2\alpha}$ injection but a smaller percentage of CL with large follicles compared to the control group. Follicle development would have been better synchronized in response to GnRH of the Resynch group and less synchronized in the Control group with a wider array of ovulatory and non-ovulatory follicles at the time of GnRH injection. Consequently there were a greater number of Control cows with a CL and large follicle at the time of $PGF_{2\alpha}$ injection. Although ovarian dynamics were distinctly different for the Resynch and Control groups approaching the second TAI, there were no significant differences in pregnancy per AI or pregnancy loss between the groups.

A reduction in the interval between first and second services has been the stimulus for the development of different resynchronization strategies (Bartolome et al., 2005c; Chebel et al, 2003; Fricke et al, 2003). Resynchronization programs are used normally in dairy herds as a reproductive management tool to improve the re-

insemination rate of nonpregnant cows, and reduce the interval from diagnosis of nonpregnancy to conception (Caraviello et al., 2006). An increase in the number of days between calving and conception (i.e., days open) is typically associated with reduced profitability partly due to increased breeding cost, increased risk of culling and replacement costs, and reduced milk production (De Vries, 2006a). In the present study, use of a CIDR insert for subsequent resynchronization of nonpregnant cows resulted in fewer total days open. De Vries (2006a) calculated costs per extra day open in USA and estimated a variation from $3.19 to $5.41 per cow per year. With a total of 814 animals on the Resynch group, the cost of using a CIDR insert from d 18 to d 25 after AI and a GnRH injection at the time of CIDR removal was estimated as $8,359 (i.e., CIDR = $8.43 and GnRH injection = $1.84). In addition, cows of the Resynch group diagnosed nonpregnant (n = 441) at d 32, were resynchronized receiving a $PGF_{2\alpha}$ (i.e., $2.03) and a GnRH injections, which had an additional cost of $1,706. Thus, the total cost for the use of a CIDR insert and a GnRH injection and the resynchronization program used for the Resynch group were estimated in $10,065. The number of pregnant cows in the Resynch group (n = 532) multiplied by the estimate of fewer total days open (i.e., 3.3 d ; $3.19 per cow per day) detected in the Resynch group resulted in a saving of $5,602, which when subtracted from the total cost gives a net cost estimate of $4,463 or $8.39 per pregnant cow. Cows diagnosed nonpregnant on the Control group were resynchronized with a GnRH injection at d 32, $PGF_{2\alpha}$ injection at d 39 and a subsequent GnRH injection 56 h later. Resynchronization of Control cows had a total cost of $2,358 or $4.59 per pregnant cow. With these estimations, there was no benefit of using a CIDR insert and a GnRH injection for all cows in an earlier resynchronization program,

which reduced days open. Cows in the Resynch group had a higher net cost compared to cows in the Control group ($8.39 vs $4.59 per ultimate pregnant cow).

Early assessment of pregnancy status and fetal viability identifies cows that fail to conceive and allows for an improvement in reproductive efficiency by possibly decreasing the interval between artificial inseminations. The combination of ultrasound technology with new reproductive technologies such as chemical diagnosis of early pregnancy provides for the further development of integrated reproductive management systems (Fricke, 2000). The measurement of PAGs in maternal blood at the time of early implantation of the conceptus is used as the basis for pregnancy diagnosis in cattle (Zoli et al., 1992; Green et al., 2005; Silva et al., 2007). The ELISA assay used in the present study was effective in identifying pregnant cows at d 30 of pregnancy with a sensitivity and specificity of 100% and 90.6%, respectively. Overall mean concentrations of PAGs in plasma did not differ between pregnant cows of the Resynch and Control group. Plasma concentrations of PAGs increased beginning at approximately 24 d, peaked at approximately 30 d, and underwent a decline that occurred 1 week earlier in the Resynch group. Perhaps the slightly earlier decline of PAGs in the Resynch group was due to increased progesterone from insertion of the CIDR insert and GnRH injection (Figure 4-4). The early rise and fall of PAGs in plasma has been reported in early pregnancy (Green et al., 2005) and is followed by a second phase rise throughout pregnancy to term. In cows diagnosed pregnant at d 32, 7 underwent late embryo mortality (LEM) between d 32 and d60 of gestation and had lower plasma concentrations of PAGs at d 30 of pregnancy compared to cows that sustained their pregnancies to d 60. However, concentrations of PAGs were non-

discernable between d 37 to 60 during the descending phase of PAGs in cows experiencing LEM and those that maintained a pregnancy. Lower concentrations of PAGs in plasma of pregnant cows at d 30 may be a predictor of LEM. This is in accordance with a series of studies reporting that measurement of plasma concentrations of PAGs during pregnancy can most likely reflect fetal well-being (Patel et al., 1995, Szenci et al, 2003; Kornmatitsuk et al, 2002). In addition, Scenzi et al. (2000) reported that in cows diagnosed with LEM both bPSPB and bPAG1 concentrations started to decline, while the corpus luteum still produced progesterone. In addition, on d 30 of pregnancy, the ELISA test used for the measurement of plasma PAGs concentrations for diagnosis of pregnancy had a sensitivity and specificity of 100% and 90.6%, respectively when cows with plasma concentrations > 0.33 ng/mL were considered pregnant. The sensitivity indicated that 100% of the cows diagnosed as pregnant at d 30 by plasma PAGs concentrations were indeed pregnant by ultrasound at d 32. Moreover, 90.6% of the cows diagnosed nonpregnant by ultrasound at d 32 were detected as nonpregnant by plasma PAGs concentrations at d 30. Thus, 9.4% of the cows checked nonpregnant at d 32 by ultrasound were diagnosed as pregnant at d 30 by the measurement of plasma PAGs concentrations. Consequently, these animals (9.4%), in the present experiment, would not have been treated with $PGF_{2\alpha}$ until diagnosed nonpregnant by ultrasound (d 32) for the Resynch group or would not have started a resynchronization program with GnRH in the Control group. The role of PAGs during pregnancy has not been elucidated to date. In the present study, in a subsample of 103 cows sampled for measurement of PAGs on the day of parturition, concentrations were only related to sex of calf with maternal plasma PAGs

being higher in cows bearing a male calf. This is likely due to greater size of male calves that reflected a possibly greater size placental-fetal unit that secreted greater amounts of PAGs throughout pregnancy. Others have reported greater concentrations of PAGs associated with male fetuses (Zoli et al., 1992).

Previous studies documented that progesterone supplementation after AI reduced pregnancy loss (El-Zarkouny and Stevenson, 2004; Chebel et al., 2006). Therefore, supplementation of progesterone after AI might benefit pregnancy in lactating dairy cows. Interestingly, in the present study, there were no differences in pregnancy per AI and pregnancy loss between cows that received a CIDR insert compared to cows of the Control group. The mean increase in plasma concentrations of progesterone in lactating dairy cows following insertion of a CIDR device was approximately 0.8 ng/mL (Cerri et al., 2009) and that might not have been enough to significantly increase pregnancy per AI or reduce pregnancy loss in the Resynch treatment group. However, cows that received a CIDR insert and a GnRH injection maintained plasma progesterone concentrations higher even after the removal of the progesterone insert. The slightly sustained concentrations of progesterone during the perimplantation period may attenuate LEM and such a benefit may have been achieved with only a GnRH injection at d 32 in the Control group. The present experiment did not have an experimental group of pregnant cows that did not receive an injection of GnRH to examine LEM. On a relative basis, overall pregnancy losses were low (i.e., 5.4%) for pre-enrolment pregnancies compared to 12.8% summarized across numerous studies (Santos et al., 2004).

Conclusions

Although distinct differences in CL and follicle development were observed between the two treatment groups, the use of progesterone supplementation did not influence pregnancy per AI for first and second TAI services, nor improved embryo survival. However, the use of a CIDR insert and subsequent resynchronization of nonpregnant cows resulted in fewer total days open. The present study showed that different health occurrences decreased pregnancy per AI to first TAI service and emphasizes the importance of transition cow management. PAGs did not differ between the two treatment groups although cows experiencing LEM had lower concentrations of PAGs at d 30 of pregnancy. Plasma concentration of PAGs at the day of parturition was higher for cows that delivered male calves. Measurement of PAGs at d 28 or 30 was sensitive and specific predictors of pregnancy at d 32 as determined by ultrasonography. Progesterone concentrations were higher in the Resynch treatment group for pregnant cows.

Table 4-1. Pregnancy per AI at 32 and 60 d after first and second service timed inseminations for all experimental cows according to treatment groups

	Pre-enrolment AI		Resynchronized AI	
	Control	Resynch	Control	Resynch
Preg/TAI 32d, % (no./no.)	45.9 (351/764)	45.8 (373/814)	39.5 (163/413)	36.0 (159/441)
Preg/TAI 60d, % (no./no.)	44.1 (337/764)	42.7 (348/814)	36.9 (152/412)	33.8 (149/441)
Preg Losses 32 to 60d, % (no./no.)	4.0 (14/351)	6.7 (25/373)	6.7 (11/163)	6.3 (10/159)

Table 4-2. Effect of resynchronization treatments on the distribution of nonpregnant cows with either no CL, 1 or 2 CL at the time of GnRH injection on d 25 Resynch and d 32 Control

	No CL[c]	1 CL[c]	2 CL	Mean[b]	Total Cows
Control	18.9% (15)[a]	70.8% (56)	10.1% (8)	0.92*	79
Resynch	48.5% (34)	48.5% (34)	2.8% (2)	0.61*	70

[a] Number of cows in parenthesis.
[b] Least Square Mean number of CL per treatment.
[c] 1CL vs No CL*Trt ; Odds Ratio: 3.73, 95% Confidence Interval 1.778 -7.841; P < 0.01.
*P < 0.01

Table 4-3. Effect of resynchronization treatments on the distribution of nonpregnant cows with either small, medium, or large follicles at the time of GnRH injection on d 25 Resynch and d 32 Control

	Follicles			Mean[b]	Total Cows
	Small	Medium[a]	Large[a]		
Control	14% (11)	58.2% (46)	35.4% (28)	0.94	79
Resynch	5.7% (4)	38.5% (27)	77.1% (54)	1.16	70

[a]Medium vs Large*Trt; Odds Ratio: 3.29, 95% Confidence Interval 1.700-6.350; P < 0.01

[b]Treatment means for number of Medium and Large follicles per cow.

Table 4-4. Effect of resynchronization treatments distribution of nonpregnant cows with No CL, CL or CL + Large follicle at the time of PGF2α injection on d 32 Resynch and d 39 Control

	No CL	CL[c]	CL + Large Follicle[3]	Mean[b]	Total Cows
Control	12.6% (10)[a]	46.8% (37)	40.5% (32)	0.88	79
Resynch	18.5% (13)	70.0% (49)	11.4% (8)	0.82	70

[a]Number of cows in parenthesis.

[b]Least Square Mean number of CL per cow.

[c]CL vs CL+Large Follicle*Trt; Odds Ratio: 0.189, 95% Confidence Interval 0.078 – 0.457; $P < 0.01$.

Table 4-5. Effect of resynchronization treatments on the presence of CL of nonpregnant cows at the time of pre-enrolment pregnancy diagnosis on d 32 after AI

	No CL[c]	CL[c]	Mean[b]	Total Cows
Control	26.6% (110) [a]	73.3% (303)	0.73	413
Resynch	19.9% (88)	80.0% (353)	0.80	441

[a] Number of cows in parenthesis.
[b]Mean number of CL per cow.
[c]CL vs No CL *Trt; Odds Ratio: 0.687, 95% Confidence Interval 0.499 – 0.945; P < 0.02.

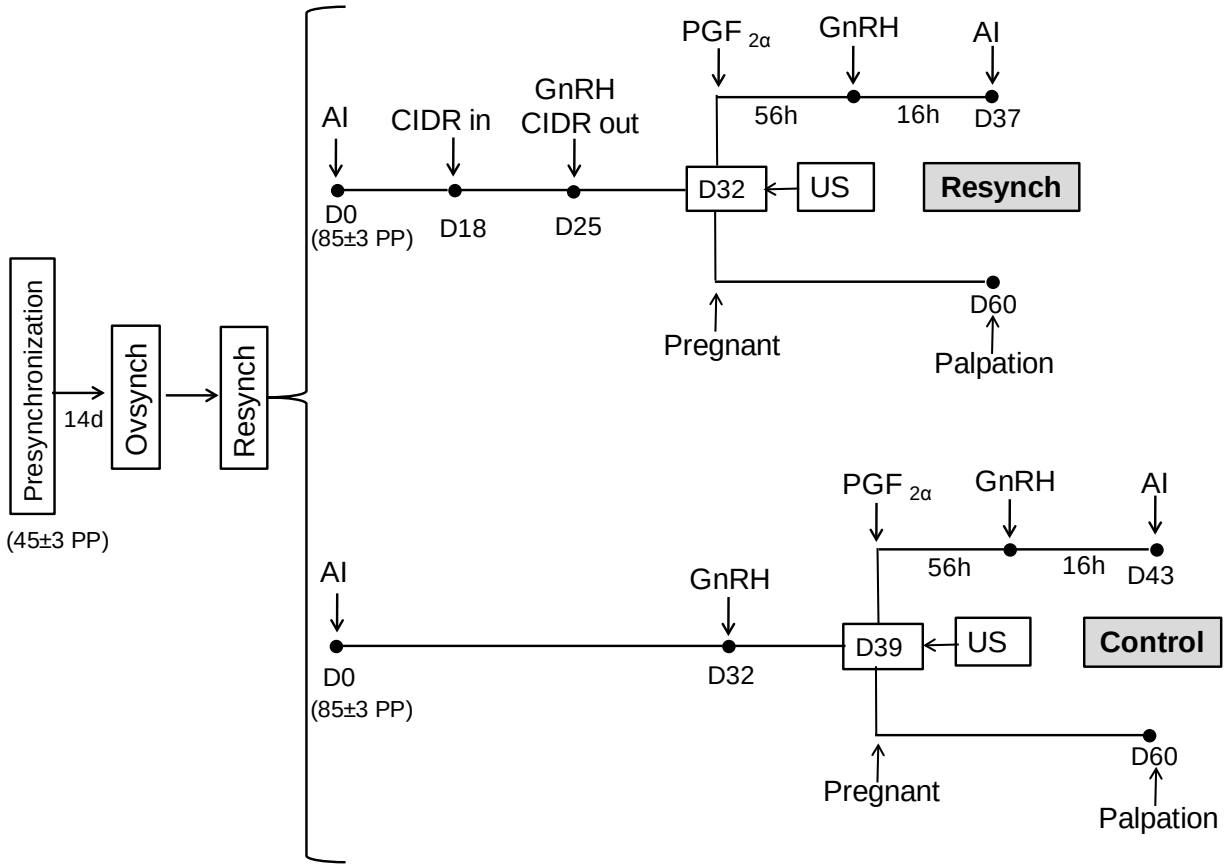

Figure 4-1. Experimental design; AI= artificial insemination; PP= days post partum; CIDR= controlled internal drug releasing containing progesterone; GnRH= injection of gonadotropin releasing hormone; PGF2α= prostaglandin F2α injection; Ovsynch= protocol for synchronization of ovulation; US= ultrasonographic examination.

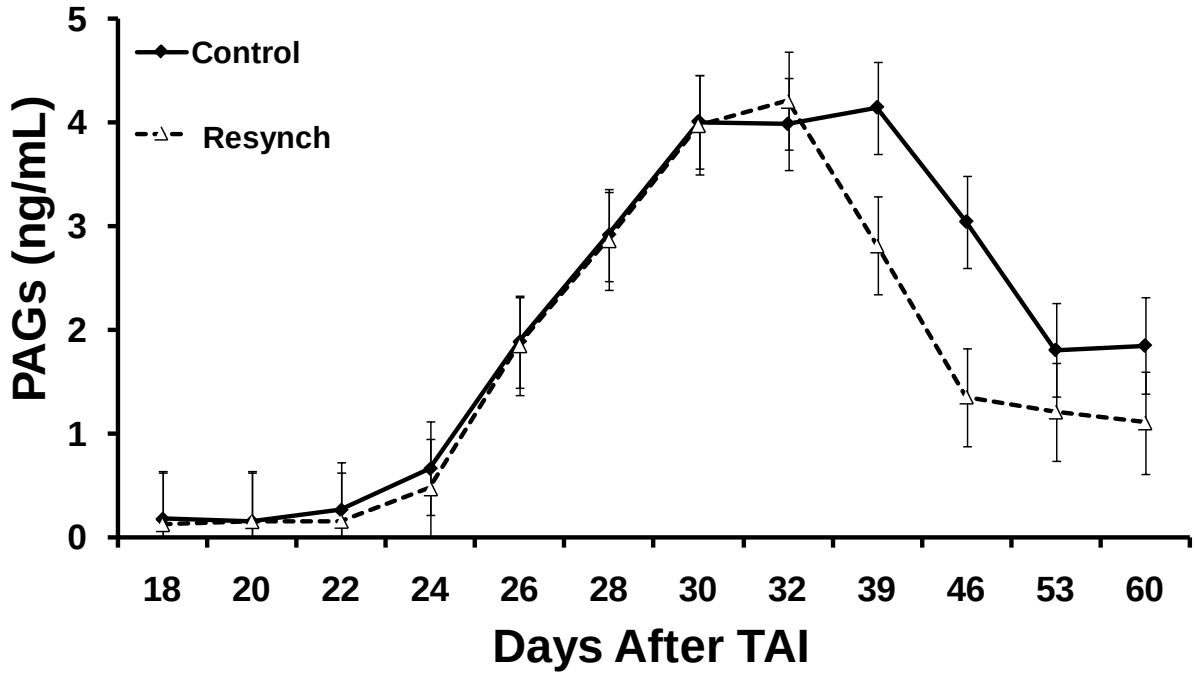

Figure 4-2. Plasma concentrations (ng/mL) of pregnancy associated glycoproteins (PAGs) in pregnant cows from d 18 to 60 of pregnancy for the Control (n = 23) and Resynch (n = 20) groups. Significant differences were detected on d 39 ($P < 0.04$) and 46 ($P < 0.01$) of pregnancy. The LSM ± S.E.M. for PAGs plasma concentrations for Control and Resynch groups were 2.06 ± 0.25 and 1.68 ± 0.27 respectively.

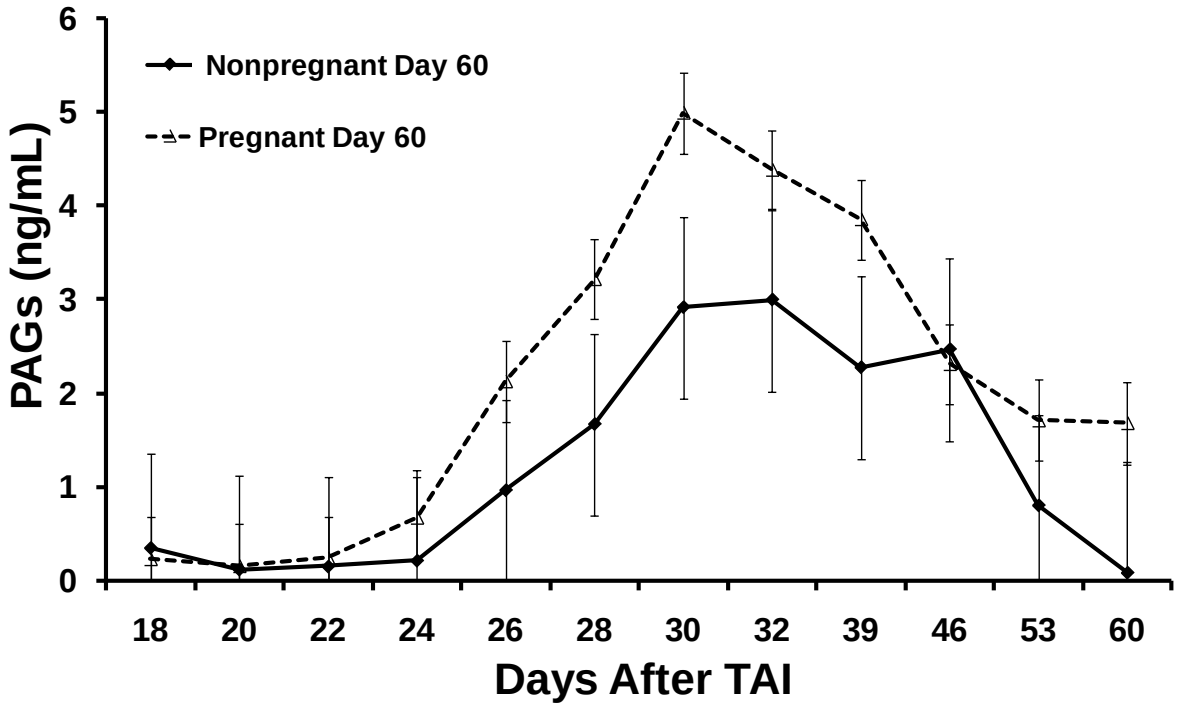

Figure 4-3. Pregnancy associated glycoproteins (PAGs) concentrations of cows (n = 36) that maintained their pregnancy until d 60 after TAI and cows (n = 7) that had pregnancy loss. Cows that experienced pregnancy loss by d 60 after TAI had significant ($P < 0.05$) lower concentrations of PAGs at d 30 after TAI compared to cows that maintained pregnancy (2.91 ± 0.96 vs 4.98 ± 0.42 ng/mL). The overall LSM ± S.E.M. for PAGs plasma concentrations for cows that maintained and lost pregnancy were 2.12 ± 0.24 and 1.24 ± 0.54 respectively.

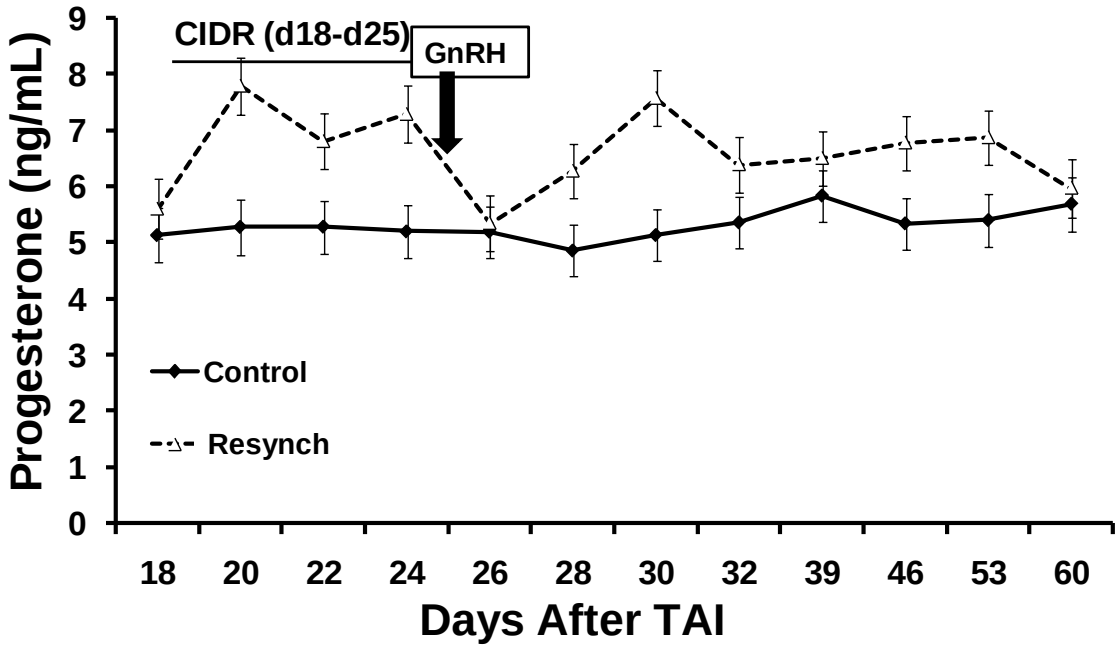

Figure 4-4. Profiles of progesterone plasma concentrations (ng/mL) of pregnant cows from d 18 to 60 of pregnancy of the Control (n = 23) and Resynch (n = 20) groups. Cows in the Resynch group maintained plasma progesterone concentrations higher ($P < 0.003$). The LSM ± S.E.M. for progesterone plasma concentrations for Control and Resynch groups were 5.30 ± 0.28 and 6.59 ± 0.30 respectively. Cows in the Resynch group were treated with a CIDR insert from d 18 to 25 after AI followed by a GnRH injection at the time of CIDR removal (d25). Nonpregnant cows in the Control group received a GnRH injection at d 32 of pregnancy.

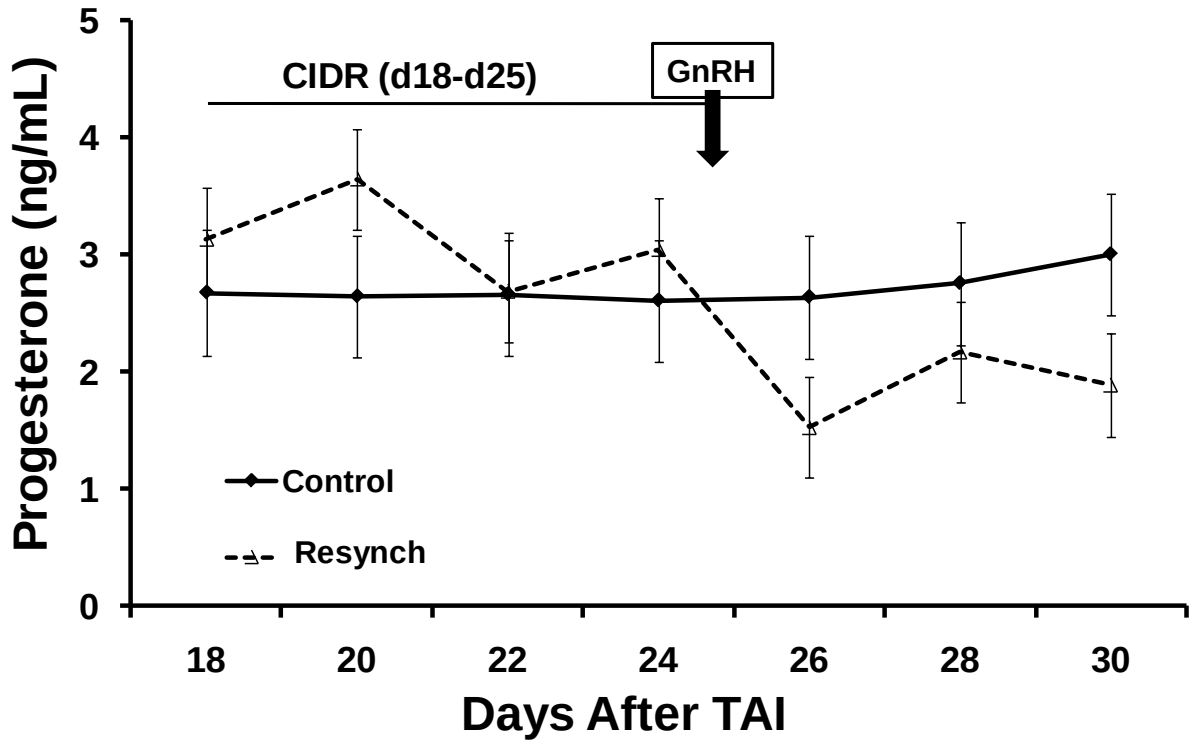

Figure 4-5. Profiles of progesterone plasma concentrations (ng/mL) of nonpregnant cows from d 18 to 30 of pregnancy of the Control (n = 23) and Resynch (n = 34) groups. There was no differences in progesterone plasma concentrations between the treatment groups, however, an interaction between treatment by day was detected ($P < 0.03$). The LSM ± S.E.M. for progesterone plasma concentrations of nonpregnant cows for Control and Resynch groups were 2.71 ± 0.36 and 2.57 ± 0.29 respectively. Cows in the Resynch group were treated with a CIDR insert from d 18 to 25 after AI followed by a GnRH injection at the time of CIDR removal (d25). Nonpregnant cows in the Control group received a GnRH injection at d 32 of pregnancy.

CHAPTER 5
GENERAL DISCUSSION AND CONCLUSION

Synchronization of embryonic development and remodelling of the endometrium are crucial to support fetal-placental development throughout gestation. Establishment of the placenta involves implantation, when fetal derived placental cells (trophoblasts) fuse to the endometrium and deliver secretory products into the maternal system. In ruminants, among the molecules recognized as being synthesized and secreted by trophoblast cells are the pregnancy associated glycoproteins (PAGs). Even though the functional role (s) of these molecules is still unclear, their function (s) may be associated with their spatial and temporal expressions throughout pregnancy in association with processes such as adhesion, implantation and remodeling of the fetal-maternal unit during placental development (Wooding et al., 2005), immune regulation (Dosogne et al., 1999; Hoeben et al., 1999; Austin et al., 1999) and prostaglandin synthesis and regulation (Del Vecchio et al., 1990; Weems et al., 1998 and 2003).

In the past few decades, the shift toward more productive cows has been associated with a decline in fertility. Elucidating the mechanisms that control embryo and endometrial development in early gestation are important for identification of genes regulating implantation, placentogenesis and maintenance of pregnancy. The development of microarray analyses characterizing tissue transcriptome expression has provided an opportunity to study early gestation expression of several thousand genes simultaneously.

In chapter 3, it was hypothesized that lactation would cause a differential expression of endometrial and conceptus expression of genes at day 17 after AI in both cyclic and pregnant cows. Objectives were to characterize postpartum metabolic and

hormonal differences between nonlactating and lactating dairy cows, evaluate lactation and pregnancy effects on endometrium and conceptus gene expression, and characterize associations between conceptus and endometrial expression of genes in early pregnancy (d 17).

Various metabolomic responses such as plasma concentrations of NEFA, BHBA, BUN and glucose are indicative that L cows undergo marked metabolic changes associated with homeoretic processes in response to lactogenesis and galactopoeisis. The alterations in metabolites reflect mobilization of lipids and proteins during a period of negative energy balance (NEB) postpartum. In chapter 3, lactational status did not affect overall postpartum NEFA concentrations ($P < 0.20$); although cows in the NL group had significantly higher ($P < 0.01$) plasma NEFA concentrations in the first week postpartum. This was attributed to a more acute negative energy balance probably related to an adaptation to the maintenance ration during the first week postpartum. L cows had higher plasma concentrations of BHBA compared with NL cows, reflecting the higher energy demand associated with lactation. In addition, lactation impacted BUN plasma concentrations with higher BUN concentrations in L compared to NL cows. This is likely attributed to the greater amounts of dietrary protein fed to the L cows throughout the study period (Table 3-1). Furthermore, L cows had lower plasma concentrations of glucose likely due to a greater utilization of glucose by the mammary gland compared to NL cows. Lactation also had a negative impact in the resumption of postpartum ovulation. NL cows cycled earlier than L cows ($26.4 \pm 2.01 < 34.7 \pm 3.09$ days). In addition, L cows had lower plasma concentrations of progesterone and greater CL weight compared with NL cows during a synchronized post-ovulatory period

approaching the day of slaughter at day17. Effects of lactation, pregnancy and their interaction were detected in plasma progesterone concentrations from day 0 (GnRH/TAI) to day 8 during the period of early CL development after GnRH injection prior to slaughter. NL cows had a greater daily increase in plasma progesterone concentrations compared to L cows. However, L pregnant cows had a lower increase in plasma progesterone concentrations per day compared with L cyclic cows. In contrast there was no difference in the progesterone curves between cyclic and pregnant cows of the NL group. Microarray analyses detected lactation by pregnancy interaction on day 17 for endometrial expression of CYP39A1 which is a gene involved in cholesterol and steroid synthesis. The CYP39A1 participates in cholesterol catabolism and is involved in the conversion of cholesterol into bile acids. CYP39A1 oxysterol 7α hydroxylase preferentially catalyzes the 7 α hydroxylation of 24 hydroxycholesterol for subsequent cholesterol clearance (Figure 5-1). A subsequent ring structure modification occurs, where the 7α-hydroxylated intermediates are converted into their 3-oxo, Δ^4 forms by a microsomal 3β-hydroxy-Δ-C_{27}–steroid oxireductase (C_{27} 3β-HSD). The reaction catalized by these enzymes involves isomerization of the double bond from 5 to the 4 position and the oxidation of 3β-hydroxyl to a 3-oxo group. Products of the C_{27} 3β-HSD take one of two routes in subsequent steps of bile acid synthesis. If the intermediate is acted upon by sterol 12α hydroxylase (CYP8B1), then the resulting product will be converted into cholic acid. If 12α hydroxylation is absent, chenodeoxycholic acid or another bile acid is formed. The 12α hydroxyalted intermediates and those produced by C_{27} 3β-HSD enzyme that escape 12α hydroxylation, are subjected to reduction of the double bond in the A-ring by the enzyme $\Delta^{4\text{-}3\text{-}}$oxosteroid 5 β-reductase. The final step of

the ring modification involves reduction of the 3-oxo group to an alcohol in the alpha stereochemical configuration and is catalyzed by 3α-hydroxysteroid dehydrogenase. Products of ring modification next undergo progressive oxidation and shortening of the sterol side chain, which is performed by CYP27A1 through the formation of 27-hydroxycholesterol. Subsequently, the terminal step in bile acid synthesis involves conjugation of bile acids to an amino acid, which increases the amphipathicity and enhances the solubility of the molecules, making them impermeable to cell membranes. L pregnant cows had higher expression of CYP39A1 compared with cyclic L cows and NL cyclic and pregnant cows. A preferential difference of greater CYP39A1 expression in pregnant L cows than cyclic L cows may contribute to the lower plasma progesterone concentrations observed in L pregnant cows. If greater cholesterol is cleared by the endometrium and liver in lactating dairy cows than less cholesterol would be available for uptake by the ovary for the synthesis of progesterone by the CL in lactating dairy cows. Even though, it is unknown whether the endometrium is metabolizing cholesterol to a degree that will affect liver cholesterol clearance, CYP39A1expression in the endometrium may be involved in local cholesterol clearance and control of local gene expression. High CYP39A1 expression in the endometrium may result in subsequent increase of cholesterol clearance, which may affect the availability of cholesterol for CL synthesis of progesterone and processing of specific proteins such as Hedgehog, which is involved in proper early embryo development.

In chapter 3, an array of genes known to be important and expressed during early pregnancy was identified (Table 3-2). The following classes of genes were identified in both conceptuses and endometrial tissues that were deemed important for early embryo

development and endometrial programming to sustain a pregnancy: prostaglandin regulatory genes (i.e., prostaglandin synthases and prostaglandin receptor genes), trophoblast cell specific genes (i.e., interferon tau and trophoblast kunitz domain proteins), genes involved in invasion and implantation (i.e. dickkopf homolog and pentraxin-related gene), immune system related genes (i.e., serpins, interferons, interleukins, tumor necrosis factors), growth factor genes (i.e., fibroblast growth factors, transforming growth factors), genes involved in cholesterol and steroid metabolism (i.e., cytochrome P450, family 39, subfamily A, polypeptide 1) and progesterone regulatory genes (i.e., progesterone receptor genes, progesterone immnunodulatory binding factor). Prostaglandin related genes expressed in the conceptus were not affected by lactation. Moreover, no effect of pregnancy or lactation was observed in endometrial expression of HPGD and PGRMC2. However, lower endometrial expression of PTGDS was observed in pregnant cows. In contrast, pregnancy enhanced endometrial expression of PIBF1, DKK1 and PTX3. In addition, DKK1 expression was inhibited by lactation. Genes expressed exclusively by trophoblast cells, such as TP1, TKDP1 and PAG11, were present in the endometrium of pregnant cows, and their expression did not differ between L and NL cows.

Expression of PAG genes was examined specifically at day 17 of pregnancy. Different PAG family genes were identified in both conceptuses and endometrial tissues at d 17 of pregnancy. To date it has been documented that PAG genes are expressed exclusively by the placenta. However, the present study detected expression of PAG genes in endometrial tissue at d 17 of pregnancy. All PAG genes observed in the endometrium (i.e., PAG 2, PAG 8, PAG 11 and PAG 12) were members of the ancient

PAG group (i.e., expressed throughout the trophectoderm). Presence of PAG genes and other genes expressed exclusively by trophoblast cells (i.e., TP1, TKDP1) in endometrial tissue at d 17 of pregnancy may reflect the presence of trophoblast cells in the endometrium tissue due to the intimate relationship between the trophectoderm and the endometrium at this time of pregnancy. Based on studies that reported possible functional roles for PAG genes, a series of both simple and partial correlation analyses between PAG genes expressed by the conceptus and endometrial tissues at d 17 of pregnancy and various candidate genes of interest were undertaken (Figures 3-13 and 3-14). The candidate genes included those expressed exclusively by trophoblast cells and genes expressed in the endometrium of both pregnant and cyclic animals. Correlation analyses were conducted with the intent of identifying associations that might be important functionally for pregnancy and to provide a basis for future studies to identify the possible function (s) of PAG genes. PAG11 was the PAG gene most highly expressed in both conceptus and endometrial tissues at d 17 of pregnancy and became the focus for the correlation analyses on this PAG family gene. Proposed roles in which PAG genes act in adhesion, implantation and remodeling of the fetal-maternal unit during placenta development (Wooding et al., 2005) and immune regulation (Dosogne et al., 1999; Hoeben et al., 1999; Austin et al., 1999) have been reported in various studies. In chapter 3, no correlations between conceptus expressed PAG11 and conceptus genes involved in immune control were detected at d 17 of pregnancy. However, simple correlation analyses showed that endometrial PAG11 was correlated with PTX3, a gene that is produced by various tissues in response to pro-inflammatory signals and has been linked with implantation and decidualization processes in mice

(Tranguch et al., 2007). Moreover, endometrial PAG11 of the present study was correlated with DKK1, a gene that antagonizes Wnt/β-catenin signaling, by inhibition of the Wnt coreceptors Lrp5 and Lrp6. In addition, DKK1 has high affinity for the transmembrane proteins Kremen1 and Kremen2, which are also involved in the modulation of Wnt signaling. In addition, DKK1 is involved in early development of head structures anterior to the midbrain and it has been documented to function as a suppressor of tumor growth in colon cancer cells in humans (Niehrs, 2006; Aguilera et al., 2006). Furthermore, it has been documented that DKK1 promotes trophoblast cell invasion in mice (Peng et al. 2008). The associations identify these genes as possible candidates for future studies involving the role of PAGs during embryo development, trophoblast cell invasion and implantation. Furthermore, PTX3 was correlated positively with DKK1, suggesting that PTX3 might be acting in combination with DKK1 in the establishment of implantation. In addition, PTX3 was correlated negatively with prostaglandin D2 synthase gene (PTGDS). It has been documented that prostaglandin D 2 is converted to a biologically active $PGF_{2\alpha}$ stereoisomer (9α, 11β $PGF_{2\alpha}$) through the action of an enzyme called 11-ketoreductase (Theodore et al., 1985). This PGD2 metabolite, via a receptor, mediates cell migration and activation and antagonizes the effects of $PGF_{2\alpha.}$ This association might suggest an alternative way to attenuate $PGF_{2\alpha}$ like effects at this stage of pregnancy. Moreover, results of standard partial correlation analyses showed a negative association between PTGDS and HPGD. Hydroxyprostaglandin dehydrogenanse is known to be involved in the degradation of prostaglandins such that a sequential reduction in PTGDS would enhance HPGD and increase $PGF_{2\alpha}$ metabolism in early pregnancy, which might be a link between

formation and destruction of $PGF_{2\alpha}$ in the endometrium. This tight regulation possibly allows an adequate functionality of reproductive events such as luteolysis, maintenance of the CL in pregnancy, implantation and even parturition.

In addition, HPGD was correlated positively with expression of the PGRMC2 gene, which encodes a protein that binds progesterone. Thus, this correlation indicates that endometrial progesterone binding might be associated with degradation of prostaglandins in early pregnancy. In addition, simple correlation analyses showed that endometrial PAG 11 was positively correlated with HPGD and PGRMC2. Moreover, standard partial correlations indicated a positive association between endometrial PGRMC2 and PIBF1. Szkeres-Bartho et al. (2001) documented that PIBF1 inhibition of natural killer (NK) cell activity is demonstrated by an altered cytokine production both in vitro and in vivo. Furthermore, PIBF1 inhibits arachidonic acid release by exerting a direct effect on inhibiting the phospholipase A2 enzyme. Thus, the mechanisms above describe PIBF1 as an anti-abortive agent. The positive correlation between gene expressions of PGRMC2 and PIBF1 in the endometrium may implicate such cellular regulatory events are involved in normal pregnancy outcomes in the cow. In addition, PTGDS, which is antagonizes the effects of $PGF_{2\alpha}$, was correlated negatively with PIBF1 at d 17 of pregnancy. This negative correlation would actually enhance PIBF1 since PTGDS was decreased due to the negative association with the gene expression of PTX3.

Simple correlation analyses showed that endometrial PAG 11 was correlated highly with trophoblast expressed genes such as TKDP1 and TP1 (i.e., interferon tau). Moreover, standard partial correlations holding TP1 as constant resulted in a high

association between TKDP1 and PAG 11. Even though TKDPs function is not yet established, most TKDPs are expressed during early pregnancy and may be important regarding the interactions of the conceptus-maternal unit (Maclean et al., 2003). Association of PAG11 with trophoblast cell specific genes at d 17 of pregnancy is important in elucidating the role that these molecules are playing in the ruminant placenta during early pregnancy.

It has been proposed that PAG genes might be involved in prostaglandin synthesis and regulation (Del Vecchio et al., 1990; Weems et al., 1998 and 2003). Conceptus expression of PAG11 was correlated positively with PGH2 synthase, PTGES, and PTGES3, and negatively correlated with $PGF_{2\alpha}R$ genes of the conceptus. However, standard partial correlation analyses holding PGH2 synthase as a constant showed a decrease in the correlations between PAG11 and downstream (i.e., distal to PGH2 synthase) enzymes and receptors such as PTGES, PTGES3 and $PGF_{2\alpha}R$. Consequently, PAG11 may primarily affect upstream expression of PGH2 synthase that than leads to downstream regulation of PGES, PGES3 and $PGF_{2\alpha}R$ expression. These correlations between PAG11 and genes involved in the prostaglandin cascade in early pregnancy within the conceptus may implement a role of PAG11 with mechanisms involved in maintenance of pregnancy, invasion of the endometrium, maintenance of the CL through the endometrium, immunoregulation within the endometrial environment and placentation.

Acceptable pregnancy per AI per AI have been achieved with the use of ovulation synchronization programs for TAI. Moreover, early detection of nonpregnant cows combined with the initiation of a resynchronization of ovulation protocol will improve

reproductive efficiency of dairy herds. Protocols for resynchronization of ovulation can increase the effective AI service rate and reduce the interval between AI services (Fricke. 2002). In addition, the use of an intravaginal device for the release of progesterone (CIDR, controlled internal drug-release, Eazi Breed™ CIDR® Pfizer Animal Health, New York, NY) after insemination has become available as an additional tool for synchronizing the return of estrus (i.e., resynchronization) of nonpregnant cows.

In the second experiment (Chapter 4), a resynchronization program was developed, using a CIDR insert (Resynch) from d 18 to d 25 after TAI followed by a GnRH injection at the time of CIDR removal, ultrasound pregnancy diagnosis at d 32 and administration of $PGF_{2\alpha}$ injection in non pregnant cows, GnRH 56h after and TAI 16 h later (d 35), with the aim to reduce the interval between AI services and improve pregnancy per AI and embryo survival in lactating dairy cows. Cows in the Control group were diagnosed for pregnancy at d 32 and nonpregnant cows received GnRH, d 39 $PGF_{2\alpha}$, GnRH 56 h after, and TAI 16 h later (d 42). Examination of ovarian structures of the Resynch and Control groups at the time of GnRH and $PGF_{2\alpha}$ injections showed clear differences in ovarian structures between the two groups. Cows in the Resynch group had a lower occurrence of CL and higher percentage of large follicles at the time of GnRH injection compared with cows in the Control group. Nonpregnant cows of the Control group likely had undergone spontaneous CL regression associated with development and ovulation of a preovulatory follicle. Thus, a developing first wave follicle would be present at the time of GnRH injection (d 32), which accounted for the greater frequency of CL and medium size follicles at d 32. The GnRH injection at d 25 appeared to induce greater synchronization of large follicles in the Resynch group. This

is suggested based on equal frequency of CL at the time of $PGF_{2\alpha}$, but smaller percentage of CL with large follicles compared to the Control group. Follicle development was less synchronized in the Control group, with a wider array of ovulatory and non-ovulatory follicles at the time of GnRH injection, which resulted in a greater number of Control cows with a CL and a large follicle at the time of $PGF_{2\alpha}$ injection. Even though, distinct differences in ovarian dynamics were observed between Resynch and Control groups, pregnancy per AI for first and second TAI services was similar for both groups. However, the use of a CIDR implant allowed a shorter interval between inseminations. The use of a CIDR insert and a GnRH injection after insemination in 814 cows of the Resynch group and administration of a $PGF_{2\alpha}$ and a GnRH injection for resynchronization of nonpregnant cows (n = 441) had a cost of $8.39 per pregnant cow of the Resynch group. In contrast, administration of a $PGF_{2\alpha}$ and two GnRH injections for resynchronization of nonpregnant cows of the Control group (n = 413) had a cost of $4.59 per pregnant cow of the Control group. With these estimations, there was no benefit on using a CIDR insert in a resynchronization program, which reduced days open. Pregnant cows in the Resynch group had a higher cost per day compared to pregnant cows in the Control group ($8.39 vs $4.59).

Previous studies documented that progesterone supplementation after AI reduced pregnancy loss (El-Zarkouny and Stevenson, 2004; Chebel et al., 2006). In the second experiment (Chapter 4), there were no differences in pregnancy loss between cows that received a CIDR insert compared to cows of the Control group; even though cows that received a CIDR insert and GnRH at CIDR insert removal maintained plasma progesterone concentrations higher even after the removal of the progesterone insert.

Reproductive inefficiency is associated with the occurrence of periparturient diseases. Several studies documented a decrease in fertility for cows affected with disorders of the reproductive tract, lameness, mastitis and metabolic disorders (LeBlanc, 2008; Garbarino et al., 2004; Schrick et al., 2001; Opsomer et al., 2000). In accordance with previous studies, we observed that the occurrence of digestive problems, mastitis, dystocia and retained fetal membranes decreased pregnancy per AI for the pre-enrolment in a Presynch-Ovsynch program.

The combination of ultrasound with new reproductive technologies for diagnosis of early pregnancy allows for the development of integrated reproductive management systems (Fricke, 2000). Even more important is the potential to diagnose non-pregnant cows that can be re-programmed for a repeat service in a timely manner. Several studies documented the measurement of PAGs, a gene family temporally expressed by both mononucleated and binucleated trophoblast cells throughout pregnancy, in maternal blood as the basis for pregnancy diagnosis (Zoli et al., 1992; Green et al., 2005; Silva et al., 2007), and identification of both embryo/fetal well being and pregnancy loss in cattle (Patel et al., 1995, Kornmatitsuk et al, 2002; Szenci et al, 2003). In the experiment from Chapter 4, circulating concentrations of progesterone and PAGs were evaluated and compared between Resynch and Control groups. Results showed that plasma concentrations of PAGs did not differ between the two treatment groups. Although cows that underwent LEM between d 32 and 60 of gestation had lower plasma concentrations of PAGs at d 30 of pregnancy. In addition, on d 30 of pregnancy, the ELISA test used for the measurement of plasma PAGs concentrations for diagnosis of pregnancy had a sensitivity and specificity of 100% and 90.6%,

respectively when cows with plasma concentrations > 0.33 ng/mL were considered pregnant. The sensitivity indicated that 100% of the cows diagnosed as pregnant at d 30 by plasma PAGs concentrations were indeed pregnant by ultrasound at d 32. Moreover, 90.6% of the cows diagnosed nonpregnant by ultrasound at d 32 were detected as nonpregnant by plasma PAGs concentrations at d 30. Thus, 9.4% of the cows checked nonpregnant at d 32 by ultrasound were diagnosed as pregnant at d 30 by the measurement of plasma PAGs concentrations. Consequently, these animals (9.4%), in the present experiment, would not have been treated with $PGF_{2\alpha}$ until diagnosed nonpregnant by ultrasound (d 32) for the Resynch group or would not have started a resynchronization program with GnRH in the Control group. Therefore, there was no inefficiency obtained with 90.6% specificity for a nonpregnancy diagnosis with measurement of plasma PAGs concentrations at d 30 under the reproductive management program of an ultrasound confirmation at d 32 after pre-enrolment. Moreover, evaluation of plasma PAGs concentrations on the day of parturition showed that concentrations were related to sex of calf, with higher concentrations for cows that delivered male calves. This result agrees with a study done by Zoli et al. (1992) and may be explained by a possibly greater size placental-fetal unit of cows carrying male calves, which secreted greater concentrations of PAGs throughout pregnancy.

In conclusion, in Chapter 3, lactation\diet altered metabolic status even though BW and BCS were the same between L and NL. Lactation delayed initiation of cyclicity and lowered concentrations of progesterone in pregnant L cows during a programmed period following an induced ovulation. Early expressions of PAG genes within the conceptus and endometrium of pregnant cows and its association with other genes infer

a possible role of PAGs in pregnancy maintenance and implantation by regulation of prostaglandin biosynthesis and identification of possible candidate genes for future studies. In Chapter 4, Resynch and Control protocols had comparable pregnancy per AI for first and second TAI services, but pregnancy occurred 3.3 d earlier in the Resynch group. Plasma progesterone was greater for pregnant cows in the Resynch group. CIDR and/or GnRH increased progesterone during pregnancy. Dynamics of PAGs were indicative of pregnancy status and pregnancy loss.

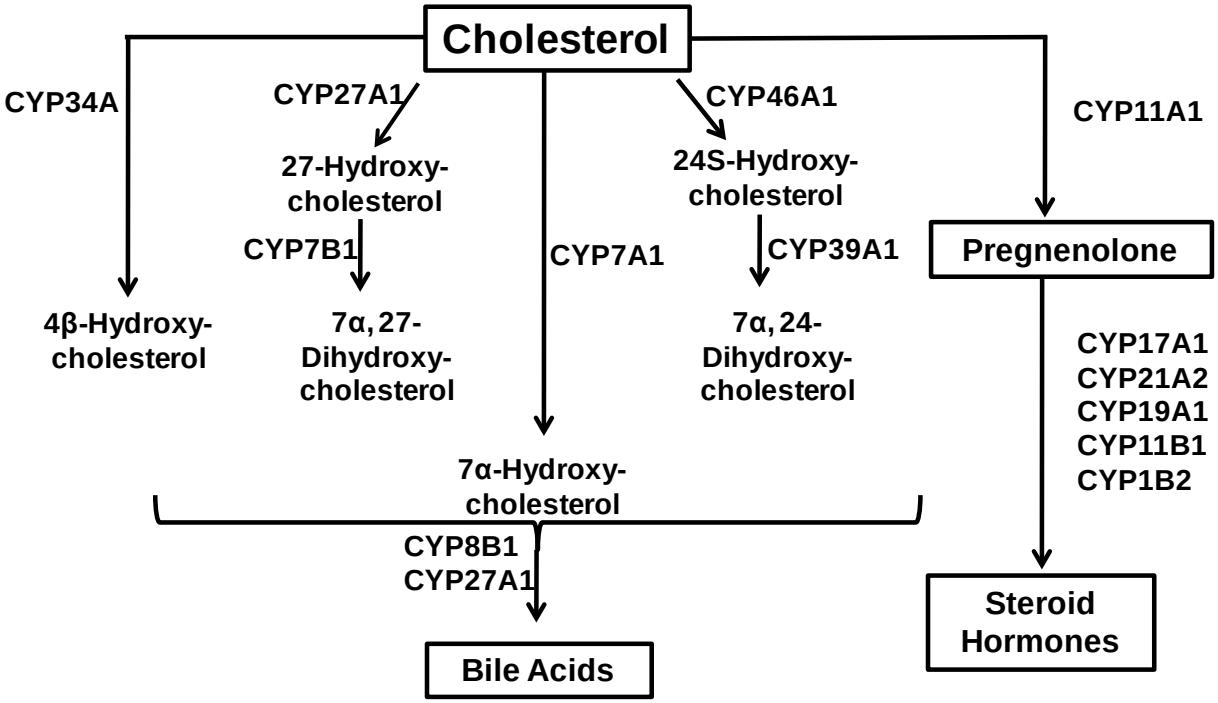

Figure 5-1. Cholesterol metabolism

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BIOGRAPHICAL SKETCH

Izabella Maria Thompson was born in Ponta Grossa, Paraná, Brazil. In 2000, she moved to USA for a year of internship in dairy cattle. In 2002 she received her degree in veterinary medicine from the Pontifícia Universidade Católica do Paraná, Brazil. After her degree, Izabella moved to Gainesville with her husband Bryan and worked at the Clinical Microbiology/ Serology/ Parasitology service at the University of Florida Veterinary Medical Center. In the Fall of 2007, Izabella began her Master's program in Animal Molecular and Cellular Biology with emphasis in dairy cattle reproductive physiology under the supervision of Dr. William W. Thatcher. In the Fall of 2009 Izabella will pursue her doctoral degree with Dr. Geoffrey Dahl.