

MANIPULATING OVARIAN FUNCTION AND UTERINE HEALTH WITH THE AIM OF
IMPROVING FERTILITY IN DAIRY CATTLE

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2013

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To my wife Bianca, my parents Gaspar and Dolores, my siblings, and God that unconditionally supported me with love and comprehension under all circumstances providing me strength and focus necessary to surpass all challenges and successfully accomplish this important goal of my career

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor Dr. José Eduardo Portela Santos, for the opportunity of pursuing a PhD degree at the University of Florida, for his unconditional support, encouragement, inspiration, and enthusiasm during the undertaking of this achievement. I vastly admire his outstanding knowledge, work ethics, dedication and passion for research, which has been a role model for young scientists.

I extended my appreciation to my committee members, Dr. Carlos. A. Risco, Dr. Klíbs. N. Galvão, Dr. Peter J. Hansen, and Dr. William W. Thatcher, for their insightful contribution throughout the 4 years of my program helping me to develop as scientist and person. I feel extremely honored for having some of most prestigious reproductive biologists and theriogenologists in the world as part of committee. I am genuinely thankful to each of you for the countless hours spent guiding me through design of studies, interpretation and discussion of results and overall development of critical thinking skills to become a better scientist. I have been always passionate about research and the interactions with this superb group of scientists magnify my admiration for science and how it can enrich lives and how it advances knowledge.

I would like to extend my appreciation to Dr. Marcel Amstalden from Texas A&M University for his collaboration helping me with sample analysis for luteinizing hormone. I also would like to thanks Dr. Xiaoling Wang and Dr. Mary Reinhard from the College of Veterinary Medicine at the University of Florida for their help with laboratory procedures and analyses.

I owe a special thanks to all of my labmates, Rafael S. Bisinotto, Eduardo S. Ribeiro, Leandro F. Greco, Natalia P. Martinez, Letícia D. P. Sinedino, and Gabriel C.

Gomes. Their innumerable hours helping designing studies, discussing ideas, solving problems and conducting research in the lab, in the farm and in our offices were essential to conclude successfully all the work presented in this dissertation.

I extend my appreciation to the Animal Sciences graduate students Guilherme Marquezini, Izabella Thompson, Paula Morelli, Sha Tao, Davi Araújo, Dan Wang, João Henrique Bittar, Vitor Mercadante, and Milerky Perdomo; and visiting students Maurício Favoreto, Henderson Ayres, Rafael Marsola, Mariana Carvalho, Ana Lúcia Sevarolli, Ricardo Surjus, Alana Calaça, Andressa Ranieri, Bianca Libanori, Erika Ganda, Luis Fernando Vilela, Vinicius Rezende, Wedson Costa, José Tiago Neves Neto, Thiago Villar, Angélica Pedrico, Achilles Vieira Neto, Guilherme Vasconcellos, Rodolfo Mingoti, Tony Bruinje, Eduard Sole, Javier Juarez, Pedro Monteiro, André Dias, Raylon Maciel, Jéssica Felice, and Alberto Zerlotini for their valuable help with the experiments.

I would like to extend my appreciation to the Animal Sciences' graduate program at the University of Florida for all support and for the alumni fellowship that funded my PhD program. I also would like to thank the faculty and staff of the Department of Animal Sciences at the University of Florida. In particular, Dr. Alan Ealy and Dr. Joel Yelich for allowing me to use their laboratories, Sergji Sennikov and Joyce Hayen for their assistance with sample processing and general laboratory procedures. I would like to thank Joann M. Fischer for all her help with the paperwork throughout my PhD program. Finally, I would like to thank Eric Diepersloot, Jay Lemmermen, and Sherry Hay at the University of Florida Dairy Unit for their assistance with animal handling. I also would like to thank Dr. Carlos Risco and Dr. Klibs Galvão at the College of Veterinary Medicine at the University of Florida, for sharing their laboratory.

I am very grateful to the owner of Alliance Dairies, Ronald St. John, and staff for use of their cows and facilities. Special thanks to Dr. Nilo Francisco, Pete Hetherington, Guy Wayne, Antonio, Franklin, Geraldo, Ricardo, Felino, Tino and Amadeo de Paz for assistance with the experiments. I am also very grateful to the owners of Dairy Production System, Dave Samural and Michael Pedreiro, and staff for use of their cows and facilities in some of my experiments.

I thank all my friends in Gainesville that throughout the last 4 years were like family to me and helped me overcome the distance from home. I especially thank Rafael Bisinotto, Leandro Greco, Eduardo Ribeiro, and Natalia Martinez whom, in addition to the uncountable days of hard work with the design and conduct of all experiments, also made me feel at home in Gainesville and were by my side through all challenges of life. I really appreciate their friendship.

I owe special recognition to my entire family in Brazil. To my parents, Gaspar and Dolores, for their sacrifice, prayers, and unconditional support. I am so grateful to have a model of humbleness and moral that centered my principles and built my character throughout the years. They are the foundation of who I am and I can only imagine how much sacrifice and pain they have been through to provide me an opportunity to pursue my dream and acquire a PhD degree in another country far away from home. I would like to especially thank my brother and sisters, Eliton, Elaine, and Andressa, for their friendship and great support during my journey.

Final and special thanks go to my wife Bianca Martins that supported me with love, patience, complicity and comprehension during this very important stage of my career.

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LIST OF ABBREVIATIONS

3 β -HSD	3-beta-hydroxysteroid dehydrogenase / Δ 5, Δ 4 isomerase
AI	artificial insemination
AMH	anti-Mullerian hormone
Ang	angiotensin
ANOVA	analysis of variance
AHR	adjusted hazard ratio
AOR	adjusted odds ratio
ATP	adenosine tri-phosphate
BCS	body condition score
BHBA	β -hydroxybutyrate
BMP	bone morphogenetic protein
Ca	calcium
cbp	collagen binding protein
CARTPT	cocaine and amphetamine regulated transcript
cAMP	cyclic adenosine monophosphate
CD	cluster differentiation
CCL20	chemokine C-C motif ligand 20
CIDR	control internal drug release
CL	corpus luteum
COS72	cosynch timed artificial insemination protocol with 72h of proestrus
CV	coefficient of variation
CXCL1	chemokine C-X-C motif ligand 1
DAG	1,2-diacylglycerol
DIM	days in milk

DMI	dry matter intake
EDTA	ethylenediaminetetraacetic acid
END	endothelin
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
fim	type I fimbriae
FSH	follicle stimulating hormone
fyu	ferric yersiniabactin uptake
GDF	growth differentiation factor
GH	growth hormone
GnRH	gonadotropin-releasing hormone
IFN τ	interferon tau
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL	interleukin
ISG	interferon stimulant genes
LH	luteinizing hormone
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MD2	myeloid differentiation factor 2
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
MYD88	myeloid differentiation factor 88
NEFA	nonesterified fatty acid
NF κ B	nuclear factor kappa B

NO	nitric oxide
OVS56	Ovsynch time artificial insemination program with 56h of proestrus
P/AI	pregnancy per artificial insemination
PAPP-A	plasma-associated pregnancy protein-A
PG	prostaglandin
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
pyo	pyolysin
PVD	purulent vaginal discharge
RIA	radioimmunoassay
StAR	steroidogenic acute regulatory protein
SCE	subclinical endometritis
TGF	transforming growth factor
TIMP	tissue inhibitor metalloproteinase
TLR4	toll-like receptor 4
TMR	total mixed ration
TNF	tumor necrosis factor
US	United States
VEGF	vascular endothelial growth factor
VF	virulence factors

Abstract of Dissertation Presented to the Graduate School
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Requirements for the Degree of Doctor of Philosophy

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August 2013

Chair: José Eduardo Portela Santos
Major: Animal Sciences

The objectives of this dissertation were to determine reproductive management strategies and hormonal manipulations of ovarian responses to optimize fertility of dairy cattle and to investigate the efficacy of treatments of uterine diseases and its subsequent effects on fertility of dairy cows. An additional objective was to determine mechanisms of induced infection with *T. pyogenes* on length of the estrous cycle.

Chapter 3 describes effects of 1 (1TAI) or 3 timed AI (3TAI) before natural service in lactating dairy cows not detected in estrus on pregnancy, and revealed that 3TAI had greater hazard of pregnancy and 21-day cycle pregnancy rate than 1TAI.

Chapter 4 evaluated the effects of the first gonadotropin-releasing hormone (GnRH) injection of the 5-d timed AI program on ovarian responses and pregnancy per AI (P/AI) and the effect of timing of the final GnRH to induce ovulation relative to AI on P/AI in dairy heifers. Use of GnRH on the 1st day of the 5-d timed AI program resulted in low ovulation and no improvement in P/AI when a single prostaglandin (PG) F_{2α} injection on d 5 was used. Extending proestrus by delaying the final GnRH from 56 to 72 h concurrent with AI benefited P/AI of heifers not in estrus at AI.

Chapter 5 investigated the effects of GnRH at the initiation of the 5-d timed AI program combined with two injections of PGF_{2α} on ovarian responses and P/AI in dairy heifers, and the role of progesterone on LH release and ovulation in response to GnRH. Increased ovulation with GnRH given at initiation of 5-d timed AI program combined with two doses of PGF_{2α} on d 5 and 6 optimized P/AI. High progesterone at GnRH administration suppressed LH release and impaired ovulation.

Chapter 6 investigated the efficacy of PGF_{2α} as a therapy to reduce the prevalence of subclinical endometritis and to increase P/AI in cows subjected to a timed AI program. The results of this study revealed that treatments with PGF_{2α} before initiation of the timed AI program were unable to improve uterine health and P/AI.

Chapter 7 investigated if intrauterine infusion of *T. pyogenes* influence uterine expression of genes related to inflammation, luteolytic cascade and luteal lifespan. Although *T. pyogenes* reduced luteal lifespan in half of the cows, only minor changes in uterine gene expression were identified.

Chapter 8 investigated the efficacy of ampicillin trihydrated for treatment of metritis and subsequent effects on fertility. Ampicillin was an efficacious therapy for metritis but had no differential effects on P/AI when compared with ceftiofur hydrochloride.

CHAPTER 1 INTRODUCTION

The evolution of the dairy industry in the United States with development and implementation of genetic selection of dairy cattle, refinements in housing, physiological and nutritional aspects and coordinated management strategies improved productivity per animal and overall efficiency in dairy operations. However, the historical trend for fertility of lactating dairy cows followed a different path with worsening reproductive responses and a negligible genetic selection for reproductive and health traits (Lucy et al., 2001; Norman et al., 2009). Some issues related with this decline in reproductive performance include aspects of cow physiology, reproductive management and animal health, and actions in these areas are expected to reverse the decline and improve cow fertility (Lucy et al., 2001, Royal et al., 2008).

One of the most important advancements made by reproductive physiologists was the development of timed AI allowing the insemination of cows without the need for detection of estrus with acceptable fertility (Pursley et al., 1996; Schmitt et al., 1996).

The first study presented in this dissertation discusses how strategic incorporation of timed AI to breeding programs using natural service impacts reproductive performance. The studies included in Chapters 4 and 5 were conducted to understand mechanisms underlining reproductive physiology of dairy heifers, and how to improve hormonal manipulation of the estrous cycle to maximize fertility when 5-d timed AI program is used. The studies presented in Chapters 6 and 8 contain an additional focus of this dissertation with evaluation of the efficacy of therapies for subclinical endometritis and metritis with the aim of mitigating the negative impact of these uterine diseases on fertility. Finally, Chapter 7 of the dissertation discusses the

effects of intrauterine infusion of *T. pyogenes* on uterine gene expression of inflammatory mediators, luteolytic factors and luteal lifespan.

Inadequate or inaccurate detection of estrus are critical factors responsible for poor reproductive performance in dairy cows (Senger, 1994; Roelofsa et al., 2010). Timed AI and natural service (NS) are common methods to manage reproduction in dairy herds in the United States (Champagne et al. 2002, NAHMS, 2002; Smith et al. 2004; De Vries et al., 2005; Caraviello et al., 2006; Lima et al., 2009) and can be used successfully without detection of estrus (Lima et al., 2009). Although AI hastens genetic progress, controls venereal diseases, and provides a safer environment for cows and farm personnel, breeding programs relying primarily on NS are still widely used by dairy producers. Several studies and surveys conducted in different regions of the US reported that 43% to 84% of the dairy farms use NS either alone or combined with AI in the United States (Champagne et al. 2002, NAHMS, 2002; Smith et al. 2004; De Vries et al., 2005; Caraviello et al., 2006). In many cases, NS is incorporated into breeding programs after cows have received one or more AI, which is commonly named as a “clean up” program (Caraviello et al., 2006). Conversely, for herds that decide not to use detection of estrus and still incorporate AI, continuous synchronization of ovulation for insemination at fixed time is an option (Lima et al., 2009). Timed AI has been shown to be an economical option to manage reproduction in high-producing dairy cows that experience a reduction in estrous intensity (Lima et al., 2010; Risco et al., 1998).

The reproductive performance (Lima et al., 2009) and costs (Lima et al., 2010) of cows bred by timed AI or NS have been compared directly and, although cows exposed to NS had similar 21-d cycle pregnancy rate compared with cows receiving only timed

AI (Lima et al., 2009), the economic evaluation favored those receiving AI (Lima et al., 2010). The economic advantage of timed AI was even greater when genetic progress was considered, and when marginal and feed cost and increased. Interestingly, the 21-d cycle pregnancy rate, a common metric used to evaluate reproduction in dairy herds, was similar between cows bred by timed AI or NS (25.0 and 25.7%, respectively), and they were both superior when compared with average values for high-producing dairy herds that ranges from 15.0 to 17.9% (De Vries et al., 2005; LeBlanc, 2010). The improvement in hazard of pregnancy for NS was attributed to a greater number of breeding opportunities, as NS cows were exposed continuously to bulls. In contrast, timed AI cows could only be inseminated after diagnosed nonpregnant, which created inter-AI intervals of 35 d (Lima et al., 2009). It is possible that a combination of timed AI and NS might benefit reproductive performance of dairy cows not observed for detection of estrus, as timed AI allows for all cows to be inseminated on the first day past the voluntary waiting period and exposure to NS after that will likely shorten the interval between breedings.

In many dairy farms using a combination of AI and NS, cows initially are inseminated one or more times and then moved to bull breeding groups (Overton and Sischo, 2005); however, it is unclear how many inseminations cows should receive before exposed to bulls to maximize pregnancy rate. This is particularly important in herds managing reproduction without the aid of estrous detection, as the interval between inseminations is determined by when a cow can be resynchronized for AI. In a previous study, the benefit of NS over TAI was only observed after 150 d postpartum, when timed AI cows had already received three inseminations (Lima et al., 2009).

Therefore, it is plausible to suggest that three timed AI may result in a similar reproductive performance when compared with one timed AI, despite the long inter-insemination interval. In fact, Overton and Sischo (2005) concluded that in herds using both AI and NS, allowing cows more opportunities for AI may benefit reproduction and that was the goal of experiment 3.

The use of timed AI programs in dairy heifers is low compared with that for lactating dairy cows (NAHMS, 2009). Programs to synchronize ovulation of dairy heifers based on GnRH and PGF_{2α} resulted in low pregnancy per AI (P/AI) compared with insemination performed after detection of estrus (Schmitt et al., 1996, Pursley et al., 1997 and Rivera et al., 2004). The depressed P/AI for most timed AI programs based on GnRH and PGF_{2α} and the perception by dairy producers that heifers become pregnant easily without the need for intervention explains the low use of ovulation synchronization protocols for management of reproduction in heifers.

Recently, a 5-d timed AI protocol investigated by Rabaglino et al. (2010a) resulted in P/AI ranging from 52.2 to 61.0% in dairy heifers in the first two inseminations, which resembled the reproductive performance obtained when heifers are artificially inseminated after detected estrus (Kuhn et al., 2006). In fact, additional work by the same investigators evaluating anti-luteolytic strategies with 325 heifers synchronized with the 5-d timed AI program observed P/AI of 59.5% on d 45 after insemination (Rabaglino et al., 2010b). Therefore, it is possible to achieve acceptable P/AI in dairy heifers following synchronized ovulation with the 5-d timed AI protocol.

The 5-d timed AI program is comprised of an injection of GnRH and insertion of a controlled internal drug-release (CIDR) intravaginal device containing progesterone,

followed 5 d later by CIDR removal and an injection of PGF_{2α}, and AI concurrent with a second GnRH injection 72 h after PGF_{2α} (Rabaglino et al., 2010a). Only 23% of the heifers had multiple corpora lutea (CL) 5 d after the injection of the GnRH (Rabaglino et al., 2010a), suggesting that ovulation to the initial GnRH was probably low. In fact, heifers receiving a single injection of PGF_{2α} 5 d after GnRH had similar luteolysis and P/AI to those receiving two injections given 12 h apart (Rabaglino et al., 2010a). The same was not true when lactating dairy cows were subjected to a similar program with a 5-d interval between GnRH and PGF_{2α} (Santos et al., 2010a). Therefore, the low incidence of ovulation induced by the first GnRH combined with more rapid turnover of follicles in heifers (Sirois and Fortune, 1988) might result in little benefit from the initial GnRH in the 5-d timed AI program in dairy heifers and the need for the initial injection of GnRH was explored in study one of Chapter 4.

In the Ovsynch protocol altering the timing of the final GnRH to induce ovulation relative to AI influences P/AI in lactating dairy cows. Brusveen et al. (2008) reported that GnRH administered 56 h after PGF_{2α} increased P/AI compared with GnRH given concurrent with timed AI at 72 h. In a series of experiments with beef cows subjected to the 5-d timed AI program, extending the proestrus from 60 to 72 h was beneficial to fertility (Bridges et al., 2008). In dairy cows subjected to the 5-d timed AI program, P/AI did not differ when the final GnRH was administered either 16 h before or concurrent with AI at 72 h after PGF_{2α} (Bisinotto et al., 2010). Although inducing ovulation 16 h before AI benefits fertility of dairy cows in the standard 7-d timed AI Ovsynch program, it is unclear if a similar benefit would occur in dairy heifers when follicle dominance is reduced such as in the 5-d timed AI protocol. Therefore, Experiment 2 of Chapter 4 was

designed to investigate which would be the ideal interval between GnRH injection to induce ovulation and insemination.

Reproductive efficiency in dairy heifers directly affects age at first calving and, therefore, has major impacts on rearing costs and subsequent productive life (Gabler et al., 2000; Ettema and Santos, 2004). Most dairy operations in the United States use AI after observed estrus to manage reproduction in heifers (NAHMS, 2009), which requires daily labor and adequate detection of estrus. Nevertheless, advances in protocols for synchronization of the estrous cycle have supported the use of timed AI as an alternative method to control reproduction in dairy heifers and improve economics when detection of estrus is less than ideal (Ribeiro et al. 2012a). Recent studies have consistently reported P/AI ranging from 50 to 60% in dairy heifers subjected to the 5-d timed AI program (Rabaglino et al., 2010; Lima et al., 2011). These results are comparable to those observed in heifers inseminated at detected estrus (Kuhn et al., 2006). Further optimization of such programs to either simplify or result in improved fertility will likely increase acceptance by dairy producers.

Despite the acceptable P/AI in heifers subjected to the 5-d timed AI protocol, important physiological aspects of ovarian responses and respective impacts on fertility have not been elucidated completely. Ovulation in response to the initial GnRH injection in timed AI programs enhances synchrony of estrous cycle, shortens follicle dominance, and improves embryo quality and P/AI (Vasconcelos et al., 1999; Chebel et al., 2006; Cerri et al., 2009a). Nevertheless, only 15 to 35% of heifers ovulate when treated with GnRH at random stages of the estrous cycle (Stevenson et al., 2008; Lima et al., 2011). In addition, heifers that ovulate in response to the initial GnRH will have a newly formed

CL 5 d later, which is generally refractory to a single treatment with PGF_{2α} (Rowson et al., 1972; Henricks et al., 1974). Eliminating the first GnRH in the 5-d timed AI program reduced ovulation at the beginning of the synchronization protocol, but increased the proportion of heifers that underwent luteolysis at AI when a single PGF_{2α} injection was used (Lima et al. 2011). Because the benefits associated with follicle turnover were offset by a less effective CL regression, P/AI did not differ between heifers that received or not GnRH at the initiation of the timed AI program (Lima et al. 2011). These results indicate that the initial GnRH is not necessary when a single PGF_{2α} is used, which simplifies and reduce costs associated with the synchronization protocol.

Heifers that receive GnRH in the beginning of 5-d timed AI program may require multiple PGF_{2α} injections to optimize luteolysis and P/AI. Results from lactating dairy cows subjected to the 5-d timed AI program indicate that the use of two injections of PGF_{2α} administered 24 h apart improved CL regression and P/AI (Santos et al., 2010a), particularly when ovulation to initial GnRH is high and more cows present a newly formed CL (Ribeiro et al., 2012b). Shorter intervals between PGF_{2α} treatments, ranging from 7 to 12 h, have been shown to increase P/AI compared with a single injection in beef cows (Kasimanickam et al., 2009), although preliminary results in dairy heifers did not confirm such a benefit (Rabaglino et al., 2010). Therefore, in Experiments 1 and 2 of Chapter 5 we hypothesized that the combination of the initial GnRH and the administration of PGF_{2α} on d 5 and 6 of the protocol would improve follicle turnover and luteal regression, which is expected to result in increased P/AI in dairy heifers.

Presumably because of less catabolism of steroid hormones in heifers than lactating dairy cows as a result of differences in splanchnic blood flow (Sangritavong et

al., 2002), progesterone concentrations in the plasma of dairy heifers are nearly 1.5 ng/mL greater than those in lactating cows during mid diestrus (Sartori et al., 2004). Because of reduced catabolism, the increase in progesterone concentrations with a CIDR is expected to be greater in nonlactating cows (Zuluaga and Williams, 2008) than in lactating cows (Cerri et al., 2009b). Progesterone affects LH secretion, which might compromise ovulatory response to GnRH treatment, and partially explain the low ovulatory response to GnRH in dairy heifers. In fact, results from beef heifers support the idea that progesterone compromises LH release and impairs ovulation following an injection of GnRH. Experiment 3 of Chapter 5 explored the concept that LH release might be hindered by high plasmatic concentration of progesterone (Colazo et al., 2008; Dias et al., 2010).

Another focus of the dissertation was uterine health. Uterine diseases are prevalent in dairy cows and they are associated with reduced reproductive performance, which ultimately affects herd profitability (Gilbert et al., 2005; LeBlanc, 2008). Uterine diseases are often classified according to clinical presentation and defined based on their impacts on P/AI or time to pregnancy (Sheldon et al., 2006). Among them, clinical endometritis is defined as presence of inflammation in the reproductive tract visible by the type of vaginal discharge that typically contains pus and persists after 21 DIM (Leblanc et al., 2002a; Sheldon et al., 2006). More recently, clinical endometritis as diagnosed by presence of pus in the vagina was classified as purulent vaginal discharge (PVD) because of the large proportion of cows without concurrent neutrophil infiltration in the endometrium (Dubuc et al., 2010). On the other hand, a large proportion of cows not diagnosed with any clinical signs of uterine disease have

presence of inflammatory cells in the endometrium, and usually more than 5% PMNL in endometrial cytology reduces P/AI and extends the interval postpartum to pregnancy (Gilbert et al., 2005; Galvão et al., 2009a).

In the United States, no particular treatment is labeled for use in cows that have either PVD or subclinical endometritis, although use of intrauterine infusion of 500 mg of cephalixin as benzathine has demonstrated efficacy in reducing interval to pregnancy in cows with PVD (Leblanc et al., 2002b) and in improving pregnancy at first AI in cows with subclinical endometritis (Kasimanickam et al., 2005). In those studies, cows were not subjected to standardized timed AI programs for first postpartum AI and many were inseminated following detection of estrus. When cows were subjected to a presynchronized timed AI program, use of intrauterine antibiotics did not benefit P/AI of dairy cows (Galvão et al., 2009b), even in those with previous diagnosis of PVD. An alternative therapy is the use of PGF_{2α} in an attempt to induce estrus and eliminate bacterial contamination that might be causing the inflammatory process in the endometrium. Use of PGF_{2α} in cows during diestrus results in luteolysis and induces cows to return to estrus, which has been suggested to enhance uterine immunity by removal of immunosuppressive effects of progesterone (Lewis, 2004). Kasimanickam et al. (2005) suggested that the improvements in P/AI caused by PGF_{2α} in postpartum cows were caused by inducing estrus and concurrent opening of the cervix and myometrium contractions that might enhance mechanical cleansing of the endometrium.

When PGF_{2α} is administered in early lactation, it is possible that the benefits to fertility might not be related to enhancing uterine health, but confounded with effects of presynchronizing the estrous cycle before timed AI programs (Moreira et al., 2001;

Galvão et al., 2007). It is known that the stage of the estrous cycle when cows initiate timed AI protocols based on GnRH is critical for fertility (Vasconcelos et al., 1999), and treatment with PGF_{2α} 11 to 12 d before the initiation of the timed AI increased P/AI (Moreira et al., 2001; Galvão et al., 2007). In fact, in most studies evaluating PGF_{2α} as therapy for treatment of uterine diseases and subsequent impacts on fertility, uterine health was not evaluated after treatment to justify the increase in P/AI (Leblanc et al., 2002b; Kasimanickam et al., 2005). In some cases, when uterine health was evaluated after PGF_{2α} treatment, P/AI at first AI improved, but the benefits were not linked to a reduction in the prevalence of subclinical endometritis in treated cows (Galvão et al., 2009a).

Timed AI programs are used commonly for reproductive management of dairy herds for first and resynchronized inseminations to mitigate the negative impacts of poor estrous detection in lactating dairy cows (Caraviello et al., 2006). An alternative presynchronization treatment, in which stage of the estrous cycle is synchronized in cyclic and anovular cows, is called Double Ovsynch (Souza et al., 2008). When PGF_{2α} is administered before the Double Ovsynch protocol, the effects on uterine health or measures of fertility are not expected to be mediated by altering the stage of the estrous cycle when cows are subjected to the timed AI protocol. The goal of the study in Chapter 6 was to demonstrate an improvement in P/AI in dairy cows with the systematic use of PGF_{2α} by enhancing uterine health based on the reduction in the prevalence of subclinical endometritis.

Uterine diseases affects nearly half of the dairy cows after parturition leading to disruption of uterine and ovarian function which frequently results in hindered fertility,

culling and remarkable economic losses for dairy producers (Sheldon et al., 2009). The economic losses caused by metritis alone are striking and calculated to be \$380 per affected cow due to reduced milk production, delayed conception, treatment and increased culling (Drillich et al., 2001). Thus, if we consider a conservative incidence rate of 20% for the 8.5 million dairy cows in US, the annual cost of metritis alone is \$650 million. Endometritis is another manifestation of uterine diseases with remarkable detrimental effects on fertility (Dubuc et al., 2011). Therefore, understanding the mechanism by which microbes subvert host innate immunity to disrupt ovarian and uterine function is fundamental to develop preventatives to mitigate the negative impacts of uterine diseases.

Trueperella pyogenes is considered one of the most relevant pathogens involved in uterine diseases, especially endometritis. This is due to its relative high prevalence in the environment, persistence in the uterus, severity of lesions on the endometrium, resistance to treatment, and synergistic action with gram-negative anaerobes (Ruder et al., 1981; Huszenicza et al., 1999; Mateus et al., 2002a, b; Williams et al., 2005,). However, the mechanism by which *T. pyogenes* affects the endometrium and reproductive events in dairy cows such as length of the estrous cycle and concentration of ovarian steroids remain elusive (Williams et al., 2007; Kaneko and Kawakami, 2009; Kaneko et al., 2013).

Several studies reported that intrauterine infusion of live *T. pyogenes* disrupts luteal development leading to early demise of the CL and ovulation of a first wave dominant follicle (Kaneko and Kawakami, 2007; Kaneko and Kawakami, 2007; Kaneko et al., 2013). Cows receiving an intrauterine infusion of *T. pyogenes* on d 3 after

ovulation had a peak of prostaglandin F metabolite (PGFM) 3 d later, followed by the regression of the newly formed CL and ovulation of the dominant follicle of the first follicular wave in approximately 50% of the time (Kaneko and Kawakami, 2008; Kaneko and Kawakami, 2009; Kaneko et al., 2013).

The explanation of how *T. pyogenes* induces short estrous cycle to disrupt normal ovarian and luteal function is unclear. Culture of endometrial cells with bacteria free filtrate of *T. pyogenes* induced synthesis of PGF_{2α} (Miller et al., 2007). This bacterium possesses a number of virulence factors that may contribute to its pathogenic potential. One of the most important is a cholesterol-dependent cytolysin, pyolysin, which is a hemolysin cytolytic for macrophages (Jost and Bilington, 2005). A second important virulence factor is peptidoglycan, which is a pathogen associated molecular pattern molecule that induces pro-inflammatory cytokines such as tumor necrosis factor α, interleukin 1β and interleukin 6 (Timmerman, et al., 1993; Stewart et al., 2003; Bromfield and Sheldon, 2011), which can stimulate endometrial synthesis of PGF_{2α} (Davidson et al., 1995; Hansen et al., 2004; Skarzynski et al., 2000). However, a possible stimulation of inflammatory mediators and their direct relationships with luteolytic cascade factors has never been investigated with an *in vivo* model of intrauterine induced infection with *T. pyogenes*.

Therefore, the possible molecular mechanisms by which intrauterine infusion with live *T. pyogenes* lead to shortening of luteal phase in dairy cows remains to be elucidated. The hypothesis investigated in Chapter 7 is that intrauterine inoculation of live *T. pyogenes* in cows with newly formed CL would increase endometrial expression

of genes affecting the inflammation and the luteolytic cascade leading to an acute endometrial production of PGF_{2α} and early demise of the newly formed CL.

Metritis is a prevalent postpartum disease in lactating dairy cows characterized by abnormally enlarged uterus and a fetid, watery red-brown fluid to viscous off-white purulent uterine discharge that can be accompanied or not with fever within 21 days postpartum, but more frequently diagnosed in the first week postpartum (Sheldon et al., 2006). The incidence rates of dairy cows developing metritis ranges from 10 to 36% (Goshen and Shpigel, 2006; Santos et al., 2010b; Chapinal et al., 2011).

The economic losses caused by metritis are striking ranging from to \$328 to \$380 per affected cow due to reduced milk production, delayed conception, cost of treatment and increased culling and death (Drillich et al., 2001; Overton and Fetrow, 2008). Additionally, cows diagnosed with metritis have an increased risk to develop both clinical and subclinical endometritis (Galvão et al., 2009; Martinez et al., 2012). The main bacteria isolated from cases of uterine infection include *Escherichia coli*, *T. pyogenes*, formerly known as *Arcanobacterium pyogenes*, and anaerobic bacteria such as *Prevotella spp.*, formerly known as *Bacteroides spp.*, and *Fusobacterium necrophorum* (Griffin et al., 1974; Noakes et al., 1989; Sheldon et al., 2002). Recently, the expression of some specific virulence factors by these bacteria were associated with increased risks for development of uterine diseases (Bicalho et al., 2012). *Escherichia coli* expressing the adhesin type I fimbriae *fimH* identified in the uterus of cows in the first 3 days postpartum was associated significantly with development of metritis and endometritis. *Fusobacterium necrophorum* expressing the leukotoxin/hemolysin *lktA* in the first 3 days or between days 8 and 12 postpartum was associated with endometritis.

Trueperella pyogenes expressing the type I fimbriae adhesin *fimA* and the pyolysin *ply* between 8 and 10 d or between 34 and 36 d postpartum was associated with endometritis (Bicalho et al., 2012). Therefore, it has been suggested that the presence of *E. coli* expressing virulence factor *fimH* in the uterus of cows in the first few days postpartum paves the way for the other bacterial infections coordinating the initial process of tissue damage and development of uterine diseases. Thus, it is reasonable to suggest that a reduction on the extent of *E. coli* load in the uterus of metritic cows might mitigate the negative impact of the disease and minimize the risk of subsequent chronic uterine infections such as clinical and subclinical endometritis.

Ampicillin is a beta-lactam antibiotic that acts as an irreversible inhibitor of dd-transpeptidase, an essential enzyme that bacteria use to make their cell walls. Therefore, ampicillin generally inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to bacterial cell lysis. Ampicillin has received FDA approval for its mechanism of action and it has been shown effective against *E. coli* (Burrows, 1993; Lehtolainen et al., 2003). However, to date, no published study has evaluated efficacy of ampicillin treatment of metritis in dairy cows.

Thus, it was hypothesized in Chapter 8 that ampicillin would be an effective therapy for metritis having similar efficacy to ceftiofur, one of the major antibiotics used to treat metritis in the United States and the objectives were to evaluate the efficacy of ampicillin trihydrate for treatment of metritis in dairy cows compared with ceftiofur hydrochloride and subsequent effect on P/AI to the first service.

CHAPTER 2 LITERATURE REVIEW

Estrous Cycle in Dairy Cattle

The estrous cycle in dairy cattle is characterized by a rhythmic pattern of cyclic ovarian activity initiated with attainment of puberty by heifers with first ovulation enabling sexual receptivity and repeated opportunities for mating, and establishment of pregnancy. The activities within the estrous cycle in dairy cattle are controlled mostly by the interplay among ovarian steroid hormones (progesterone and estradiol), hormones of the hypothalamus (gonadotropin-releasing hormone; GnRH), the anterior pituitary (follicle-stimulating hormone; FSH and luteinizing hormone; LH), and the uterus (prostaglandin $F_{2\alpha}$; $PGF_{2\alpha}$), which through a mechanism of negative and positive feedback, modulate ovarian activity (Roche, 1996). The normal length of the estrous cycle in dairy cattle ranges from 18 to 24 days (Peter et al., 2009). The average inter-ovulatory period was calculated based on series of studies as 20.8 days for dairy heifers and 23.0 days for dairy cows (Sartori et al., 2004).

Changes in ovarian dynamics are dictated to a large extent by patterns of release and concentrations of regulatory hormones. The bovine estrous cycle is divided into two phases, the follicular and luteal phases; and four periods, proestrus, estrus, metestrus and diestrus (Peter et al., 2009). The follicular phase encompasses the proestrus and estrus periods going from regression of the corpus luteum (CL) to ovulation with a shift from progesterone to estradiol dominance lasting 4 to 6 days. The proestrus phase is the period in which structural and functional CL regression initiates and a substantial increase in the synthesis of estradiol by pre-ovulatory dominant follicles occurs leading to an onset of sexual receptivity that defines inception of estrus (Wattemann et al.,

1972). The period of estrus comprises the initiation of sexual receptivity and ovulation of the dominant follicle. From the day of occurrence of luteolysis to the day preceding ovulation the maximal serum estradiol concentrations increases about 3-fold (~2.5 pg/mL to 7.9 pg/mL) and the maximal serum estradiol concentration preceding ovulation is greater for dairy heifers than for dairy cows (11.3 pg/mL vs. 7.9 pg/mL, Sartori et al., 2004). The concurrent increase in estradiol concentrations in blood and reduced concentrations of progesterone leads to stimulation of arcuate nucleus, ventromedial nucleus, and the preoptic area of hypothalamus allowing the display of behavioral estrus (Frandsen et al., 2003, Molenda-Figueira et al., 2006).

The duration of estrus in modern dairy cows have been reported to be approximately 7 hours with 9.1 standing events or mounts recorded during standing estrus (Drasfield et al., 1998; Lopez et al., 2004, Diskin et al., 2008). A few decades earlier, the duration of estrus was twice as long with 14.9 hours (Esslemont and Bryant, 1976). Milk production, the major trait used for genetic selection of dairy cattle, has been negatively associated with duration of estrus. Cows producing 55 kg/day had 2.8 hours of estrus duration, whereas cows producing 25 kg/day had 14.7 hours of estrus duration. The duration of estrus duration in heifers ranged from 12 to 14 hours (Diskin et al., 2008). A recent study reported heritability estimates for estrus duration and intensity to be low (2% to 8%; Lovendahl and Chagunda, 2009), but genetic control of estrous behavior remains elusive. It has been suggested that changes in the underlying molecular mechanisms that regulate estrous behavior could be manifested by altered gene expression patterns, although very little is currently known in dairy cattle (Boer et al., 2012). Duration of estrus is likely to also be influenced by environmental factor other

than milk production. Cow footing, heat stress, and lameness all influence the ability of cows to display and maintain normal duration of estrous expression (Lucy, 2001)

The luteal phase begins with ovulation and ends with CL regression comprising the metestrus and diestrus periods with duration of 14 to 18 days. The period of metestrus initiates after ovulation and typically lasts 3 to 4 days being characterized by the formation of the CL from the collapsed ovulated follicle (corpus haemorrhagicum) and metestrus bleeding in some cows. After ovulation, progesterone concentrations begin to increase because of the formation of the CL with luteinization of granulosa and theca cells forming large and small luteal cells, respectively. Increased progesterone concentrations in plasma prepare the uterus for the establishment and maintenance of pregnancy, or in case of lack of pregnancy establishment re-occurrence of the estrous cycle (Peter et al., 2009). Diestrus is the period in which the CL matures, becomes responsive to $\text{PGF}_{2\alpha}$, and sustains secretion of progesterone, ending with its demise during luteolysis. Transition from metestrus to diestrus is marked by a sharp increase of progesterone on day 4 after ovulation (Stabenfeldt et al., 1969). During diestrus, progesterone concentrations in the plasma increase more than 3-fold (1.5 to 5.5 ng/mL) from days 4 to 8 after ovulation. Subsequently, the rate of increase is reduced and reaches the maximal value on day 16 of estrous cycle at approximately 7 ng/mL (Stabenfeldt et al., 1969). Dairy heifers have greater maximal serum progesterone concentration than dairy cows (7.3 ng/mL vs. 5.6 ng/mL, Sartori et al., 2004) and this difference has been attributed to the increase hepatic catabolism of steroids caused by increased hepatic catabolism of ovarian steroids as splanchnic blood flow is markedly increased by increased dry matter and caloric intake in dairy cows (Sangsrivong et al.,

2002). During the diestrus, progesterone concentrations remain elevated and recurrent follicular waves occur due to periodic release of FSH from the anterior pituitary gland. Nevertheless, the dominant follicles that develop throughout the luteal phase do not ovulate because progesterone induces a negative feedback on LH, thus only allowing the secretion of greater amplitude but lesser frequency LH pulses that are not sufficient for ovulation and a block of the preovulatory surge of LH (Rahe et al., 1980). If pregnancy does not occur in dairy cattle, pulsatile release of PGF_{2α} by the endometrium will lead to spontaneous luteolysis between days 17 and 19 of the estrous cycle (Ginther et al., 2010).

The development of ultrasonography and its application to bovine reproduction lead to major advancements in the understanding and characterization of ovarian dynamics during the estrous cycle. It is well established that dairy cattle have between 2 to 3 waves of follicular development within each estrous cycle with only the last wave of the cycle leading to ovulation (Sirois and Fortune, 1988; Sartori et al., 2004). In dairy heifers occurrence of 3 follicular waves ranges from 33 to 84% of the cycles (Savio et al., 1988; Sirois and Fortune, 1988; Sartori et al., 2004). Approximately 50% of the estrous cycles are 2-wave cycles and 50% are 3-wave cycles (Table 2-1). On the other hand, 79% of cycles of high-producing dairy cows have 2 follicular waves (Sartori et al., 2004). Each wave of follicular development consists of emergence, selection and dominance followed by either atresia or ovulation of the dominant follicle.

Table 2-1. Characteristics of the estrous cycle of heifers

Reference	Follicle wave		Emergence of 2 nd wave		Estrous cycle length		Progesterone peak ng/mL
	2-wave % (n)	3-wave % (n)	2-wave Day of the cycle	3-wave Day of the cycle	2-wave Day	3-wave Day	
<i><u>Beef heifers</u></i>							
Savio et al. (1988)	16 (4)	84 (21)	10	~10	20.5 ± 1.3	21.3 ± 1.5	N/A
Bong et al. (1993)	25 (3)	75 (9)	N/A	N/A	20.8 ± 0.9	21.3 ± 0.5	N/A
<i><u>Dairy heifers</u></i>							
Sirois and Fortune (1988)	20 (2)	80 (8)	11	9.4 ± 0.5	20 ± 1	20.7±0.4	~7.5
Ginther et al. (1989)	82 (18)	18 (4)	9.6 ± 0.2	9.0 ± 0.0	20.4 ± 0.3	22.8 ± 0.6	N/A
Knopt et al. (1989)	90 (9)	10 (1)	10 ± 0.4	10	21	23	N/A
Ko et al. (1991)	75 (9)	25 (3)	~10	~8	~20	~23	N/A
Kulick et al. (2001)	57 (13)	43 (10)	~10	~9	~19.5	~23.0	N/A
Sartori et al. (2004)	56 (15)	44 (12)	9.7 ± 0.2	8.2 ± 0.4	20.7 ± 0.3	23.1 ± 0.7	7.3 ± 0.4
Wolfenson et al. (2004)	70 (14)	30 (6)	~10	~10	~22	~22	~10
Overall	54 (87)	46 (74)	10.1 ± 0.2	9.3 ± 0.3	20.6 ± 0.3	22.2 ± 0.3	8.3 ± 0.9

N/A = not applicable

The beginning of gonadotropin-dependent follicle development is characterized by the emergence of a follicle cohort typically consisting of 5 to 20 follicles ≥ 4 mm and is associated with a transient increase in FSH concentrations peaking at 0.7 days before follicle emergence (Ginther et al., 1989; Adams et al., 1992; Sunderland et al., 1994). Although follicle emergence has been described as when the cohort of follicles attain 4 mm in diameter, detailed ultrasonographic examination demonstrated that even smaller follicles ranging from 1 to 3 mm develop in wave pattern associated with FSH surge (Jaiswal et al., 2004). The dominant follicle emerges in humans from the cohort of recruited follicles based initially on its morphology as the largest developing follicle (Gougeon and Lefevre, 1983; Ginther et al., 1996). This increase in size is paralleled by an increase in follicular fluid estradiol and inhibin concentrations, which suppresses FSH concentrations from the anterior pituitary gland via negative feedback (Sunderland et al., 1994; Ginther et al., 2000). The next event in follicular dynamics is called deviation. This is characterized by the differentiation in growth rates between the upcoming dominant follicle and its largest subordinate follicle, which in dairy cattle occurs when the largest follicle achieves approximately 8.5 mm of diameter and the second largest follicle is approximately 7.2 mm of diameter (Ginther et al., 1996). The hallmarks of deviation are inability of subordinate follicle to develop if the dominant follicle is ablated and nadir concentrations of FSH (Adams et al., 1992; Mihm and Evans; 2008). Before deviation, the elimination of the largest follicle leads to development of the second largest as the dominant follicle (Ginther et al., 1997). Moreover, follicular deviation has been associated temporally with an increase in the expression of LH receptors in granulosa cells and in LH responsiveness in some studies

(Sartori et al., 2001; Mihm et al., 2006; Nogueira et al., 2007), but not in all studies (Evans and Fortune, 1997). In fact, treatment of cows with an ovulatory dose of LH will only cause ovulation in follicles that have grown past the time of deviation (Sartori et al., 2001), suggesting that the development of LH responsiveness associated with deviation is required for ovulatory capacity.

After deviation, dominance is established and the dominant follicle becomes LH dependent and it can either develop and ovulate or enter in atresia if progesterone concentrations are high. Therefore, only second or third follicular wave dominant follicles undergo ovulation in cows having normal estrous cycle and no exogenous hormonal manipulation. The emergence of the second follicle wave occurs at around days 9 to 11 in cows with a two wave pattern, and around days 8 or 9 in those a with three wave pattern, whereas the third follicle wave arises around day 15 to 16 of estrous cycle (Adams et al., 2008).

Follicle Development and Function

A pre-determined number of primordial follicles are established during fetal development with ovarian follicle growth taking a period of approximately 3 to 5 months with distinct gonadotropin-independent and dependent stages (Webb et al., 2004). Gonadotropin-dependent follicle growth in cattle occurs in wave pattern with occurrence of 2 to 3 follicular waves in each estrous cycle (Savio et al., 1988). Temporal histological evaluation of the bovine ovary revealed 6 distinct types of structural and developmental follicles (Braw-Tal and Yossefi, 1997). Follicles were classified in primordial, transitory, primary, small pre-antral, large pre-antral and antral follicles. Primordial follicles are composed of the oocyte surrounded single layer of flattened granulosa cells generally with a size smaller than 40 μm . Transitory follicles resemble the primordial follicles with

the only difference being the granulosa cells are cuboidal and multiplying. Primary follicles have the oocyte bordered for 1 or 1.5 layer of cuboidal granulosa cells with a size ranging from 40 to 80 μm . Small pre-antral follicles have 2 to 3 layers of cuboidal granulosa cells around the oocyte and ranges in size from 81 to 130 μm . It is the earliest follicle type to present the zona pellucida. Large pre-antral follicles have 4 to 6 layers of cuboidal granulosa cells surrounding the oocyte with size ranging from 131 to 250 μm and with a zona pellucida that for the first time forms a complete ring around the oocyte. Lastly, the antral follicle characterized by the oocyte bordered by more than 6 layers of cuboidal granulosa cells with a size greater than 250 μm and with the presence of an antrum.

Cattle are born with a finite number of primordial follicles (Fortune et al., 2010) High variation in ovarian size, ovarian reserve, and number of follicles recruited in each follicular wave in the bovine is reported to be positively associated with fertility (Ireland et al., 2011). The interval from the activation of primordial follicles to the formation of preovulatory follicle have been estimated to be approximately 180 days with 138 days spent in the pre-antral stages and the remaining 42 days in the antral stages (Lussier et al., 1987). The delay between the appearance of the first primordial and the first primary follicles is 50 days in cattle and is associated with progression through to meiotic prophase I and arrest at diplotene (McNatty et al., 2007).

Although there have been many advances on the understanding about the molecular and biochemical factors regulating follicle development, the pivotal factors controlling the activation of primordial follicles remains elusive. Primordial follicles have transforming growth factor (TGF)- β ligand, other growth factor ligands and receptors

expressed in oocytes and granulosa cells. Activin-like kinase (ALK) 3, 5 and 6, betaglycan, bone morphogenic protein (BMP) 6, bone morphogenic protein receptor (BMPR) II, growth differentiation factor (GDF) 9, connexin 37, kit-ligand-c-kit, and estradiol receptor (ER) β , are some of growth factors and related genes expressed in primordial follicles that potentially are part of the interplay regulating activation of primordial follicles (McNatty et al., 2007). It is likely that several of these growth-promoting factors are involved, both negatively and positively, with the initiation of follicular growth. The results of *in vitro* studies using the cortical portion of the bovine ovary revealed that the majority of primordial follicles are activated within a few days of isolation from the ovary, suggesting that inhibitory factors may play a key role on regulation of exit from the pool of primordial follicles.

It has been suggested that a paracrine communication with the ovarian stroma is required to prevent activation of primordial follicles and control the pace of follicular development (Fortune et al., 1998). Additionally, Fortune (2003) reported that anti-Müllerian hormone (AMH) inhibits follicle activation and early follicular growth in bovine ovaries. It is important to notice that activation of primordial follicles is not dependent on gonadotropic stimulus (Braw-Tal and Yossefi, 1997; Fortune et al., 1998).

Once follicles transition from the pool of primordial follicles, they are still committed to a gonadotropin-independent growth. As the follicle grows to the primary stage, the granulosa cells increase in number and change shape, becoming uniformly cuboidal. The oocyte also enlarges, with 3- to 10- fold increases in the volume of smooth endoplasmic reticulum, mitochondria, ribosomes and lipid droplets, and the zona pellucida, absent in primordial follicles, is deposited (Lundy et al., 1999).

Analysis of expressed sequence tags from primary follicles shows that several hundred genes not found in primordial follicles are activated in primary follicles, including some involved in mitochondrial function, cell signaling and communication, and the synthesis of the zona pellucida. With the advance to the primary stage, the follicle becomes responsive to progestins, estrogens, androgens, and gonadotropins; however, gonadotropin-dependent regulation is not observed until the antral follicle stage. In addition, several factors related to regulation of the follicular growth begin to be synthesized by the oocyte (BMP-6, BMP-15, GDF-9), granulosa (inhibin, follistatin), and theca cells (TGF- β 1 and 2, androgens; McNatty et al., 2007).

The current view is that GDF9 and BMP15 secreted by the oocyte act in a concentration-dependent paracrine manner on adjacent cumulus and granulosa cells. Furthermore, reduced concentrations of BMP15 and GDF9 alter only slightly the responsiveness of granulosa cells to gonadotropins, whereas the absence of BMP15 or GDF9 arrest follicular growth at primordial or primary stages (McNatty et al., 2007). Interaction between GDF9 and BMP15 produced by the oocyte induces cell proliferation and controls responsiveness to gonadotropins in a spatial-dependent manner because cells that diverge to become the cumulus and mural granulosa cells are affected differently (McNatty et al., 2007). GDF9 and BMP15 are produced as pre-pro-proteins consisting of a signal peptide, a large pro-region and a mature region (Shimasaki et al., 2004), and they signal to granulosa and cumulus cells through the TGF- β superfamily receptor BMP-RII (Juengel and McNatty, 2005). When GDF9 binds to BMP-RII, ALK5 is activated and phosphorylates SMAD2/3. Thereafter, SMAD2/3 associates with the common SMAD4 and this complex translocates to the nucleus where it interacts with

specific DNA motifs and transcriptional regulators leading to the transcription of target genes. When BMP15 binds to BMP-RII, it causes recruitment and activation of ALK6, leading to signaling through the alternative BMP pathway mediated by SMADs 1, 5 and 8 (Juengel and McNatty 2005).

Although FSH receptors are present in granulosa cells since the primordial follicles stage in many species (Cortvrindt et al., 1997; Findlay and Drummond, 1999), the transition from primary to small antral follicles seems to remain independent of gonadotropins (Knight and Glister, 2001), but influenced by many factors such as: testosterone, fibroblast growth factor (FGF) 7 and vascular endothelium growth factor (VEGF) that directly or indirectly influence granulosa cells and oocyte development (Fortune et al., 2010; Buratini and Price, 2011).

Once follicles transit from pre-antral to antral stage they become gonadotropin dependent. At this stage, follicles undergo cyclic recruitment and develop either to the pre-ovulatory state or undergo atresia. The transition from pre-antral to antral stages is characterized by a reduction in somatic cell proliferation and improved differentiation into cells responsive to gonadotropins that can produce increased amounts of steroids (Webb and Campbell, 2007).

The gonadotropins FSH and LH secreted by gonadotroph cells in the anterior pituitary act on granulosa cells and theca cells dictating the fate of the antral follicles. The onset of antral follicle growth is stimulated by FSH that binds to granulosa cells and initiates the recruitment of a new follicular wave. Luteinizing hormone binds to granulosa and theca cells inducing the development and final maturation of the selected dominant

antral follicle. The two gonadotropins act in concert promoting steroidogenesis in follicular somatic cells

The secretion of FSH and LH is regulated by GnRH, and the release of GnRH is modulated by ovarian steroids. It has been reported that GnRH neurons have no receptors for progesterone and estradiol (Skinner et al., 2001; Herbison and Pape, 2001); therefore, steroid modulation of GnRH secretion occurs indirectly through intermediary neurons that possess the relevant steroid receptors and respond to their stimulation (Clarke and Pompolo, 2005). Neurons with receptors to ovarian steroids and communication with GnRH neurons are classified as stimulatory and inhibitory neurons. Kisspeptin, dopamine, and glutamine are neurotransmitters that stimulate release of GnRH while aminobutyric acid, nitric oxide and opioids are neurotransmitters that inhibit GnRH secretion (Clarke and Pompolo, 2005). Kisspeptin neurons express ER α and progesterone receptor and, therefore, have the potential to relay feedback effects on the GnRH neuron. Evidence now suggests that reduced activity of kisspeptin neurons in the arcuate nucleus of sheep is responsible for translating estrogen negative feedback to GnRH neurons. Ovariectomized sheep have an increased level of KiSS-1 mRNA in the neurons compared to controls. Also, if estrogen replacement is given to ovariectomized female sheep, then KiSS-1 mRNA levels are reduced to control levels (Roa and Tena-Sempere, 2007). This suggests that steroids are negatively regulating KiSS-1 mRNA in the arcuate nucleus, hence reducing stimulation of GnRH neurons. The interplay between ovarian steroids and GnRH is defined as a bimodal system. Thus, GnRH is released as either tonic mode (low frequency and low amplitude pulses) at luteal phase or surge mode (high frequency and high amplitude) at the follicular phase.

The intra-ovarian regulation of both gonadotropin-responsive and gonadotropin-dependent follicles has been largely attributed to the IGF system. Studies have shown that IGF-I increases the sensitivity of small follicles (5 mm in cattle) to gonadotropin stimulation and simulates their transition from the gonadotropin-responsive to the gonadotropin-dependent stages (Mazerbourg et al., 2003). In ovarian tissue *in vitro*, IGF-I stimulated steroidogenesis by thecal cells and both proliferation and differentiation of granulosa cells (Mazerbourg et al., 2003). The secretion of estradiol by granulosa cells in culture was also stimulated by IGF-I (Campbell et al., 1996). Therefore, it seems that IGF-I can stimulate either the proliferation or the differentiation and differentiated functions of granulosa cells, depending on the stage of development of the follicle. The IGF binding protein (IGFBPs) are important modulator of the IGF system. The supply of IGF-I to the follicle is outside the control of the reproductive axis, therefore intra-follicular IGF activity is regulated locally, primarily by the IGFBPs. The low-molecular weight IGFBPs (BP-2, -4 and -5) are inhibitory to IGF actions because they bind IGF, preventing it from binding to its receptor. Therefore, what dictates changes during folliculogenesis is the bioavailability of IGF-I and not the total concentration. In the cow, intrafollicular concentrations of IGFBP-2 and IGFBP-4 decrease as follicles grow to pre-ovulatory size and of IGFBP-5 increase in follicles, as they become atretic (Mazerbourg et al., 2003).

The pregnancy-associated plasma protein-A (PAPP-A) is a pivotal factor modulating the concentration of IGFBPs. The low concentrations of IGFBPs in healthy growing follicles are caused by increased rates of proteolytic degradation of IGFBP-2, -4 and -5 by PAPP-A and by low rates of gene transcription for IGFBP-2 (Mazerbourg et

al., 2003). In bovine granulosa cells, the expression of the mRNA for PAPP-A is the highest in ovulatory follicles and is positively correlated with aromatase and LH receptor (Mazerbourg et al., 2001). Furthermore, PAPP-A activity was increased in dominant follicles in comparison with subordinate follicles on days 2 and 3 of the first follicular wave of the estrous cycle and the levels of activity correlated positively with estradiol and negatively with low-molecular weight IGFBNs in follicular fluid (Rivera et al., 2001). Cattle were treated with low doses of recombinant bovine FSH for 2 days shortly after wave emergence, two co-dominant follicles were selected, both with higher PAPP-A activities and estradiol concentrations and lower amounts of IGFBN-4 in their follicular fluid than subordinate follicles (Rivera and Fortune, 2001). These results suggest that the action of FSH on dominant follicles is to increase PAPP-A activity. Careful analyses of various characteristics of follicles just before and after follicle emergence showed that an increase in PAPP-A activity in one follicle of the wave was detected before any detectable difference in diameter or in the concentrations of estradiol or IGFBN-4 or -5 in follicular fluid (Rivera and Fortune 2003).

Recently, a new postulated theory suggested that acquisition of LH receptors on granulosa cells occurs prior to changes in PAPP-A and the subsequent increased bioavailability of IGF-1 (Luo et al., 2011). Dominant follicles expressed LH receptors in the granulosa cells of 12 h before follicle deviation, but an increase in PAPP-A expression was only observed at follicle deviation (Luo et al., 2011). Authors speculated that LH receptors in the granulosa cells were induced by LH secretion itself. Additionally, LH signaling also increased expression of steroidogenic enzymes in theca (steroidogenic acute regulatory protein - StAR, cholesterol side-chain cleavage –

P450scc) and granulosa cells (aromatase – P450arom). Before deviation, LH receptors are only expressed in theca cells through which LH stimulates expression of steroidogenic enzymes essential for production of androgens that are then used by granulosa cells to produce estradiol. After deviation, expression of LH receptors in the granulosa cells allows the follicle to respond to LH and undergo further development (Luo et al., 2011). Acquisition of LH receptors by the granulosa cells is the hallmark for acquisition of ovulatory capacity in response to LH surge, which is supported by occurrence of an inducible ovulation when using exogenous GnRH or LH only after follicle deviation.

Another important factor recently reported to be involved with health status of follicles is the cocaine and amphetamine regulated transcript (CARTPT) and its peptide CART (Lv et al., 2009). Results revealed that granulosa cell CARTPT mRNA expression and follicular fluid CART concentration are higher in estrogen-inactive atretic follicles than in estrogen-active healthy follicles collected at pre-deviation stage and immediately after selection (early dominance stage) and relatively low in the remaining stages of the follicular wave (Lv et al., 2009). Additionally, CART negatively regulates FSH and IGF-1 actions on granulosa cells *in vitro*, reducing CYP19A1 expression and inhibits estradiol production *in vivo* (Lv et al., 2009). Moreover, CART concentrations in healthy follicles decrease after dominance (Lv et al., 2009).

Follicular dominance is largely acknowledged as an evolutionary mechanism to control number of offspring per pregnancy in monocotous species. Interestingly, in polycotous species such as pig and mice that have a less strict control of follicle dominance, CARTPT is not expressed in their ovaries suggesting that CART could be a

pivotal functional modulator of selection of a single dominant follicle in monocotous species (Smith et al., 2010).

Throughout the diestrus stage of the estrus cycle, cattle will have dominant follicles that will not undergo ovulation because progesterone blocks pulsatile LH release. Once the CL regresses and progesterone concentrations fall, LH secretion rises and the dominant follicle is capable of undergoing ovulation. However, if subluteal or low concentrations of progesterone are present, dominant follicles may continue to develop in size and remain dominant longer without occurrence of ovulation due to progesterone blocks of the LH surge (Mihm et al., 1994). Regression or ovulation of the dominant follicle lead to reduced concentrations of estradiol and inhibin allowing plasmatic concentrations of FSH to increase inducing the emergence of a new follicular wave.

Corpus Luteum Formation, Function and Regression

The CL is an ephemeral endocrine gland that throughout its lifespan undergoes a period of extremely rapid growth that involves hypertrophy, proliferation and differentiation of the steroidogenic cells, as well as extensive angiogenesis. The major function of the CL is to produce progesterone that is essential for the maintenance of pregnancy. The CL lifespan in normal estrous cycle non-pregnant cows is approximately 17 days (Ginther et al 2010, Sartori et al., 2004).

After ovulation, the remaining cells in the ruptured follicle undergo a developmental phase within 8 to 10 days that is marked initially by a period of tissue remodeling with intense sprouting of endothelial cells up to the first third of cycle, with the mature CL being characterized by a dense network of vessels critical to support the differentiation of follicular cells. The alterations toward the development of a functional

CL are named luteinization and involve cellular structural changes, such as an increase in the cytoplasmic to nuclear area ratio and an accumulation of lipid droplets (Brännström and Fridén, 1997). After achieving maturity, the bovine CL is composed of vascular endothelial cells (50%), large and small steroidogenic luteal cells (30%) and the remaining 20% of cells are constituted by pericytes, fibrocytes, nerves, immune and smooth muscle cells (Farin et al., 1986; O'Shea et al., 1989; Lei et al., 1991). The population of immune cells in the CL is diverse with presence of macrophages, neutrophils, eosinophils, CD4⁺ T lymphocytes, and CD8⁺ T lymphocytes that play important role on CL formation, protection and demise (Penne et al., 1999; Bauer et al., 2001). The mature CL engages in an unmatched production of steroids, resulting in extremely high metabolic activity within the tissue, with the greatest blood flow per unit of tissues, and oxygen consumption on a cell basis estimated to be 2 to 6 times greater than that for the liver, kidney, or heart (Niswender et al., 2000; Robinson et al., 2008).

Once CL formation is initiated, one of the most important events that take place is the shift from estradiol to progesterone production by large and small steroidogenic luteal cells that are formed from granulosa and theca cells, respectively. For small luteal cells LH is the major stimulator of progesterone production through increase of cAMP that activates protein kinase A (PKA) leading to up regulation of P450_{scc}, StAR, and 3-beta-hydroxysteroid dehydrogenase / Δ^5 , Δ^4 isomerase (3 β -HSD). Large luteal cells are unresponsive to LH relying on constitutive activated PKA and perhaps other luteotropic hormones such as growth hormone (GH), IGF, FGF, and oxytocin to stimulate synthesis of progesterone (Miyamoto et al., 2010).

Additionally, it seems that progesterone self-stimulates its own synthesis through progesterone nuclear receptors and through non-genomic pathways, which additionally protects luteal cells from apoptosis (Okuda et al., 2004; Liszewska et al., 2005, Rekawiecki et al., 2008). During the peri-ovulatory period, follicular steroidogenesis by theca and granulosa is dramatically down-regulated, but the transport of cholesterol across the outer mitochondrial membrane remains unaltered during the late stages of development of the ovulatory follicle based on StAR mRNA expression (Nimz et al., 2009). Granulosa cells expression of P450scc, 3 β -HSD, P450arom and theca cells expression of P450c17 and 3 β -HSD in the theca cells were downregulated within 18 hours preceding the pre-ovulatory surge of LH (Voss and Fortune, 1993a; Voss and Fortune, 1993b). Consequently, androstenedione, testosterone, and estradiol concentrations in the follicular fluid were reduced between 4 and 10 hours after the pre-ovulatory surge of LH (Komar et al., 2001). Accordingly, estradiol concentrations declined 50% within the first 5 hours of the pre-ovulatory surge of LH, and returned to basal values within the next 9 hours (Chenault et al., 1974). After 72 hours of culture, the expression of P450scc and 3 β -HSD, but not P450c17 and P450arom, was reestablished in follicular cells (Voss and Fortune, 1993a; Voss and Fortune, 1993b). These results are consistent with increasing production of progesterone *in vitro* by granulosa and theca cells (i.e.: luteinization) between 24 and 72 hours of culture not associated with an increase in androgen production (Voss and Fortune, 1993a; Voss and Fortune, 1993b).

The development of the CL is dependent largely on the vascular system, which has two main types of functional luteal blood vessels: arteriovenous vessels (ie,

arteriola, diameter ~40 μm) and venula vessels (diameter ~30 μm) that have a smooth muscle layer; and capillary vessels, which do not have a smooth muscle layer. In the bovine CL, the number of arteriolo-venous and microcapillary vessels drastically increases from the early to midluteal phase (Bauer et al., 2003; Hojo et al., 2009; Shirasuna et al., 2010). Angiogenesis in the bovine CL is controlled mostly by vascular endothelial growth factor A (VEGFA) and FGF-2 and its respective receptors VEGFR-2 and FGF receptor (Ferrara et al., 1997; Connolly, 1991). The expressions of VEGFA, VEGFR-2, FGF2 and FGF receptor are upregulated in early luteal phase, but remarkably down-regulated during midluteal to the regression phases in the bovine (Schams et al., 1994; Berisha et al., 2000). Robinson et al. (2008) using a system of luteal cell culture to stimulate angiogenesis induced endothelial cell network formation with FGF2 and VEGFA at physiological dose of 1 ng/mL. Furthermore, addition of VEGFR2 inhibitor to this culture system reduced endothelial cell networks formation by 60%. When a FGFR1 inhibitor was added to the medium, even at the presence of VEGFA, endothelial cell network formation was reduced by 90%. These results suggest that FGF2 is more important than VEGFA for the formation of luteal vascular networks (Woad et al., 2009).

The effects of VEGFA and FGF2 on the bovine CL to influence progesterone production go beyond angiogenesis. Studies using microdialysis system revealed VEGFA and FGF2 stimulate progesterone secretion in the bovine CL (Miyamoto et al., 1992; Kobayashi et al., 2001). Moreover, Yamashita et al. (2008) reported that the injection of specific antibodies for VEGFA or FGF2 antibodies directly into the cow CL reduced progesterone secretion, CL volume, and down-regulated luteal mRNA

expression of VEGFA, FGF2 and up regulated expression of angiopoietins confirming the pivotal importance of VEGFA and FGF2 on CL development.

The immune system is also critically involved with CL development. In cows, the developing CL has an increased number of macrophages and monocytes (Penny et al., 1999). Macrophages are the most abundant immune cells in the bovine ovary deriving from differentiation of monocytes arriving at the ovaries as a response to local cytokines such as granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, tumor necrosis factor α and interferon gamma (Zhao et al., 1995; Townson et al., 2003; Zhang et al., 2008). Macrophage elimination was associated positively with increased amounts of ovarian hemorrhage, which lead to a remarkable depletion of endothelial cells and an increase in the number of erythrocytes in the bovine ovary (Turner et al., 2011). Removal of macrophages prompted the disruption of the critical pericyte-endothelium interaction, compromising dramatically endothelial function (Turner et al., 2011). It seems that macrophages play a critical role in maintaining the integrity of ovarian vasculature.

Polymorphonuclear leukocytes (PMNL) such as eosinophils and neutrophils are detected in the CL during the estrous cycle. Eosinophils infiltrate into the CL soon after occurrence of ovulation in the bovine CL (Reibiger and Spanel-Borowski, 2000). Studies using human ovaries suggested that P-selectin expressed on endothelial cells are responsible to recruit eosinophils into the developing CL (Aust et al., 2000). A large number of neutrophils and elevated concentration of the neutrophil specific chemokine interleukin 8 (IL-8) are present in the CL of cows during early luteal phase (Jiemtaweeboon et al., 2011). The formation of early CL lead to PMNL migration *in vitro*

when IL-8 and the supernatant of activated PMNL were used; and IL-8 stimulated the formation of capillary-like structures of CL-derived endothelial cells suggesting that PMNL and IL-8 may be involved in regulation of angiogenesis in the developing CL (Jiemtaweeboon et al., 2011).

The process of CL maintenance when pregnancy occurs involves endocrine, vascular and immunological factors that orchestrate a response critical to the fate of the CL. The abrogation of luteolysis involves blocking of the pulsatile release of PGF_{2α} in the uterus by molecules secreted by developing conceptus. A body of evidence gathered throughout the last few decades suggest that the type I interferon tau (IFN_τ) is the primary embryonic signal in ruminants (Bazer et al., 2008) involved with maternal recognition of the pregnancy by suppressing pulsatile uterine release of PGF_{2α}. The mechanism of suppressing the pulsatile release of PGF_{2α} is mediated by the suppression of the expression of estrogen receptor-α (ESR1) and oxytocin receptor (OXR) mRNA in uterine tissue (Spencer & Bazer, 1996). Maximum secretion of IFN_τ occurs on day 17 of gestation at the same time of maternal recognition of pregnancy (Bazer et al. 1997). Interferon tau mediates its effects in bovine endometrium by binding to endometrial type-I IFN receptors (Li and Roberts 1994) activating the janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Binelli et al. 2001). A number of genes have already been described as induced by IFN_τ in the endometrium of cattle or sheep, such as IFN-stimulated gene 15 (ISG15), 20-50-oligoadenylate synthetase, bovine ubiquitin activating E1-like enzyme, members of the 1–8 family, mixovirus resistance protein 1 (MX1), granulocyte–macrophage colony-stimulating factor-1, IFN regulatory factors 1 and 2 and signal transducer and activator

of transcriptions 1 and 2 (Thatcher et al. 2001, Wolf et al. 2003). The conceptus secretes more than one type I IFN (Cochet et al., 2009) and a number of interferon-responsive genes are upregulated on the endometrium of cows in response to IFNs, which may induce other important factors related to endometrial function. Moreover, IFN τ can upregulate IFN-responsive genes in circulating immune cells, which could possibly be involved with rescuing the CL for maintenance of pregnancy (Gifford et al., 2007; Ott and Gifford 2010). Interferon-stimulated gene 15 and MX1 are upregulated in CL of pregnant cows (Yang et al., 2010), and there is now evidence that IFN τ is released into the uterine vein and may itself exert endocrine effects on the CL (Spencer et al., 1999; Hansen et al., 2010). Yang et al. (2010) was unable to identify IFN τ -induced stimulation of ISG15 in cultured bovine luteal cells, suggesting that the effects of IFN τ on the CL may be mediated by immune or endothelial cells not present in the culture media. However, previous studies demonstrated that IFN α , which acts on the same type I interferon receptor as IFN τ , inhibited both cytokine-stimulated prostaglandin production and expression of class II major histocompatibility molecules in cultured luteal steroidogenic cells, indicating that these cells can respond to type I IFN (Pate, 1995).

The amount of blood vessels remains unchanged from late luteal phase to early pregnancy, but number of pericytes and smooth muscle cells undergoes reduction in CL of early pregnant cows (Beindorff et al., 2010). Furthermore, in early pregnancy VEGFA luteal expression remain unchanged and the vasculature becomes stable with the decreased angiopoietin-2/angiopoietin-1 mRNA abundance ratio (an index of instability of vasculature) in the CL compared with the CL in the late luteal phase of a nonpregnant

cycle (Beindorff et al., 2010). Another system apparently involved with CL maintenance is the lymphatic system. Recent studies demonstrated upregulated expression of lymphatic endothelial hyaluronan receptor 1, a lymph vessel marker, in luteal tissues at d 40 of pregnancy (Nita et al., 2011). This study also showed that lymphatic endothelial cells treated IFN γ proliferated and formed capillary like tubes *in vitro* (Nitta et al., 2011). Moreover, lymphangiogenic factors such as VEGFC and lymphatic endothelial hyaluronan receptor-1 were also up regulated luteal tissue at d 16 of pregnancy (Nitta et al., 2011). Taken together, these results suggest that perhaps it is the lymphatic system, and not the blood vascular system of the bovine CL, that is rebuilt during early pregnancy.

During early pregnancy the CL is more resilient to the luteolytic effects of PGF $_{2\alpha}$ than the CL on the same day of the normal estrous cycle (Pratt et al., 1977; Silvia and Niswender, 1986). This suggests that perhaps intraluteal factors can influence what occurs within the bovine CL. The sensitivity of the early developing CL (fewer than 5 days) also has been exploited. Tsai and Wiltbank (1998) suggested that the early CL, in spite of presenting functional PGF $_{2\alpha}$ receptors, is unable to stimulating intra-luteal synthesis of PGF $_{2\alpha}$ via prostaglandin-endoperoxidase synthase 2, increasing expression of monocyte chemoattractant protein 1, and inhibiting progesterone production through StAR. Miyamoto et al. (2009) suggested a site restricted action of PGF $_{2\alpha}$ depending on the stage of the estrous cycle. In the mid-cycle CL (d 8 to 12), PGF $_{2\alpha}$ induces an acute increase in blood flow in the periphery of the CL concurrent with expression of endothelial nitric oxide synthase, but the same phenomenon is not observed in the early-cycle CL (d 4). Moreover, Atli et al. (2012) reported that although the initial pulse

of $\text{PGF}_{2\alpha}$ upregulates mRNA expression of many pathways related to luteolysis, the second and later pulses of $\text{PGF}_{2\alpha}$ are actually responsible for a distinct pattern of gene expression that result in luteolysis.

Luteolysis is a phenomenon coordinated by increased pulsatile release of $\text{PGF}_{2\alpha}$ in the bovine uterus, which reaches the ovaries through a countercurrent exchange mechanism between the utero-ovarian vein and ovarian artery (Hixon and Hansel, 1974). The synthesis of $\text{PGF}_{2\alpha}$ occurs mostly in epithelial endometrial cells that have the pool of phospholipids replenished by exposure to progesterone during diestrus. The exposure to progesterone during the diestrus cycle downregulates its own receptor in the reproductive tract and hypothalamus and upregulates estrogen and oxytocin receptors previously unresponsive to progesterone (Spencer and Bazer, 1995; Wathes et al., 1996, McCracken et al., 1999). Simultaneously, estradiol production by the dominant follicle of the last follicular wave stimulates pulsatile release of oxytocin that binds its receptor in the endometrial cells and activates phospholipase A_2 (PLA_2). The synthesis of $\text{PGF}_{2\alpha}$ in endometrial cells occurs through hydrolysis of ester bonds between phospholipids and arachidonic acid by PLA_2 . The released arachidonic acid is converted by prostaglandin G/H synthase to PGH_2 ; and then PGH_2 is converted to $\text{PGF}_{2\alpha}$ through the action of prostaglandin F synthase.

The blood vascular system of the CL also plays a key role on luteal regression. Treatment with $\text{PGF}_{2\alpha}$ leads to acute increase in blood flow at the periphery of the CL, progesterone increases, and LH changes followed by a gradual decrease in luteal blood flow in the d 10 midcycle CL. However, the same events are not observed in the d 4 early CL in cattle (Acosta et al., 2002; Ginther et al., 2007). Moreover, each peak of

uterine PGF_{2α} during spontaneous luteolysis leads to increased luteal blood flow in cattle (Miyamoto et al., 2005; Shirasuna et al., 2008). Nitric oxide (NO) is a potent vasodilator and has been reported to directly inhibit progesterone secretion and induce apoptosis in bovine luteal cells (Skarzynski et al., 2000; Skarzynski et al., 2003). In the bovine, treatments with PGF_{2α} up regulated expression of endothelial NO synthase (NOS) and increased luteal blood flow in mature CL 30 min after PGF_{2α} injection (Shirasuna et al., 2008). Treatment with the NO donor S-nitroso-N-acetyl-D,L-penicillamine into CL led to an acute increase in luteal blood flow and shortened the estrous cycle, whereas injection of the NOS inhibitor L-NG-nitroarginine methyl ester into CL completely blocked the acute increase in luteal blood flow induced by PGF_{2α} and delayed the beginning of luteolysis (Shirasuna et al., 2008). Therefore, it seems that the increased blood flow in the mature CL is modulated by NO, indicating that an acute elevation in peripheral blood flow to CL is likely one of the first physiological indicators of NO action in response to PGF_{2α}.

Endothelial cells are the primary type of cells to undergo apoptosis. It has been suggested that some capillaries disappear earlier than larger vessels with smooth muscle in the CL during luteolysis (Hojo et al., 2009). The strong vasoconstrictive factors endothelin-1 (EDN1) and angiotensin II (Ang II) are involved in the process of luteal regression in ruminants (Miyamoto et al., 1997; Hayashi and Miyamoto, 1999). Indeed, EDN1, Ang II, and NO inhibited progesterone secretion and PGF_{2α} upregulated expression of EDN1 in bovine CL *in vitro* (Miyamoto et al., 1997). Therefore, luteolytic PGF_{2α} is likely responsible for the regulation of endothelial and vascular functions

through induction of angiolysis and vasoconstriction to limit the oxygen and nutrient supply during luteolysis.

During luteolysis, leukocytes, particularly macrophages and T lymphocytes, significantly increase in number, and 70% of proliferating cells in the bovine CL are CD14⁺ macrophages (Bauer et al., 2001). In cows, large numbers of CD4⁺ and CD8⁺ T cells were found in regressing CL (Bauer et al., 2001), indicating an active role for these immune cell types in luteolysis. Furthermore, inflammatory cytokines, such as TNF- α , IL-1 β , and IFN- γ , and chemokines, such as monocyte chemoattractant protein 1 are involved in luteal regression (Pate and Keyes, 2001; Okuda and Sakumoto, 2003; Neuvians et al., 2004). These immunomodulatory factors may stimulate the accumulation of T lymphocytes and macrophages within the CL to support the luteolytic cascades. Afterwards, the CL regresses mostly through the loss of cells by apoptosis (Juengel et al., 1993), and apoptotic luteal cells are phagocytized by macrophages (Kato et al., 2005).

Luteal cells express both class I and class II major histocompatibility complex (MHC) molecules. These MHC molecules are essential for the recognition of cells by T lymphocytes as either self or nonself (Fairchild and Pate, 1989; Khoury and Marshall, 1990). The expression of MHC class II on luteal cells increased when luteal regression was induced by PGF_{2 α} suggesting that the demise of the CL might be involved in local autoimmune response mechanisms facilitated by increased expression of class II MHC molecules at the time of luteolysis (Benyo et al., 1991). Furthermore, bovine luteal cells can stimulate T-cell proliferation and this response is increased in the presence of cells from regressing CL as compared to responses in cells from midcycle CL in cows. This

results suggests that luteal cells may act as antigen-presenting cells, initiating a transient autoimmune response during luteolysis (Petroff et al., 1997).

Macrophages and T lymphocytes can produce several cytokines, such as TNF- α , IFN- γ , and prostaglandins to escalate the immune inflammatory response and to connect with peripheral resident cells depending on the stimulatory conditions. The cytokine TNF- α is present in large and small luteal cells, endothelial cells, and immune cells in the bovine CL and inhibits progesterone secretion and induces IFN- γ and Fas-mediated apoptotic cell death in bovine luteal and endothelial cells by increasing caspase-3 activity (Taniguchi et al., 2002; Pru et al., 2003). Interferon gamma inhibits LH-stimulated progesterone production, increases prostaglandin synthesis, upregulates MHC class I and II molecules, and induces cell death (Fairchild and Pate, 1989, Fairchild and Pate, 1995). The interaction between leukocytes and endothelial cells for leukocyte recruitment implies an overlapping succession of adhesive events encompassing leukocyte induction, rolling, and firm adhesion onto endothelial cells. P-selectin and E-selectin on endothelial cells can interact with leukocytes to promote leukocyte rolling and transient adhesion (Sako et al., 2003)

The bovine luteolytic cascade can be compared to an acute process with massive immune infiltration and vascular alteration with increased angiogenesis and blood flow. Initially, there is vasoconstriction likely induced by PGF_{2 α} and simultaneously the cell adhesion molecule P-selectin is acutely translocated to endothelial cell membranes, on which P-selectin strongly interact with its receptor present in neutrophils promoting rolling and transit adhesion of neutrophils (Shirasuna et al., 2012). In fact, it was demonstrated that after 5 min of the administration of PGF_{2 α} , P-selectin expression

and neutrophil adhesion increased in luteal endothelial before any chemoattractants of neutrophils were increased. This suggests that P-selectin actively contribute to infiltration of neutrophils in CL during PGF_{2α} induced luteolysis. At 10 to 30 min after the beginning of acute inflammation, vascular dilatation starts in local arterioles, and the velocity and volume of blood flow increases, resulting in the induction of high levels of neutrophil adhesion. This acute increase in blood flow peaks several hours after the start of acute inflammation, and blood flow disappears approximately 1 d later (Shirasuna et al., 2012).

Reproductive Management, Efficiency and Timed Artificial Insemination

Reproductive efficiency has been acknowledged widely as a major contributor for herd profitability in dairy operations, which makes the adoption of adequate reproductive management practices a critical component for the success of dairy farms (Britt, 1985; Giordano et al., 2011; Ribeiro et al., 2012a; Galvão et al., 2013). Failure to obtain proper reproductive efficiency results in reduced percentage of cows at the early stages of lactation, increased costs with AI, and delayed genetic progress (Santos et al., 2010b). Furthermore, low reproductive performance leads to reduced total milk sales, increased number of culled cows, and reduced number of replacement heifers born (Britt, 1985; De Vries, 2004; Meadows et al., 2005; Ribeiro et al., 2012a). Other factors influenced by the selection of a specific reproductive management program and its efficiency include feed cost, labor cost, and veterinary expenses that are generally associated with lactation length, dry period length, and number of services to conceive (Lima et al., 2010; Giordano et al., 2011; Ribeiro et al., 2012a). Therefore, the selection a long-term reproductive management strategy that improves reproductive efficiency is a critical decision for dairy operations sustainability.

Recently, a body of evidence in the literature suggests that incorporation of timed AI in reproductive programs either as sole strategy or in combination with AI at estrous detection can lead to economic benefits for lactating dairy cows and heifers (Lima et al., 2010; Giordano et al., 2011; Ribeiro et al., 2012a; Galvão et al., 2013). Lima et al. (2010) compared the cost of timed AI and natural service using as inputs reproductive results and economical information from a field study that evaluated these two reproductive programs (Lima et al., 2009), and reported that the cost of a pregnancy was \$9.73/cow less for timed AI than natural service. Furthermore, sensitivity analysis using increased cost of feeding or increased P/AI resulted in an even greater economic advantage of timed AI compared with natural service (Lima et al., 2010).

In another study, the profitability of three different reproductive programs (100% estrous detection vs. two programs with 100% timed AI) on a specific dairy farm were estimated using Markov chain simulation with partial budgeting and sensitivity analysis performed to assess the effect of varying specific reproductive parameters on profitability (Giordano et al., 2011). The results of this study revealed two reproductive programs with 100% timed AI, the Double-Ovsynch and d 32 resynch at first and second or subsequent service, or Double-Ovsynch and Double-Ovsynch resynch at first and second or subsequent service, were superior to the 100% estrous detection program. For programs with 100% timed AI, Double-Ovsynch for resynch resulted in increased P/AI for resynchronized services and it was economically superior despite having higher costs and a longer interbreeding interval. A 4% increase in P/AI for resynchronized AI was sufficient for the Double-Ovsynch resynch to outperform the program with day 32 resynch for subsequent services. Adding estrous detection to the

100% timed AI programs was only beneficial for the program with the lower P/AI. The improvement in service rate required for the 100% estrous program to have the same profitability as the superior 100% timed AI program was 12%, which ultimately suggests that well-managed timed AI is superior to estrous detection.

A third study compared the economic outcome of reproductive programs using estrous detection, timed AI, or a combination of both timed AI and estrous detection using a stochastic dynamic Monte-Carlo simulation model (Galvão et al., 2013). The programs evaluated were 100% estrous detection; timed AI with Presynch-Ovsynch for first AI, and Ovsynch for resynchronization of open cows at 32 d after AI; or timed AI-estrous detection with Presynch-Ovsynch for first AI, but estrous detection and AI after first AI, and cows diagnosed open 32 d after AI were resynchronized using Ovsynch. Sensitivity analysis was performed using estrous detection rates of 40 vs. 60%, accuracy of estrus of 85 vs. 95%, and compliance with timed AI of 85 vs. 95%. For programs with an estrous detection rate of 40%, timed AI program with 95% of compliance was more profitable than the 100% estrous detection or the timed AI-estrous detection programs. For programs with estrous detection rate of 60%, the program timed AI-estrous detection resulted in the greatest profit followed by 100% estrous detection programs and 100% timed AI program, respectively. Combining timed AI and estrous detection increased profits within each level of accuracy or compliance. Adding timed AI to estrous detection would increase overall profit/cow per year by \$46.8 to \$74.7 with 40% estrous detection rate, and by \$8.9 to \$30.5 with 60% estrous detection rate. Therefore, the combination of timed AI with estrous detection program

was the reproductive management program that maximized profit for dairy producers (Galvão et al., 2013).

An economic analysis of reproductive programs for dairy heifers with timed AI and estrous detection was performed using a simulation to calculate pregnancy rates, average time to pregnancy, total costs per AI, and pregnancy for four reproductive management strategies with breeding period of 84 days allowing approximately four estrous cycles (Ribeiro et al., 2012a). The four breeding programs used were: 1) 100% timed AI; 2) 100 % detection of estrus; 3) timed AI for first breeding and detection of estrus for the remaining services; and 4) timed AI for first breeding followed by insemination upon detected estrus or resynchronized insemination after nonpregnancy diagnosis. Sensitivity analyses were performed for four estrous detection rates of 50, 60, 70 and 80%. The results of this study revealed that incorporation of timed AI for first service lowered the cost per pregnancy compared with estrous detection alone, although the benefits were less as estrous detection rates increased. Likewise, programs with 100% timed AI were less expensive than programs with exclusive use of insemination at detected estrus only if detection of estrus were below 70%. When additional timed AI were incorporated into the breeding program to resynchronize nonpregnant heifers that had not been detected in estrus, it further benefited the program with low estrous detection rates, of 50 and 60%. However for higher estrous detection rates, of 70 and 80%, the benefits were minor or inexistent. Incorporation of detection of estrus after one timed AI was superior to timed AI alone only when estrous detection rate was 60% or more. Most of the changes in costs per pregnancy resulted from feed costs associated with heifers becoming pregnant later in the breeding period.

In summary, combination of timed AI program for first insemination with estrous detection and additional timed AI for nonpregnant heifers maximized the economic success of a reproductive program; however when estrous detection rates were equal or greater than 70% benefits of additional timed AI for nonpregnant heifers was negligible (Ribeiro et al., 2012a).

Although reproductive efficiency is critical for economical sustainability of dairy operations, there is substantial evidence that measurements of reproductive performance declined in the last four decades in the US until the mid-2000's (Washburn et al., 2002; Butler, 2003; VanRaden et al., 2004, De Vries and Risco et al., 2005; Norman et al., 2009). Several factors have contributed to the impairment of reproductive efficiency including cow physiology, nutritional and reproductive management and genetics (Lucy et al., 2001; Weigel et al., 2006). The evolvement of the dairy industry in the US have been marked by intensive genetic selection for production traits and consolidation of farms resulting in increased herd size, housing of cows on concrete floor with smaller area per cow and reduced time detecting estrus at individual cow basis (Senger, 1994; Roelofsa et al., 2010). Two major problems aroused with the modernization of dairy industry: 1) milk production, the major trait used for genetic selection, has an antagonistic relationship with reproduction in dairy cows and estrous behavior (Laben et al., 1982; Pryce et al., 1997, Butler, 2003; VanRaden et al., 2004; Cochran et al.2013), and 2) the new management practices and housing of dairy farms are associated with impaired expression of estrous behavior and reduced estrous detection rates (Senger, 1994; Lopez et al., 2004; Roelofsa et al., 2010).

Although some studies evaluating the association of milk production and fertility within a herd and among many herds did not reveal the same trend of a negative relationship between production and fertility (Santos et al., 2009; LeBlanc, 2010), it is a general consensus that milk production modifies behavior, metabolic, nutritional and health demands in lactating dairy cows (Wiltbank et al., 2006). In fact, Lopez et al., (2004) reported high-producing cows (averaging 46.4 kg/day) in comparison with low producers (averaging 33.5 kg/day) had shorter duration of estrus (10.9 h vs. 6.2 h), reduced number of total standing events (6.3 vs. 8.8) and reduced standing time (21.7 s vs. 28.2 s). These results clearly exemplify how behavioral estrus, a key component for proper detection of estrus, is hindered by milk production.

In the last 20 years, research efforts were devoted to the development of knowledge, techniques and strategies to reverse this trend of declining fertility in dairy cows (Lucy et al., 2001). One of the most important reproductive techniques developed in the recent years was timed AI, which is a program to synchronize ovulation characterized by a time sensitive sequential use of hormonal treatments to induce occurrence of follicle turnover, luteolysis and synchronous ovulation allowing cows to inseminated in pre-determined optimum time. Timed AI was instrumental and added value to the classic estrous synchronization programs based on PGF_{2α} to control luteal lifespan because it overcomes issues related to cow factors (anovulation, lameness, low intensity and duration of estrous behavior), environmental factors (heat stress, poor flooring) and human errors that limit estrous detection rates and subsequent reproductive performance (Wiltbank et al., 2006).

The first stride towards development of timed AI occurred when GnRH was given seven days prior PGF_{2α} injections leading to increased control of follicle development and acceptable fertility (Thatcher et al., 1989). The use of GnRH resulted in occurrence of LH surge within two hours and ovulation 28 hours later in dairy cattle (Bodensteiner et al., 1996). The decisive stride towards the development of timed AI program was the addition of second GnRH injection 48 hours after the injection of PGF_{2α} followed by insemination 24 to 32 hours later (Pursley et al., 1995; Burke et al., 1996). This program was later named Ovsynch (Pursley et al., 1997) and it is now part of the reproductive programs in most dairy herds in the US. The proportion of synchronized breeding in the US was estimated to be between 10 and 19% depending upon region, and it increased from 2% in 1996 to as much 35% in 2005 for all cows inseminated for their first service (Miller et al., 2007).

After the development of timed AI, several studies exploited key aspects of reproductive physiology aiming to optimize the results of timed AI program for dairy cows. Pursley et al., (1998) investigated the optimal interval between second GnRH injection and AI for the Ovsynch program inseminating at 0, 8, 16, 24, or 32 h after the second GnRH treatment. This study found a quadratic effect of time of AI on P/AI, which increased from 0 to 16 h with subsequent decreases from 24 to 32 h, when the expected time of ovulation occurs. However, only insemination performed after the expected time of ovulation, 32 h after final GnRH, resulted in a statistically significant decrease in both P/AI and percentage calving per AI (Pursley et al., 1998). Analysis of calving data suggested that AI at any time between 0 and 24 h after the final GnRH resulted in similar rates of calving. Therefore, the optimal time for AI was determined to

be approximately 16 h after final GnRH, nonetheless there is some flexibility to perform timed AI before ovulation (between 0 to 24 hours), on which pregnancy outcomes should be not ideal, but acceptable (Pursley et al., 1998). The physiological basis for an optimal time for insemination at 16 h after GnRH or 12 h before ovulation is an ideal synchrony between sufficient time for optimal sperm capacitation and the presence of oocyte that is not overly aged in the reproductive tract (Saacke et al., 2000).

Another important aspect of reproductive physiology related to Ovsynch protocol is that synchronization of ovulation and fertility are optimized when cows receive the first GnRH of the protocol in early diestrus between days 5 to days 9 of the estrous cycle (Vasconcelos et al., 1999). Indeed, cows receiving the first GnRH during early diestrus are unlikely to have spontaneous luteolysis in the middle of the program, have limited follicle dominance and are benefited by the presence of a CL and optimal concentrations of progesterone, which in turn is critical for follicle development (Bleach et al., 2004, Cerri et al., 2009a, Bisinotto et al., 2013).

Using the concept of ideal timing to initiate a timed AI program, Moreira et al. (2001) developed a program named Presynch, which aimed to increase the chance of obtaining cows on early diestrus at initiation of Ovsynch protocol. The Presynch-Ovsynch program developed by Moreira et al. (2001) was composed of two injections of PGF_{2α} given 14 days apart, with the second injection given 12 days prior to the first GnRH of the Ovsynch protocol. When compared with Ovsynch alone, the Presynch-Ovsynch remarkably increased P/ AI in cyclic lactating cows (from 25% to 43%). Although the program developed by Moreira et al., (2001) was successful and other studies confirmed the benefits of this program (El-Zarkouny et al., 2004; Navanukraw et

al., 2004), it was based solely on PGF_{2α}, which has limited to no efficacy in anovular cows and cows not bearing a CL at time of the PGF_{2α} injections. Therefore, other programs were successfully developed to presynchronize the estrous cycle in dairy cows using GnRH before the PGF_{2α} injection increasing the chance of obtaining cows in early diestrus at initiation of the timed AI programs independent of cyclic status (Bello et al., 2006, Galvão et al., 2007; Souza et al., 2008).

When programs based on the incorporation of one treatment of GnRH before PGF_{2α} were compared with the presynchronization programs based on PGF_{2α} alone, they did not benefit fertility of dairy cows (Galvão et al., 2007; Ribeiro et al., 2011). The Double-Ovsynch protocol that incorporates an entire Ovsynch program as presynchronization improved P/AI in comparison with the presynchronization based on PGF_{2α} (Souza et al., 2008). Other alternative presynchronization programs used progesterone supplementation aiming to induce estrous cyclicity in of anovular dairy cows before timed AI. Despite the fact that these programs induced cyclicity in about 50% of the anovular cows no improvements in fertility were observed (Chebel et al., 2006; Bicalho et al., 2007; Stevenson, 2011).

Generally, presynchronization is only feasible to the first insemination postpartum to avoid delays in re-insemination for subsequent services. However, a recent study that compared resynchronization programs using the conventional Ovsynch to Double-Ovsynch, the latter used as a presynchronization and synchronization program all in one, revealed that cows rebred using the Double-Ovsynch program had increased synchronization rate (72 vs. 51%) and P/AI (39 vs. 30%) compared with regular Ovsynch (Giordano et al., 2012). Although this program had additional costs with

hormonal treatments and increased interval between inseminations, an economic analysis indicated that the incorporation of the Double-Ovsynch for resynchronization within a reproductive program resulted in a more profitable breeding program than use of Ovsynch alone for resynchronization (Giordano et al., 2011). Nevertheless, this benefit was observed because cows began the resynchronization before pregnancy diagnosis in an attempt to offset the lengthy resynchronization protocol with Double-Ovsynch.

Another aspect related to timed AI exploited by some recent studies was the reduction in the period of follicular dominance (Santos et al., 2010a), which previously was shown to be important to improve embryo quality (Cerri et al., 2009a). Santos et al., (2010a) shortened the interval between the initial GnRH and the injection of PGF_{2α} from 7 to 5 days, which lead to an increased P/AI in lactating dairy cows (37.9 vs. 30.9%). It is important to mention that for 5-d program an additional injection of PGF_{2α} was given on d 6 of the program to ensure that a newly formed CL in response to the initial GnRH was fully regressed (Santos et al., 2010a).

Additionally, on the 5 d program the administration of the second GnRH 56 hour after the PGF_{2α} did not improve P/AI (Bisinotto et al., 2010a) such as reported in the 7-d program (Pursley et al., 1998; Brusveen et al., 2008) The 16 hours prolonged proestrus in a 5-day program likely resulted in additional growth of the ovulatory follicle, greater concentrations of estradiol, which explained the increased percentage of cows in estrus (Bisinotto et al., 2010a). Therefore, the potential benefits of having an improved synchrony between oocyte availability and numbers of viable spermatozoa for fertilization (Saacke, 2008) encountered in programs of 7 days with GnRH given 56

hours after the PGF_{2α} is offset by prolonged proestrus in programs of 5 days (Bisinotto et al., 2010a).

Anovulation or, in some cases, the absence of a CL due to stage of the estrous cycle when a timed AI is initiated reduces the risk for pregnancy by 30%, suggesting that the concentration of progesterone on which a future dominant follicle grows is critical for fertility (Bisinotto et al., 2010b). A recent study targeting supplementation of progesterone in cows lacking CL at the initiation of the timed AI program was able to reverse the negative impact of lack of CL on P/AI (Bisinotto et al., 2013). Holsteins cows were evaluated for the presence of CL and cows not bearing a CL at initiation of 5-d timed AI program were allocated randomly to either remain as untreated control or receive two controlled internal drug release (CIDR) inserts containing progesterone for 5 days. Cows without the CL that received two CIDR inserts as a treatment had similar P/AI than cows in diestrus (46.8 vs. 49.9 %) and both had greater P/AI than cows without CL that remained untreated (30.8 %). The use of two CIDR devices was successful because elevated plasma concentration of progesterone to 2.65 ng/mL, which is greater than the 0.8 to 1.0 ng/mL identified in cows supplemented with only one CIDR insert (Cerri et al., 2009b; Lima et al., 2009b). These results suggest that one CIDR insert likely released an insufficient concentration of progesterone to optimize follicle or oocyte maturation during the final stages of development before AI. In fact, supplementation of lactating dairy cows with a single CIDR insert during timed AI programs improves fertility of those bearing a CL, but not in cows without a CL (Bisinotto and Santos, 2012).

Timed AI programs for dairy heifers have had a different historical trend than timed AI programs for lactating dairy cows. The first research projects that exploited timed AI for dairy heifers failed to obtain acceptable P/AI ranging from 25.8% to 45.5%, which were consistently inferior to results obtained for AI after detected estrus within the same studies (Schmitt et al., 1996; Pursley et al., 1997, Stevenson et al., 2000; Rivera et al., 2004; Rivera et al., 2005; Rivera et al; 2006). The first studies investigating timed AI for dairy heifers used Ovsynch or similar protocols with a 7 days interval between the first GnRH and PGF_{2α} (Schmitt et al., 1996: Pursley et al., 1997; Stevenson et al., 2000). This approach likely lead to a period of follicle dominance that potentially is too long for the 50% dairy heifers that have 3 follicular waves (Table 3-1). The next series of studies reduced the period of follicle dominance using a 6-day program (Rivera., et al., 2004) and added a CIDR insert between first GnRH and PGF_{2α} to limit follicle turnover in the middle of the protocol (Rivera et al., 2005; Rivera et al., 2006), but the results for these studies were still not comparable to AI performed after detected estrus.

The first stride toward the development of a timed AI program in heifers that resulted in acceptable fertility occurred when Rabaglino et al. (2010b) reduced the period of follicle dominance even further using a 5 day program and a CIDR insert between the 1st GnRH and PGF_{2α} injection followed 72 hours later by concurrent GnRH and AI. The results of P/AI for this study were the first comparable to insemination performed after estrous detection ranging from 53.1% to 59.5% (Rabaglino et al., 2010b; Kuhn et al., 2010). Another study subsequently investigated the effects of the addition of a second PGF_{2α} 12 hours after the first treatment in the 5 day timed AI program for dairy heifers finding no improvements in P/AI and luteolysis (Rabaglino et

al., 2010a). However, an interesting finding from this study was that only 23% of the heifers had more than one CL 5 d after the injection of GnRH suggesting that ovulation to the initial GnRH was probably low (Rabaglino et al., 2010a).

The importance of the proestrus length and follicle dominance period was investigated in recent study that compared a modified 5 day timed AI program with GnRH injection and insemination performed concurrently from 53 to 60 hours after the PGF_{2α}, with a 7 day timed program using also 53 to 60 hour interval from PGF_{2α} (Lopes Jr. et al., 2013). The P/AI for the modified 5 day timed AI with shorter than usual proestrus was only 44.8%, but was still greater than the P/AI for the 7 day timed AI program (35.7%) suggesting the shorter proestrus might be detrimental to P/AI and a longer period of follicle dominance associated may aggravated even more the detrimental effect on fertility of dairy heifers.

In contrast with most of the literature, two recent studies compared 5-d timed AI program with 7-d timed AI in dairy heifers reporting no differences in P/AI (Colazo and Ambrose, 2011; Mellieon Jr. et al., 2012). Although, these studies were well designed, caution is required on their interpretation, because Colazo and Ambrose (2011) used only 64 heifers which makes it difficult to have sufficient power to identify difference in P/AI. Mellieon Jr. et al. (2012) used sexed semen and had very low P/AI, approximately 30%, which also can mask the ability to identify a statistical difference among treatments.

Uterine Diseases Relevance and Characterization

Uterine diseases affect half of the dairy cows after parturition leading to disruption of uterine and ovarian function which frequently hinders fertility, increases culling, and contributes to remarkable economic losses for dairy producers (Sheldon et

al., 2009). The most common forms of uterine diseases includes retained fetal membranes (RFM), metritis and endometritis.

Retained fetal membranes are characterized as failure to release the placenta within 12 (Van Werven et al., 1992) or 24 h post calving (Kelton et al., 1998). Ninety five % of the cows that released the placenta within 24 hours did so within 12 hours; therefore, this distinction between 12 or 24 hours seems to be of little, if any relevance (Van Werven et al., 1992). Once RFM occurred, fetal membranes tend to be retained for 7 days on average (Eiler, 1997). Retained fetal membranes have detrimental impacts on fertility and are a major risk factor for metritis, clinical endometritis, and other periparturient diseases that ultimately compromise survival, production, and reproductive performance (Grohn et al., 1990; Oltenacu et al., 1990; Laven and Peters, 1996). Retained fetal membranes affect 7.8% of the US dairy cow population (NAHMS, 2009). There are a number of risk factors associated with RFM, including induced parturition, shortened gestation, abortion, dystocia, fetotomy, cesarean section surgery, twinning, nutritional deficiencies such as vitamin E and selenium, and immunosuppression (Muller and Owens, 1974; Terblanche et al., 1976; Julien and Conrad, 1986; Jooston et al., 1987; Rajala and Grohn, 1998; Laven and Peters, 1996).

The impact of RFM ranges from impaired reproductive performance to development to severe metritis with loss of milk production and reproductive performance and increase risk of culling. A meta-analysis revealed that the daily rate of pregnancy decreased by 16% in cows diagnosed with RFM relative to unaffected cows (Fourichon et al., 2000), which extended interval to pregnancy by up to 51 days (Borsberry and Dobson, 1989). The estimated cost of each case of RFM ranges from

US \$355 to \$464 (Kossaibati and Esslemont, 1997; Laven, 2006), and the major cause of this economic loss is reduced milk yield and consequent reduction of milk sales. In fact, losses of milk production are associated with progression to metritis. It has been estimated that cows affected by RFM have a 3 kg/d reduction in milk yield for the first 5 d after calving, and cows with a RFM have 2 to 4-fold increase relative risk of developing metritis (Santos et al., 2010b). Cows affected by RFM that develop metritis have a cumulative loss of milk of approximately 110 kg during the first 7 weeks of lactation (Deluyker et al., 2012). When left untreated, RFM alone reduced milk yield in primiparous and multiparous cows by 412 and 537 kg/lactation, respectively. Similarly, when left untreated, metritis alone reduced milk yield in primiparous and multiparous cows by 338 and 498 kg/lactation, respectively.

Metritis is characterized by an abnormally enlarged uterus and a fetid, watery red-brown fluid to viscous off-white purulent uterine discharge within 21 days postpartum, but more frequently diagnosed in the first week postpartum (Sheldon et al., 2006). Metritis can be classified according to the severity of the disease as: grade 1 if they have an abnormally enlarged uterus and a purulent uterine discharge without any systemic signs of disease; grade 2 when additional signs of systemic illness such as decreased milk yield, dullness and fever >39.5 °C are present; and grade 3 when signs of toxemia such as inappetence, cold extremities, depression and/or collapse are present, which generally is associated to a poor prognosis (Sheldon et al., 2006). Grades 2 and 3 can also be called acute puerperal metritis or toxic puerperal metritis (Drillich et al., 2011). The incidence of metritis in dairy cows range from 10 to 36% (Goshen and Shpigel, 2006; Santos et al., 2010b; Chapinal et al., 2011). In many

studies, the reported incidence of metritis was low, likely because only cows that develop fever concurrent with metritis were classified as having metritis. However, approximately 50 to 60% of the cows diagnosed with metritis do not develop fever, based on rectal temperature ≥ 39.5 °C, which results in underestimation of the incidence of the disease (Benzaquen et al., 2007, Martinez et al., 2012).

The economic losses caused by metritis are striking and it has been calculated at \$380 per affected cow due reduced milk production, delayed conception, treatment and increased culling (Drillich et al., 2001). Thus, if a conservative incidence rate of 20% for the 8.5 million dairy cows in US the annual cost of metritis alone is \$650 million.

Many factors have been associated with increased risk of developing metritis. Huzzey et al. (2007) reported a strong association between feed intake and cows' behavior and the subsequent development of metritis. Cows with severe presentation of metritis consumed 2 to 6 kg of less of dry matter than healthy cows at 2 to 3 weeks before the diagnosis of metritis. Increased concentrations of nonesterified fatty acids (NEFA), β -hydroxybutyric acid (BHBA) early postpartum and neutrophils with less intracellular glycogen were associated with cows diagnosed with metritis (Galvão et al., 2010). Other factors associated with metritis were plasma concentrations of haptoglobin ≥ 0.8 g/L and plasma concentration of NEFA ≥ 0.6 mM in the first week postpartum (Dubuc et al., 2010b). Additionally, cows presenting subclinical hypocalcemia, based on serum total Ca concentration ≤ 8.59 mg/dL in one or more of the first 3 days post calving, had 3.2 and 11.5 times greater risk of developing metritis and puerperal metritis, respectively (Martinez et al., 2012). Although dystocia, twinning, stillbirth, and male offspring are all risk factor for the development of metritis, RFM is considered the

most important risk factor for metritis (Gröhn et al., 1990; Correa et al., 1993). Any condition that may impair feed intake and immune function increases the risk of metritis (Leblanc et al., 2007).

Endometritis is an inflammatory disease of the pelvic tissue diagnosed after 21 days postpartum caused by the inability of the host to eliminate microbial infection (Sheldon et al., 2006). Endometritis is classified as clinical or subclinical. Clinical endometritis is defined by presence of purulent vaginal discharge detectable 21 days or more after parturition, or mucopurulent discharge detectable in the vagina after 26 days postpartum. Subclinical endometritis is characterized by inflammation of the endometrium defined by presence of PMNL exceeding between 5.5% (Santos et al., 2009) and 10% of cells (Kasimanickam et al., 2004) in samples collected by flushing the uterine lumen or by endometrial cytobrush, in the absence of clinical signs at approximately 5 weeks postpartum (Sheldon et al., 2006). The inflammation is presumably associated with recovery of the tissues after clinical endometritis, trauma or other non-microbial disease. The nomenclature for clinical endometritis may not be the most appropriate because a large proportion of cows presenting pus in the vaginal discharge do not have concurrent neutrophil infiltration in the endometrium (Dubuc et al., 2010). Thus, purulent vaginal discharge was suggested as an alternative terminology for clinical endometritis to resemble properly what have been diagnosed in cases of clinical endometritis (Dubuc et al., 2010). The incidence of subclinical endometritis is dependent on the cut-off for diagnosis and the time after parturition but is in the order of 37 to 74% of animals (Gilbert et al., 2005). Most importantly, subclinical

endometritis affects 40% cows classified healthy at the breeding time (Gilbert et al., 2005).

Clinical and subclinical endometritis have been associated with reduced P/AI at first service, increased pregnancy loss, and prolonged time to pregnancy (Kasimanickam et al., 2005; Galvão et al., 2009a; Dubuc et al., 2011). Cows diagnosed with both clinical and subclinical endometritis had the longest interval from calving to pregnancy compared with those diagnosed with only one of the two diseases or with cows having no diagnosis of uterine diseases (Dubuc et al., 2011). Subclinical endometritis, a persistent uterine disease, leads to reduced fertilization, and compromises early embryo development and survival (Cerri et al., 2009; Hill and Gilbert, 2008; Galvão et al., 2009). Additionally, cows diagnosed with subclinical endometritis have altered endometrial and embryonic gene expression (Hoelker et al., 2012). Endometrium from cows diagnosed with subclinical endometritis had altered patterns of expression of genes involved in cell adhesion and immune modulation and embryos from cows with subclinical endometritis had altered pattern of gene expression involving pathways in cell cycle and apoptosis.

Uterine Disease Etiology: Immunological and Microbiological Aspects

Throughout the last few decades, major advances have been made to better understand how host uterine innate immunity is subverted by microbes leading to development of uterine diseases. Although, microbes are inextricably linked to uterine diseases development, a dysregulation of uterine immunity alone may also lead to development of uterine disorders that impair fertility of dairy cows.

Research conducted in the last 3 decades identified three general causes of RFM in cattle: factors around parturition maintaining blood pressure within the chorionic

villi, occurrence of uterine atony, and dysregulation at a molecular and cellular level (McNaughton and Murray, 2009). The first cause is associated with pathological changes that interfere with the detachment of fetal and maternal epithelial components within the placentome such as occurrence of villous edema as result of cesarean section surgery or uterine torsion (Grunert, 1986). Uterine atony is a second cause and it is considered very unusual occurring in 1 to 2% of the cases and it is associated with multiple pregnancies in which the myometrium had been stretched excessively and muscle tonicity is compromised (Grunert, 1986). The third and most important possible cause of RFM is dysregulated inflammation and immune function. Histological investigation of the placentome of cows with RFM revealed small areas of necrosis between the villous trophoblast and the crypt epithelium, suggesting that there may have been a more generalized disease condition, which was associated with the presence of microorganisms (Al-Sadi et al., 1994).

Recently, many other immune dysfunctional related factors have been associated with RFM including collagenase, hyaluronidase, matrix metalloproteinases (MMPs), reactive oxygen species (ROS), major histocompatibility complex (MHC), and feto-maternal steroidogenesis. Factors related to extracellular matrix components in the placentome are suggested to be involved with the cause of RFM. After a normal parturition, collagen fibers in the caruncular connective tissue have a swollen appearance with indistinct contours and a linear arrangement, whose breakdown is regulated by the enzymes collagenase and hyaluronidase. Retained fetal membranes increase the activity of these enzymes in both the maternal and fetal compartments of the placentome (Kankofer et al., 1998). Matrix metalloproteinases are a family of zinc-

and calcium-dependent endopeptidases that degrade extracellular matrix proteins and are also involved in the breakdown of extracellular matrix components such as collagen (Woessner, 1998). They are synthesized by some inflammatory and epithelial cells and secreted as inactive precursors that are activated in extracellular spaces by enzymes such as plasmin. The activities of MMP-2 and MMP-9 are regulated at the cellular level by proenzyme activation and by tissue inhibitors metalloproteinase, TIMP-1 and TIMP-2. ProMMP-9 activity is found only in the maternal compartment and has no influence on placental separation after calving. However, several active isoforms of MMP-2 are present within both compartments of the feto-maternal unit during normal stage 3 of labor, but only one, the 68 kDa form, has been detected in cows with a retained placenta. Maj and Kankofer (1997) considered that higher activities of proMMP-2 and the absence of other MMP-2 isoforms affected the hydrolysis of collagen so that an increase in rigidity of the intravillous extracellular matrix caused placental retention.

Immunological antioxidant mechanisms against ROS may also play an important role in the release of fetal membranes. Reactive oxygen species produced during normal cellular metabolism are harmful if not removed causing peroxidative damage to cell membranes and other cellular components. Alternatively, they may react with other cellular reducing equivalents, such as NADPH, which disrupt cellular biochemical processes such as glucose metabolism. Some of their negative influences include changes to the steroidogenic and arachidonic acid cascades (Staats et al., 1988). Enzymes such as glutathione peroxidase, catalase and superoxide dismutase remove ROS. The antioxidant status of placentomes, in terms of their enzyme activity for glutathione transferase, catalase and superoxide dismutase, was remarkably reduced

for up to two weeks before calving in cows that subsequently were diagnosed with RFM (Kankofer, 2001). In case these mechanisms fail, chain-breaking antioxidants are also present, such as vitamin E, which inhibits the chain reactions initiated by free ROS (Miller and Brzezinska-Slebodzinska, 1993).

Oxidative DNA damage increases cows with RFM. The enzyme 8-hydroxy- 2'-deoxyguanosine (8-OH-dG), which is associated with damage to DNA was identified in increased concentrations in both maternal and fetal compartments have been recorded in cows with RFM (Kankofer and Schmerold, 2002).

In addition to having an antioxidant role, vitamin E also regulates the activity of PLA₂, which is important in cleaving arachidonic acid from cell membranes during the synthesis of prostaglandin. The form of vitamin E, α -tocopherol, binds to and inhibits PLA specifically and effectively. A dysregulation of the metabolism of prostaglandin was also revealed in cows with RFM having an increased plasmatic concentration of PGF_{2 α} and reduced concentration of PGE₂ in comparison to unaffected cows (Chandra et al., 2002).

Another possible factor involved with RFM was a downregulation of maternal antigen recognition. Cows suffering from RFM had reduced chemotactic activity of maternal leucocytes and periparturient cows in which there was no leucocyte activity, the incidence of RFM was 100% (Miyoshi et al., 2002). MHC-I compatibility of pregnant cows and their calves was another relevant factor linked to RFM. Cows that were more homologous with their fetuses had a greater risk of RFM than cows that were more dissimilar (Jooston et al., 1991). Therefore, from an immunological perspective, the development of RFM could be associated with a reduction in the variability of MHC-I

expression between dam and fetus that reduces the production of appropriate lymphokines within the fetomaternal unit as it develops towards its mature state.

The synthesis of prostaglandins by the uterus is another possible dysregulated pathway in the bovine leading to RFM. Takagi et al. (2002) reported that the ratio of caruncular PGE₂ to PGF_{2α} at parturition and six hours later was lower in cows with RFM, suggesting a hampered role for these two prostaglandins in placental separation. Additionally, there might be an imbalance in arachidonic acid metabolism in the endometrium RFM cows, possibly involving vasoactive-related peptide systems such as endothelin-1 (Takagi et al., 2008). *In vitro* studies of the synthesis of prostaglandins in uninucleate and binucleate cells obtained from cows with a RFM produced predominantly PGE₂, on the other hand, cells from healthy cows synthesized more PGF_{2α} (Gross and Williams, 1988). Uninucleate cells synthesize more prostaglandin than binucleate cells, but binucleate cells readily convert PGF_{2α} into PGE₂. There are fewer viable binucleate cells in placentas that have been released normally than in placentas that have been retained for one hour and then removed. The enzyme PGE₂ 9-keto-reductase has been suggested to reverse the metabolism of PGF_{2α} into PGE₂ and regulating the ratio of these two hormones. Its activity is significantly higher in both the fetal and maternal compartments of placentomes obtained from cows diagnosed with RFM than in normal healthy cows (Kankofer and Schmerold, 2002).

The bovine placentome is a target organ for the steroid hormones progesterone and estrogen, and receptors for these hormones have been identified. Placental steroid hormones help to regulate placental growth and differentiation (Hoffman and Schuller, 2002). Their biosynthesis in trophoblastic cells requires the transfer of cholesterol

from the outer to the inner mitochondrial membrane, where pregnenolone is synthesized. Under the influence of fetal cortisol, the concentration of which increases as gestation advances, the activity of the enzyme 17 α -hydroxylase progresses to convert placental progesterone to estrogen. The resulting increase in the ratio of estrogen to progesterone is responsible for increasing the rate of synthesis and/or release of utero stimulatory hormones such as prostaglandin and oxytocin, which act through cell-mediated pathways to increase intracellular calcium and activate myometrial contractile fibers (Takagi et al., 2002). Boos et al. (2000) showed that the immunoreactivity of both estrogen and progesterone receptors in placentomes tended to be less in cows that expelled the placental membranes spontaneously than in cows that retained them. These authors suggested that a lower steroid hormone receptor status in the placentome 40 to 50 hours before parturition was crucial for normal placental separation to occur. Higher immunoreactivity scores in cows with retained fetal membranes, particularly in the maternal crypt epithelium, suggested a degree of immaturity in tissue that correlated with an increase in the number of epithelial cells undergoing apoptosis after calving (Boos et al., 2000). In a mature feto-maternal unit, this process should have been completed at or immediately after calving.

Binucleate cells express TIMP-2 and, with a reduction in the number of binucleate cells as parturition approaches, less is produced at the end of a normal pregnancy (Walter and Boos, 2001). Takagi et al. (2008) suggested that TIMP-2 activity and MMP-9 expression may be influenced by placental progesterone. During pregnancy, both are necessary to maintain the structure and function of a healthy feto-maternal unit by preventing epithelial separation within the placentome, a role similar to

their role in the cervix and corpus luteum of other mammals. Furthermore, enzyme activity controlling placental steroidogenesis continues after parturition, and results in significantly increased expression of MMP-9 mRNA in the cotyledons of cows with RFM. In summary, although many factors may be involved with occurrence of RFM, a clear predominant mechanism remains unidentified.

The etiology of metritis and endometritis remains partially elusive, but it has been always highly linked with microbial infections occurring primarily at time of parturition. The major pathogens commonly present and associated with uterine diseases are *E. coli* and *T. pyogenes* followed by a range of anaerobic bacteria such as *F. necrophorum*, *Prevotella melaninogenicus*, and opportunistic bacteria such as *Pseudomonas spp.*, *Streptococcus spp.*, and *Staphylococcus spp.* that were identified in a variety of combinations using conventional cultures methods (Sheldon et al., 2002; Williams et al., 2005; Santos et al., 2010c; Santos et al., 2010d).

Many studies suggest that early postpartum uterine infection with *E. coli* paves the way for subsequent infection with other bacteria or viruses (Dohmen et al., 2000; Donofrio et al., 2008; Bicalho et al., 2011). Moreover, *E. coli* infection during the first week postpartum is associated with negative effects on the ovary, hypothalamic-pituitary axis, general health, as well as uterine disease (Williams et al., 2007). Recently many studies have exploited the molecular and epidemiological characterization of bovine uterine *E. coli* (Silva et al., 2009, Sheldon et al., 2010, Bicalho et al., 2010). Silva et al. (2009) reported the genomic and phenotypic characteristics of 72 *E. coli* isolates recovered from the uterus of dairy cows with normal puerperium or metritis and evaluated 15 *E. coli* virulence factor genes identifying none associated with uterine

disease. Sheldon et al. (2010) identified 114 uterine *E. coli* isolates in dairy cows with or without metritis in the first 4 weeks postpartum and investigated the presence of 17 virulence factor genes and only the virulence factor ferric yersiniabactin uptake gene (*fyuA*) was found to be associated with uterine disease. Sheldon et al. (2010) also suggested that endometrial pathogenic *E. coli* were expressing the type I fimbriae (*fimH*) gene, because mannose treatment of *E. coli* decreased their ability to adhere to endometrial cells. Bicalho et al. (2010) used multiplex PCR protocols to screen the isolates for the presence of 32 virulence factor genes in cows with and without uterine diseases. Six virulence factors, common to extra-intestinal and entero-aggregative *E. coli* were found to be associated with metritis and endometritis: *fimH*, α -hemolysin (*hlyA*), cytolethal distending toxin (*cdt*), capsule K2 and K5 (*kpsMII*), invasion brain endothelium A (*ibeA*), and arginine succinyltransferase (*astA*). The virulence factor gene *fimH* was the most prevalent and the most significant.

The virulence factor *fimH* has been described as critical factor for bacteria adhesion on epithelial cells in models of urinary tract infection in mouse and humans and *fimH* immunization in a murine model prevented *in vivo* colonization of the bladder mucosa by 99% (Langermann et al., 1997). In bovine metritis, recent research indicates that the infected uterus is predominated by *E. coli* in the first week postpartum, which alters the intrauterine environment to support future infection by other opportunistic anaerobic bacteria starting in the second week postpartum (Dohmen et al., 2000). In another study, Bicalho et al. (2012) reported that *E. coli* *fimH* was associated significantly with metritis and endometritis when detected at 1 to 3 days postpartum.

Trueperella pyogenes is considered one of the most relevant pathogens involved in uterine diseases, especially endometritis. This is due to its relative high prevalence in the environment, persistence in the uterus, severity of lesions on the endometrium, resistance to treatment, and synergistic action with gram-negative anaerobes (Ruder et al., 1981; Bonett et al., 1991; Huszenicza et al., 1999; Mateus et al., 2002a, b; Williams et al., 2005). The strains of *T. pyogenes* isolated from the uteri of cows all expressed the virulence factor pyolysin (plo), which encodes a cholesterol dependent cytotoxin (Jost and Billington, 2005; Silva et al., 2008). Cholesterol dependent cytotoxin molecules are attracted to cholesterol-rich domains in cell membranes, where they aggregate to form a pore leading to osmotic death of the cell, and pyolysin readily kills endometrial epithelial and stromal cells *in vitro* (Miller, 2009).

Intrauterine infusion of live *T. pyogenes* on day 3 after ovulation induced a peak of PGF_{2α} metabolite 3 days later, followed by the regression of the newly formed CL and ovulation of the dominant follicle of the first follicular wave in 50% of the cows (Kaneko and Kawakami, 2008, Kaneko and Kawakami, 2009). A later study infused *T. pyogenes* 6 times, every 3 days from days 3 to 18 after ovulation inducing early demise of the CL 5 of 12 cows with a sharp rise of PGF_{2α} metabolite on day 6 after ovulation (Kaneko et al., 2013). In the same way, culture of endometrial cells with a bacteria free filtrate of *T. pyogenes* induced synthesis of PGF_{2α} (Miller et al., 2007). It has been suggested that inflammation induced by peptidoglycan gram-positive cell wall, such as the one present in *T. pyogenes*, might induce release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF- α) and IL-1β (Stewart et al., 2003; Timmerman, et al., 1993),

which could stimulate endometrial synthesis of PGF_{2α} (Davidson et al., 1995; Hansen et al., 2004; Skarzynski et al., 2000).

A genomic characterization of *T. pyogenes* was conducted to characterize field isolates recovered from the uterus of cows with and without clinical metritis, in an attempt to identify factors that might be associated with the establishment and persistence of the disease (Silva et al., 2009). Eight virulence factor genes *plp*, neuraminidase P and H (*nanP*, *nanH*), collagen binding protein A (*cbpA*), Type I fimbriae A, C, E and G (*fimA*, *fimC*, *fimE*, *fimG*) were used in this characterization. However none of them were related with development of metritis, suggesting that the type of *T. pyogenes* may not be a determinant factor in the occurrence of the disease. It was suggested that host intrinsic factors, the synergism between *T. pyogenes* and other bacteria, and the differential gene expression of virulence factor genes may play a more relevant role in the establishment of puerperal uterine infections (Silva et al., 2009).

In recent years major advancements were made on understanding the pathogenesis of *E. coli* lipopolysaccharides (LPS) in uterine innate immunity of dairy cows using endometrial cells explants and granulosa cells and the severity of metritis has been linked to its mechanism of pathogenicity (Sheldon et al., 2010, Cronin et al., 2011, Sheldon and Bromfield, 2011). Studies showed that LPS are major components of the outer membrane of gram-negative bacteria that can lead to endotoxic shock, sepsis and death (Bryant et al., 2010). The endotoxin LPS is one of the major pathogen-associated molecular pattern (PAMPs) molecules that is identified by the pathogen recognition receptor (PRR) toll-like receptor 4 (TLR4) in bovine epithelial and stromal endometrial cells, granulosa cells and professional immune cells (Cronin et al., 2011;

Sheldon and Bromfield, 2011). When LPS binds to TLR4 it forms a heterodimer complex with co-receptors cluster of differentiation 14 (CD14) and myeloid differentiation factor-2 (MD2). LPS binding activates the signaling molecule myeloid differentiation factor 88 (MYD88) inducing the phosphorylation of extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), p38 mitogen-activated protein kinase (p38-MAPK) and nuclear translocation of nuclear factor κ B (NF κ B), which in the nucleus binds DNA motifs and transcriptional regulators leading to production of proinflammatory cytokines, such as interleukin 1 β (IL-1 β), IL-6, tumor necrosis factor α (TNF α), and chemokines, such as chemokine (C-X-C motif) ligand 1 (CXCL1), chemokine (C-C motif) ligand 20 (CCL20), and IL-8 (Cronin et al., 2011; Sheldon and Bromfield, 2011). Although LPS-TLR4 inflammatory signaling pathway has been shown consistently in bovine endometrial cells (Sheldon et al., 2010; Cronin et al., 2011), hitherto there is lack of investigation of molecular LPS mediated mechanisms *in vivo* on dairy cows showing how metritis really develops. Infection of the postpartum uterus is common in dairy cows and becomes a burden to uterine health by damaging the endometrium and causing cows to become systemically ill. The prevalence of *E. coli* in the first week postpartum is high and reported in healthy cows as well (Bicalho et al., 2010). Thus, in spite of presence of sufficient LPS to induce an inflammatory response, uterine inflammation, endotoxic shock, and sepsis does not necessarily occur in dairy cows.

Another potential component of *E. coli* pathogenicity is the virulence factor fimH that was recently shown to be highly prevalent in cows in the first week postpartum, and it was associated with increased prevalence of clinical metritis and endometritis (Bicalho

et al., 2010; Bicalho et al., 2012). The virulence factor fimH, the adhesion portion of type 1 fimbriae produced by most uropathogenic *E. coli*, is a conserved protein involved in bacterial attachment to mucosal epithelial cells (Connel et al., 1996). The fimH protein generally binds to mannose receptor on epithelial cells leading to colonization of mucosa by *E. coli* (Langermann et al., 1997). Moreover, fimH can activate the innate immune system through TLR4 and its signaling molecule MYD88 in the genital mucosa leading to increased influx of PMNL cells in the urinary tract (Ashkar et al., 2008). The role fimH may play on the pathogenicity of *E. coli* contaminating the uterus of postpartum dairy cows remains unknown. However, the high prevalence of *E. coli* carrying fimH associated with uterine diseases suggest that it likely plays a role in the development of metritis and endometritis and deserves further investigation.

The general structure of bacterial LPS consists of a hydrophobic lipid A domain, an oligosaccharide 'core' and a distal polysaccharide, the O antigen (Raetz and Withfield, 2002). The lipid A moiety alone is sufficient to activate the innate immune response; adaptive (antibody) responses are generated to the O antigen polysaccharide later in the course of an infection. Lipid A consists of a diglucosamine diphosphate headgroup that is substituted with a variable number of acyl chains, ranging from 4 to 8. *E. coli* lipid A contains a diglucosamine diphosphate headgroup and six acyl chains. In general, such hexa-acyl lipid A molecules are powerful immunostimulants. Changes in the number of acyl chains and in the phosphorylation status of the headgroup can have a profound influence on the biological activity of lipid A. The synthetic compound of lipid A eritoran (also known as E5564) has four acyl chains and act an antagonist of TLR4 in all species investigated so far (Christ et al; 1995; Figueiredo et al., 2008). Eritoran

acting as TLR4 antagonist can block the excessive immune reaction triggered by this receptor. Therefore, if LPS and fimH are involved in the pathogenicity of uterine disease through TLR4, blocking the receptor might become an alternative method to minimize the risk of metritis.

An interesting study revealed that granulosa cells responded acutely to LPS with rapid phosphorylation of TLR signaling components, p38 and ERK, and increased expression of IL6 and IL8 mRNA, although nuclear translocation of p65 was not evident (Bromfield and Sheldon, 2011). Additionally, TLR4 was targeted with small interfering RNA leading to attenuated granulosa cell accumulation of IL-6 in response to LPS. Moreover, LPS stimulated IL-6 secretion and expansion by cumulus oocyte complexes and increased rates of meiotic arrest and germinal vesicle breakdown failure. In summary, LPS elicits innate immune response via TLR4 pathway, which ultimately compromise meiotic competence (Bromfield and Sheldon, 2011).

Another recent study investigated and tested the hypothesis that LPS perturbs the development of primordial ovarian follicles revealing that of bovine ovarian cortex *ex vivo* exposure to LPS reduced the primordial follicle pool associated with increased primordial follicle activation. Key intracellular regulators of follicle activation were modulated by LPS exposure with loss of the primordial follicle phosphatase and tensin homolog (PTEN) and cytoplasmic translocation of forkhead family transcription factor (FOXO3). In the same study acute exposure of mice *in vivo* to LPS also reduced the primordial follicle pool associated with increased follicle atresia and the increased follicle atresia was TLR4-dependent. In conclusion, LPS reduced the primordial ovarian follicle

pool in the bovine ovarian cortex *ex vivo* and in the murine ovary *in vivo*, which ultimately may hinder later fertility (Bromfield and Sheldon, 2013)

Although the understanding of how pathogens subvert host defenses and lead to development of uterine diseases evolved considerably in the last few years, a thorough mechanism remains elusive. One of the major factors hampering a better understanding the etiology of uterine diseases is a limited knowledge of the uterine microbiome. Current knowledge is based on the understanding of microbial communities involved in the pathogenesis of metritis and endometritis identified by traditional methods of culture, which was seriously hindered by the fact that more than 99% of the environmental microbes are not amenable to culture under standard laboratory conditions (Aman et al., 1995; Handelsman, 2004). Recently, results using DNA high-throughput pyrosequencing of uterine fluid samples of healthy, metritic, and endometritic cows at 3 day intervals after calving revealed that the core bacterial community was different in healthy cows, when compared to cows suffering from uterine diseases. Furthermore, the phylogenetic diversity in all the combined samples changed gradually over time, particularly at the 34 to 36 days postpartum, and the core community seemed to be specific for each health status (Santos and Bicalho, 2012). Although these results were enlightening, many limitations such as small sample size, cows from one single farm, sample collection in short period of time and use of antibiotics for treatment of metritis require careful consideration before any generalization are made. Thus, further investigation comparing environmental microbiota present in different farms, immunological status of the cows and the individual immune response to pathogenic and opportunistic bacteria is critical to characterize molecular pathogenesis of persistent

uterine diseases and identify entry points to development of new preventatives, prognostic tools and effective treatments.

Therapy for Uterine Diseases

Retained fetal membrane was recognized as a clinical problem occurred the first time almost two centuries ago (Knowlson, 1834). The first description discussed the manual removal of fetal membranes as an issue for cattle. Knowlson (1834) described that the forced removal of partially attached membranes could lead to 'much hurt' to the cow and, even worse, allowing them to rot would be 'a great folly of a short-sighted man for he loses five times as much in the end' (Knowlson, 1834). Therefore, it has been long known that physically tearing the membranes could be detrimental to the cow's health. Knowlson (1834) reported that the first treatment for retained fetal membranes included 1 oz of spermaceti, 1 oz of gum myrrh, 2 oz of juniper berries, 2 oz of bay berries, 1 oz of round birthwort root and 1 oz of galangal ground up with a pestle and mortar and administered to the cow with three pints of cold ale. Alternatively, the same basic formula could be given with a quart of warm gruel and a wine glass of gin or brandy (Clater, 1839).

Throughout the last 200 years, much has been learned about the etiology and risk factors for RFM, and several therapies and prevention were attempted most of them with relatively no success. Although manual removal remains a common practice, many studies failed to show any benefit of this approach on reproductive performance or milk production (Bolinder et al., 1988; Kulasekar et al., 2004; Drillich et al., 2006a; Drillich et al., 2007). In fact, manual removal can result in more frequent and severe uterine infections, when compared with more conservative treatment (Bolinder et al., 1988). Manual removal prolonged the interval from calving to cyclicity by 20 days and presence

of intrauterine pathogenic bacteria was 100% in cows with manually removed RFM versus only 37% of untreated cows at 3 weeks postpartum, and further 37% of treated versus 12% of untreated cows at 5 weeks postpartum (Bolinder et al.,1988). The possible explanation is that removal of the placenta can cause damage to the endometrium, suppress uterine leukocyte phagocytosis and leave behind necrotic portions, which together encourage bacterial invasion (Peters and Laven, 1996). While research does not support manual removal as an effective treatment for RFM, it is still commonly used both because of aesthetic benefits, including parlor hygiene and removal of offensive odors, and perceived but not realistic benefits that removing the placenta eliminates a potential source of infection and reduces likelihood of uterine diseases.

Results of use of antimicrobial to treat RFM are controversial (Peters and Laven, 1996). Cows diagnosed with RFM are more likely to develop metritis and the claim by the same researchers behind the use of antibiotics to treat RFM is to prevent or mitigate possible severity of metritis and its subsequent negative effects on fertility. Intrauterine antimicrobials, given as infusions or boluses, were unable to reduce the incidence of metritis or improve fertility (Peters and Laven, 1996). Drillich et al. (2007) used two strategies to treat RFM. In the first, cows with RFM and fever received 1 mg/kg of ceftiofur systemically for 3 to 5 consecutive days, whereas cows with RFM and no fever remained untreated. In second treatment strategy, all cows with RFM were treated with 6 g of tetracycline administered into the uterus for 3 days, and RFM cows with fever received additional 10 mg/kg of amoxicillin systemically. Although treatment with intrauterine antibiotics lowered the incidence of postpartum fever, no differences were

found among treatment groups in terms of milk yield or reproductive performance (Drillich et al., 2007).

Goshen and Shpigel (2006) assessed if treating cows with RFM and metritis with intrauterine chlortetracycline would influence reproductive performance and milk production. Benefits in reproductive performance and milk production only occurred in cows for metritis, and no difference in either milk yield or reproductive performance were found between treated and untreated RFM cows. These results indicate that although intrauterine antibiotics can benefit cows with metritis, they are unlikely to cause earlier release of membranes or prevent metritis in cows with RFM.

Another possibility explored by researchers was that intrauterine antibiotics might control local bacterial growth and interfere with the necrotizing process that is responsible for the eventual release of RFM (Roberts, 1986). Oxytetracycline, which is often used for intrauterine treatment in cattle with RFM and metritis, inhibit MMPs important for endometrial repair in other species, which potentially could interfere with the normal placental detachment mechanisms (Eiler and Hopkins, 1993). Systemic antibiotics are believed to be beneficial in RFM cases with concurrent metritis (Risco and Hernandez, 2003; Drillich et al., 2006a; LeBlanc et al., 2008). Combining systemic with intrauterine antimicrobials to treat RFM did not improve efficacy of the treatment (Drillich et al., 2006a).

Currently there are no studies on which cows with concurrent RFM and fever were left untreated, therefore, it is unclear whether the resolution of fever is caused by the antibiotics alone or to the cow's own immune defense mechanisms.

Treating all RFM cows with systemic ceftiofur irrespective of temperature was not beneficial to reduce occurrence of fever, increase shedding of RFM, or enhance subsequent reproductive performance, in comparison with selective antibiotic treatment of cows only presenting fever (Drillich et al., 2006b). The results of this study suggest that treating only cows with RFM and fever can substantially reduce unnecessary antimicrobial use (Drillich et al., 2006b). Treatment of cows with RFM for 5 days with 2.2 mg/kg of ceftiofur hydrochloride systemically was beneficial in preventing metritis when compared with estradiol cypionate or no treatment; however, no significant subsequent improvements in reproductive performance were identified (Risco and Hernandez, 2003).

Another controversial topic regarding RFM is the use of hormones to aid on release of membranes. Oxytocin and PGF_{2α} are the most commonly used hormones in cases of RFM. Although these hormones are powerful inducers of uterine contraction, it is thought that uterine atony accounts for a very small percentage of cases of retained placenta cases (Laven and Peters, 1996) and several studies have not supported their use as a general treatment for RFM (Stevens and Dissimore, 1997, Drillich et al., 2005).

A study suggested that use of collagenase, an enzyme capable of breakdown collagen, might aid detachment of the caruncle-cotyledon bond in cows with RFM (Eiler and Hopkins, 1993). The umbilical arteries of cows with retained placenta were injected with 200,000 IU of bacterial collagenase leading to earlier placental release than untreated herd mates. When applied within 24 to 72 hours after calving, collagenase led to the release of membranes in 85% of the cases within 36 hours, whereas none of the 24 control cows released their membranes within this time period. Although collagenase

therapy shows promise as an option of treating retained placenta, the cost is high (~\$75.00). Unfortunately, no studies have evaluated dosage regimens and long-term production and reproduction outcomes of collagenase treatments in cows with RFM to determine if collagenase treatments of RFM would be an alternative for therapy of RFM.

Cows diagnosed with metritis develop moderate to severe illness, therefore there is a consensus that most of metritis cases require systemic antibiotic treatment (LeBlanc, 2008). Sheldon et al., (2004) reported that 1 mg/kg of ceftiofur maintains therapeutic concentrations in uterine tissues against *E. coli* (Sheldon et al., 2004), but the same results were not observed for all cows in other studies (Okker et al., 2002; Drillich et al., 2006c). Currently, the most common treatments of choice for metritis are ceftiofur, given at dosage of 2.2 mg/kg IM once a day, or procaine penicillin, given at the dosage of 21,000 IU/kg IM once or twice a day for a period of 3 to 5 days (Smith et al., 1998; Drillich et al., 2001, 2006a; Chenault et al., 2004).

Other alternative treatments reported with efficacy similar to ceftiofur or penicillin to treat metritis include systemic use of tetracycline at a dosage of 10 mg/kg, systemic use of ampicillin at a dosage of 11 mg/kg, and intrauterine treatments with oxytetracycline and ampicillin (Schmitt et al., 2001, Smith et al., 1998; Drillich et al., 2003, 2006b). Although tetracycline at a dosage of 10 mg/kg was an effective treatment for metritis (Schmitt et al., 2001), it did not achieve therapeutic concentrations in uterine tissues (Bretzlaff et al., 1983). Uterine concentration of ampicillin in dairy cows diagnosed or not with metritis has not been reported in the literature. Addition of one dose of flunixin meglumine does not improve outcomes over the use of systemic antibiotics alone (Drillich et al., 2007).

A recent a large field study investigated the efficacy of a 2-dose regimen treatment 72 hours apart of ceftiofur crystalline free acid (CCFA) sterile suspension given at 6.6 mg/kg s.c. in the base of the ear, a long acting ceftiofur formulation, to treat puerperal metritis in dairy cows (McLaughlin et al., 2012). Clinical cure was greater for cows treated with CCFA than untreated control cows (74.3 vs. 55.3%), and average rectal temperatures were lower for CCFA than control suggesting that CCFA was an effective treatment for acute metritis in dairy cows (McLaughlin et al., 2012). A second study with CCFA investigated the efficacy of a single treatment to prevent or reduce incidence of metritis in high-risk dairy cows, those having dystocia, twins, stillbirth, or RFM (McLaughlin et al., 2013). The use of CCFA decreased the incidence of subsequent metritis and lowered rectal temperature for the first 2 days after treatment, but had no improvements in reproductive performance or milk production (McLaughlin et al., 2013).

von Krueger et al. (2013) investigated concentrations of ceftiofur derivatives in serum, endometrial tissue, and lochia of cows with fever postpartum or metritis from 4 to 6 d after treatment with a single dose of 6.6 mg of CCFA. The results of this study revealed that mean concentrations of desfuroylceftiofuracetamide, an active metabolite of ceftiofur, detected were above the reported minimum inhibitory concentrations required to inhibit relevant pathogens such as *E. coli* and *T. pyogenes* in serum on days 4, 5 and 6 and in endometrial tissue and lochia only on d 4 in CCFA-treated cows. These results support the concept that one single treatment with 6.6 mg/kg of CCFA is not sufficient to efficaciously treat metritis if the disease is not resolved within 4 days (von Krueger et al., 2013).

Lately, the focus of some studies has been on the development of effective preventatives. Machado et al. (2012) evaluated the effects of intrauterine administration of mannose or a bacteriophage cocktail and the presence of *E. coli* and *T. pyogenes* in the uterine lumen on uterine health and reproductive performance of lactating dairy cows. Their results revealed no effects on uterine health, reproduction performance, or responses in cultures for *E. coli* and *T. pyogenes*. The rationale behind this study was that mannose is a fimH antagonist that potentially could lead to elimination of pathogenic mechanism of fimH and consequently prevent endometrial colonization by pathogenic *E. coli*. Indeed, King et al. (1998) reported that mannose might be effective in reducing bacterial infection in the equine endometrium. Secondly, bacteriophages are viruses that infect bacteria being obligate intracellular parasites without their own metabolism and could potentially parasitize *E. coli* and other bacteria mitigating their potential impact on development of metritis.

An important factor related to treatment of metritis is the cost benefit to producers. A case can be made that treatment of metritis is justified to improve cow welfare and reduce the probability of death in severe cases. However, the criteria to measure efficacy of treatments are not consistent. In an ideal world, the aim would be to return cows to their normal level of production without any further issues. However, what recent studies demonstrated was an expected reduction of body temperature and remission of fever in approximately 70% with an improvement, but not complete resolution, of fetid uterine discharge (Chenault et al., 2004; McLaughlin et al., 2012). LeBlanc et al. (2008) discussed the limited data on the efficacy of treatment of metritis for prevention of subsequent related diseases; or for improvement of milk production, or

improvement of reproductive performance. Moreover, it is not known if aggressive programs aiming for early diagnosis and treatment of metritis can prevent progression to severe disease and losses in performance, or whether they result in treatment that is not medically or economically beneficial. The new trend suggests that incorporation of observation of cows' attitude, daily milk production and dry matter intake will be valuable screening tests to select cows for further examination. Cows diagnosed with RFM needs to be observed daily for potential progression to metritis until the placenta is released. Clearly, further research using large-scale field studies are necessary to established better criteria for early treatment of metritis.

Endometritis treatments reported include systemic or intrauterine administered antibiotics, intrauterine substances and systemic use of PGF_{2α}. A plethora of studies evaluated use of intrauterine substances to treat endometritis including tetracycline (Thurmond et al., 1993; Sheldon and Noakes, 1998), penicillin (Thurmond et al., 1993), chloramphenicol (Steffan et al., 1984), cephapirin (Dohmen et al., 1995; McDougall, 2001; LeBlanc et al., 2002b), gentamycin, spectinomycin, sulfonamides, nitrofurazone (Gustafsson, 1984; Gilbert and Schwark, 1992), and non-antimicrobial substances such as diluted Lugol's iodine(Callahan and Horstman, 1987), chlorhexidine (Gilbert and Schwark, 1992), enzymes (Drillich et al., 2005), and hypertonic dextrose (Brick et al., 2012). With exception of the cephapirin in two different studies (McDougall, 2001; LeBlanc et al., 2002b) and hypertonic dextrose (Brick et al., 2012), all other studies had negligible to no benefits on reproductive performance and suffered from many issues such as lack of negative controls and statistical power, diagnostic criteria for endometritis that were not validated as having an impact on reproductive performance,

and no label approval for intrauterine use with no published information on withdrawal times.

LeBlanc et al., (2002b) compared the effect of intrauterine administration of 500 mg of cephapirin benzathine or intramuscular administration of 100 µg PGF_{2α} as cloprostenol sodium on time to pregnancy in dairy cows diagnosed with clinical endometritis between 20 and 33 postpartum. No benefits of treatment between 20 and 26 days postpartum were observed, but cows treated with cephapirin between 27 and 33 days postpartum had a 60% increase in the hazard of pregnancy than untreated controls. No benefits were observed for cows treated with PGF_{2α} (LeBlanc et al., 2002b). McDougall (2001) randomly allocated cows with risk factors for uterine diseases to receive cephapirin intrauterine or remain untreated at 41 ± 14 days postpartum. Cows that delivered a dead calf, had RFM, or had purulent discharge from the vulva observed after 13 DIM and then treated with cephapirin were approximately 2 to 3 times more likely to become pregnant by 56 days into the breeding season when compared to untreated cows (McDougall, 2001). Brick et al. (2012) assessed the cure rates and P/AI in cows with clinical endometritis in cows treated with intrauterine infusion of a hypertonic solution of 50% dextrose or subcutaneous CCFA. Cows diagnosed with clinical endometritis treated with dextrose tend to have increased P/AI (29.8%) than untreated control cows (21.1%) and cows receiving subcutaneous CCFA (19.7%).

In a different approach, using a non-antibiotic substance, Drillich et al. (2005) evaluated the efficacy of proteolytic enzymes to treat chronic endometritis in comparison with PGF_{2α}. The product used in the study contained the enzymes chymotrypsin (16 mg), trypsin (16 mg), and papain (8 mg), and additionally 200,000 IU

of retinol palmitate (vitamin A) and 240 mg of α -tocopherol acetate (vitamin E). Although no differences in cure rates were identified between the proteolytic enzymes and PGF_{2 α} , conception rate to all services for cows with endometritis was higher in cows treated with PGF_{2 α} than in cows treated with proteolytic enzymes.

One of the most common treatments investigated for endometritis to date is PGF_{2 α} . Results regarding the effects of PGF_{2 α} on clinical and subclinical endometritis and subsequent reproductive outcomes are controversial. LeBlanc et al. (2002a) reported that administration of PGF_{2 α} between 20 and 26 days postpartum to cows with endometritis without a CL was associated with a significant reduction in pregnancy rate, and no differences in pregnancy rates were observed in cows treated with PGF_{2 α} or not between 27 and 33 days postpartum. Kasimanickam et al. (2005) reported that a single treatment with PGF_{2 α} between 20 to 33 days postpartum in cows diagnosed with subclinical endometritis or not improved P/AI to the first service and median days open. Moreover, cows with subclinical endometritis treated with PGF_{2 α} had a significantly increased hazard rate to pregnancy compared to control. Galvão et al. (2009a) reported that cows treated with PGF_{2 α} on days 21, 35 and 49 postpartum had no benefits on prevalence of subclinical endometritis and time to first AI, but increased P/AI to the first service. Kaufmann et al., (2010) compared effects of PGF_{2 α} with ceftiofur to treat cows diagnosed with clinical endometritis revealing no differences in AI submission rate, days to first service, first service conception rate, days open and proportion of cows pregnant. Dubuc et al. (2010) reported that treatment with PGF_{2 α} at 35 and 49 days postpartum did not affect the probability of cure of clinical and subclinical endometritis irrespective

of cyclic status and did not mitigate the negative effects of clinical and subclinical endometritis on reproductive performance.

CHAPTER 3
EFFECT OF ONE OR THREE TIMED ARTIFICIAL INSEMINATIONS BEFORE
NATURAL SERVICE ON REPRODUCTIVE PERFORMANCE OF LACTATING DAIRY
COWS NOT OBSERVED FOR DETECTION OF ESTRUS

The objectives of this study were to determine the effects of one or three timed AI before natural service (NS) in lactating dairy cows not observed for detection of estrus on hazard of pregnancy, days nonpregnant, and 21-d cycle pregnancy rate. A total of 1,050 lactating Holstein cows were subjected to a double Ovsynch program for first postpartum AI. On the day of first AI (78 ± 3 d in milk), cows were blocked by parity and randomly assigned to receive either one timed AI (1TAI, $n = 533$) or three timed AI (3TAI, $n = 517$) before being exposed to NS. Cows assigned to 1TAI were exposed to bulls 7 d after the first AI. Nonpregnant cows in 3TAI were resynchronized with the Ovsynch protocol twice, with intervals between AI of 42 d, before being exposed to NS 7 d after the third AI. Cows were evaluated for pregnancy 32 d after each timed AI or every 28 d after being exposed to NS. Pregnant cows were re-examined for pregnancy 28 d later (i.e., 60 d gestation). Exposure to heat stress was categorized based on the first AI being performed during the hot or cool season according to the temperature and humidity index. Body condition was scored at first AI. All cows were allowed a period of 231 d of breeding, after which nonpregnant cows were censored. Pregnancy to the first AI did not differ between 1TAI and 3TAI on Day 60 after insemination (30.8 vs. 33.5%). Cows receiving 3TAI had a 15% greater hazard of pregnancy and a 17% greater 21-d pregnancy rate than 1TAI and these benefits originated from the first 84 d of breeding. These changes in rate of pregnancy reduced the median and mean days nonpregnant by 9 and 10 d, respectively. Despite the long inter-AI interval in cows subjected to 3TAI, reproductive performance was improved compared with a single timed AI and

subsequent exposure to NS. In dairy herds that use a combination of AI and NS, allowing cows additional opportunities to AI before onset of breeding with bulls is expected to improve reproductive performance.

Introductory Remarks

Inadequate or inaccurate detection of estrus are critical factors responsible for poor reproductive performance in dairy cows (Senger, 1994; Roellofsa et al., 2010). Timed artificial insemination (AI) and natural service (NS) are common methods to manage reproduction in dairy herds in the United States (Champagne et al. 2002, NAHMS, 2002; Smith et al. 2004; De Vries et al., 2005; Caraviello et al., 2006; Lima et al., 2009) and can be successfully used without detection of estrus (Lima et al., 2009). Although AI hastens genetic progress, controls venereal diseases, and provides a safer environment for cows and farm personnel, breeding programs relying primarily on NS are still widely used by dairy producers. Several studies and surveys conducted in different regions of the US showed that 43% to 84% of the dairy farms use NS either alone or combined with AI States (Champagne et al. 2002, NAHMS, 2002; Smith et al. 2004; De Vries et al., 2005; Caraviello et al., 2006). In many cases, NS is incorporated into breeding programs after cows have received one or more AI, which is commonly named as “clean up” program (Caraviello et al., 2006). Conversely, for herds that decide not to use detection of estrus and still incorporate AI, continuous synchronization of ovulation for insemination at fixed time is an option (Lima et al., 2009). Timed AI has been shown to be an economical option to manage reproduction in high-producing dairy cows that experience a reduction in estrous intensity (Lima et al., 2010; Risco et al., 1998).

The reproductive efficiency (Lima et al., 2009) and costs (Lima et al., 2010) of cows bred by timed AI or NS have been directly compared and, although cows exposed to NS only showed minor improvements in reproductive performance (Lima et al., 2009), the economic evaluation favored those receiving timed AI (Lima et al., 2010). The economic advantage of timed AI was even greater when genetic progress was considered, and when marginal feed cost and milk price increased. Interestingly, the 21-d cycle pregnancy rate, a common metric used to evaluate reproduction in dairy herds, was similar between cows bred by timed AI or NS (25.0 and 25.7%, respectively), and they were both superior when compared with average values for high-producing dairy herds that ranges from 15.0 to 17.9% (De Vries et al., 2005; LeBlanc, 2010). The improvement in hazard of pregnancy for NS was attributed to a greater number of breeding opportunities, as NS cows were exposed continuously to bulls. In contrast, timed AI cows could only be inseminated after diagnosed nonpregnant, which created inter-AI intervals of 35 d (Lima et al., 2009). It is possible that a combination of timed AI and NS might benefit reproductive performance of dairy cows not observed for detection of estrus, as timed AI allows for all cows to be inseminated on the first day past the voluntary waiting period and exposure to NS after that will likely shorten the interval between breedings.

In many dairy farms using a combination of AI and NS, cows initially are inseminated one or more times and then moved to bull breeding groups (Overton and Sischo, 2005); however, it is unclear how many inseminations cows should receive before exposed to bulls to maximize pregnancy rate. This is particularly important in herds managing reproduction without the aid of estrous detection, as the interval

between inseminations is determined by when a cow can be resynchronized for AI. In a previous study, the benefit of NS over TAI was only observed after 150 d postpartum, when timed AI cows had already received three inseminations (Lima et al., 2009). Therefore, it is plausible to suggest that three timed AI may result in a similar reproductive performance when compared with one timed AI, despite the long inter-insemination interval. In fact, Overton and Sisco (2005) concluded that in herds using both AI and NS, allowing cows more opportunities for AI may benefit reproduction.

The hypothesis of the current study was that cows subjected to three sequential timed AI would have similar hazard and time to pregnancy, and 21-d cycle pregnancy rate compared with cows exposed to NS 7 d after the first postpartum timed AI. Therefore, the objective of this study was to determine the effect of one or three timed AI followed by NS on reproductive performance of lactating dairy cows not observed for detection of estrus.

Materials and Methods

Cows, Housing, and Diets

All procedures performed during this study were approved by the University of Florida Institutional Animal Care and Use Committee. The study was conducted between July of 2009 and October of 2010 in a commercial dairy farm milking approximately 2,000 Holstein cows during the study period and located in north central Florida, USA. Cows were housed in free-stall barns equipped with fans and sprinklers for forced evaporative cooling during the hot season. Primiparous and multiparous cows were housed separately. Lactating cow diets were formulated to meet or exceed the nutrient requirements established by NRC (2001) for lactating Holstein cows weighing 650 kg, consuming 24 kg/d of dry matter, and producing 45 kg/d of milk containing 3.5%

fat and 3.1% true protein. Diet was fed as totally mixed ration and composed of corn silage, annual ryegrass (*Lolium multiflorum* Lam.) silage, Tifton 85 Bermudagrass silage, ground corn, citrus pulp, cottonseed hulls, expeller soybean meal, solvent-extracted soybean meal, and a mineral-vitamin premix. Cows were milked at least three but no more than four times daily according to the farm milking throughput system that operated continuously throughout a 24 h period.

Treatments, Exclusion Criteria, and Reproductive Programs

Cows with uterine adhesions or abscesses, displaced abomasum, cesarean section or fetotomy at calving, and cows that missed any part of their experimental protocol for the first service were not included in the study. After enrollment, cows sold, dead or that missed any part of their program were censored on the respective days.

All cows were enrolled in a double Ovsynch program at 51 ± 3 d postpartum, which was designated as Day -27 of the study (Figure 3-1). All cows received 100 μ g of GnRH i.m. (2 mL Cystorelin; 50 μ g/mL of gonadorelin diacetate tetrahydrate; Merial Ltd., Iselin, NJ, USA) on Day -27, followed 7 d later by 25 mg of PGF_{2 α} i.m. (5 mL Lutalyse sterile solution; 5 mg/mL of dinoprost tromethamine; Pfizer Animal Health Inc., Madison, NJ, USA) and a second GnRH injection on Day -17. On Day -10, the breeding Ovsynch was initiated with administration of GnRH, followed by PGF_{2 α} on Day -3, and a final injection of GnRH on Day -1, approximately 56 h after the PGF_{2 α} of the breeding Ovsynch. On Day 0, 16 h after the final injection of GnRH, all cows received timed AI. All timed AI were performed by 5 technicians using 13 different sires.

On Day 0, weekly cohorts of cows were blocked by parity and, within each block, randomly assigned to one of two treatments: one (1TAI) or three timed AI (3TAI; Figure

3-2). Cows assigned to 1TAI were moved to pens with bulls for NS 7 d after the first insemination. Cows enrolled in the 3TAI treatment remained in the same group and were re-synchronized up to 2 times following a non-pregnancy diagnosis.

Resynchronization of ovulation of 3TAI cows was initiated when diagnosed not pregnant with the Ovsynch protocol including a controlled internal drug-release (CIDR) insert containing progesterone (Eazi-Breed CIDR Cattle Insert; Pfizer Animal Health Inc.) that was present on the days between injections of GnRH and PGF_{2α} (Figure 3-1). Because of the reproductive program selected for 3TAI cows, the interval between inseminations was 42 d. Seven days after the third AI, cows in the 3TAI treatment were moved to NS pens together with 1TAI cows.

Pregnancy was diagnosed by transrectal ultrasonography of the uterus and its contents 32 d after the first AI in all cows by visualization of an embryo with heartbeat. Cows with a CL and fluid in the ipsilateral uterine horn, but without a visible embryo or with an embryo without heartbeat were considered as not pregnant. Cows diagnosed pregnant were re-examined by transrectal palpation of the uterus and its contents 28 d later (i.e., 60 d of gestation) to reconfirm pregnancy and to identify pregnancy loss. The same procedure was used for pregnancy diagnoses after the second and third AI in cows in the 3TAI treatment. For nonpregnant cows exposed to NS, pregnancy was diagnosed by transrectal ultrasonography every 28 d (Figure 3-1) until detected pregnant, or culled, or dead, or until 231 d after the first AI (309 ± 3 d postpartum), which was selected as the end of the experiment. Because cows could have been bred by NS on study Day 231, a final pregnancy diagnosis was performed 28 d later, on Day 259 (337 ± 3 d postpartum) and pregnant cows were reconfirmed 28 d later. Two-

hundred and thirty-one days after the first AI was chosen as the criterion to end the study because it allowed all cows to have eleven 11 complete 21-d estrous cycles. Based on farm records, it was anticipated that more than 85% of the cows would be pregnant by 309 d postpartum; therefore, extending the study beyond those days would have little impact on the results of the experiment. The criterion of repeated pregnancy diagnosis every 28 d was chosen to allow an accurate determination of gestation age in pregnant cows exposed to NS when gestation is between 28 and 55 d. Age of pregnancy for cows bred by NS was estimated according to the diameter of the amniotic vesicle (Zemjanis, 1970; Ginther, 1998). Pregnant cows by NS were re-examined for pregnancy 28 d after the initial diagnosis.

Bull Management

Bulls assigned to the study were at least 18 months of age and negative for persistent infection caused by bovine viral diarrhea virus examined by immunohistochemistry of skin using an ear notch sample. Every bull underwent a breeding soundness evaluation according to the guidelines of the Society for Theriogenology (Chenoweth, 1992) and only those classified as satisfactory breeders were used in the study. In addition, bulls were tested for *Tritrichomonas foetus* using a smegma sample cultured in a modified diamond media (InPouch™ TF, Biomed Diagnostics, White City, OR, USA). The breeding soundness evaluation and *T. foetus* test were repeated every 6 months in each bull. All bulls were vaccinated once a year against respiratory diseases and leptospirosis (Bovi-Shield GOLD 5 L5, Pfizer Animal Health Inc.), clostridiosis (Ultrabac 8, Pfizer Animal Health Inc.), and campylobacteriosis (Vibrin, Pfizer Animal Health Inc.). The ratio of bulls per nonpregnant cows was maintained at one to twenty. Bulls were rotated every 14 d such

that they remained with cows for 14 d and were allowed to rest for 14 d. When resting, bulls were placed in Tifton 85 Bermudagrass (*Cynodon* spp.) pasture, with portable shades and trees for heat abatement. Resting bulls also receivedorts from lactating cows. Breeding bulls were fed the lactating cow diet *ad libitum* while in the breeding pens, and received an average of 16.9 kg of dry matter per day.

Body Condition Scoring

All cows enrolled had their body condition (BCS) scored at 110 ± 3 d postpartum concurrently with the evaluation of pregnancy diagnosis for the first timed AI. Cows were scored in a one to five scale (1 = emaciated, 5 = obese; Ferguson et al., 1994). For purposes of statistical analyses, cows were categorized as having BCS ≤ 2.75 or BCS ≥ 3.00.

Seasonality

The temperature (°C) and relative humidity (%) data were obtained from the Florida automated Weather Network (<http://fawn.ifas.ufl.edu/scripts/reportrequest.asp>). The data were collected from July of 2009 to October of 2010. The weather station is located in Alachua, Florida, approximately 48.3 kilometers from the experimental location. Average daily temperature-humidity index (THI) was calculated as described by the NOAA (1996). Ambient temperature was converted from °C to °F [Temperature in °F = (temperature °C x 1.8) + 32], and the following formula was used to compute the THI: temperature (°F) – [0.55 – (0.55 x relative humidity)] x (temperature °F - 58). The THI was the criterion used to determine effect of season (hot or cool) on reproductive performance. The average daily THI was categorized as cool when THI < 72, or hot when THI ≥ 72. Cows inseminated when the average daily THI on the week of first AI was ≥ 72 were categorized as being exposed to heat stress (hot season), whereas

those in which the average daily THI in the week of first AI was < 72 were considered to have not been exposed to heat stress (cool season) and this categorization was used for statistical analyses.

Experimental Design and Statistical Analysis

The study followed a randomized complete block design. Weekly cohort of cows were blocked according to parity (primiparous or multiparous) on the day of the first AI and, within each block, randomly assigned to either 1TAI or 3TAI. The sample size was calculated to allow detection of statistical effect ($\alpha = 0.05$; $\beta = 0.20$) when a 6-percentage unit difference in the proportion of pregnant cows was observed at any time in the study. It was assumed that 35% of the cows would be pregnant at the first AI in both treatments. Starting at 35% proportion of pregnancy, the sample size was calculated at 5 percentage unit intervals (35 vs. 41%; 40 vs. 46%; 45 vs. 51%; 50 vs. 56%; etc) to determine the maximum number of cows needed. The maximum number of cows needed per treatment was 543 (when the proportions of cows pregnant were 50 vs. 56%) and the minimum was 246 (when the proportions of cows pregnant were 85 vs. 91%).

The reproductive responses of interest for analyses were the proportion of cows pregnant within the first 21, 42 and 84 d of breeding, the proportion of pregnant cows at the end of the study (Day 231), the hazard of pregnancy, median d to pregnancy, and the 21-d cycle pregnancy rate. A cow was considered pregnant only when the reconfirmation of pregnancy 28 d after the initial diagnosis was positive (pregnant on Day 60 of gestation). The pregnancy per AI after the first postpartum insemination was also evaluated to determine if equal proportions of cows became pregnant when assigned to treatments. The 21-d cycle pregnancy rate was calculated in both

treatments based on the number of cows that became pregnant in an interval of 21 d divided by the number of eligible nonpregnant cows in that 21 d period.

Binary responses were analyzed by multivariate logistic regression using the logistic procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC). Binary data with repeated measurements such as 21-d cycle pregnancy rate were analyzed by the GLIMMIX procedure of SAS with cow within treatment as a random effect. The adjusted odds ratios (AOR) and respective 95% confidence intervals (CI) were calculated for binary responses. Additional analyses for binary data were performed with modified Poisson regression model with the GENMOD procedure of SAS using a log link function and correction for data dispersion (Spiegelman et al., 2005; Fang, 2011). These analyses were performed to estimate the adjusted risk ratios (ARR). For the analyses of 21-d cycle pregnancy rates, the repeated statement with an exchangeable correlation matrix was used to cluster cows within treatment to indicate a random effect of cow within treatment.

Time to pregnancy was analyzed by survival analysis with the Cox's proportional hazard model using the PHREG procedure of SAS. Cows that left the study either because they were sold or died before study Day 231 were censored. The adjusted hazard ratios (AHR) and respective 95% CI were calculated for time-dependent categorical data. Proportionality was assessed by evaluating the Kaplan-Meier curves using the LIFETEST procedure of SAS and by including an interaction between treatment and days postpartum in the Cox's model. When hazard was not proportional, then interval to pregnancy was partitioned into two periods to accommodate

proportionality. Median and mean days to pregnancy were generated by the Kaplan-Meyer method using the LIFETEST procedure of SAS.

For all responses analyzed, multivariate models were built and included the effects of treatment, covariates (sire, technician, parity, BCS, and season), and interactions between treatment and covariates. Covariates were sequentially removed from statistical models in a stepwise backward fashion if $P > 0.10$. Treatment was forced in all final statistical models.

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

Results

The mean (1TAI = 80.7 ± 0.4 vs. 3TAI = 80.3 ± 0.4 d; $P = 0.30$) and median (1TAI = 78 vs. 3TAI = 79 d; $P = 0.61$) days postpartum at first AI did not differ between treatments. Similarly, the mean (1TAI = 3.05 ± 0.02 vs. 3TAI = 3.03 ± 0.02 ; $P = 0.26$) and median (1TAI = 3.00 vs. 3TAI = 3.00; $P = 0.23$) BCS did not differ between treatments. The proportions of 1TAI and 3TAI cows exposed to heat stress, based on receiving their first AI during the hot season, were similar ($P = 0.62$) and were 39.6 and 38.1%, respectively.

Pregnancy per AI to the First Timed AI

As expected, there were no differences in pregnancy per AI on Days 32 and 60 and on pregnancy loss after the first AI in cows receiving 1TAI and 3TAI (Table3-1).

Primiparous cows were more likely to become pregnant than multiparous at the first AI on Days 32 (primiparous = 63.2% vs. multiparous = 35.2%, $P < 0.001$) and 60 (primiparous = 55.2% vs. multiparous = 29.0%, $P = 0.002$) after insemination.

Pregnancy loss did not differ ($P = 0.62$) between parities (primiparous = 12.7% vs.

multiparous = 17.8%). Cows with BCS ≥ 3.00 had greater ($P < 0.001$) pregnancy per AI evaluated on Days 32 ($\geq 3.00 = 44.7\%$ vs. $\leq 2.75 = 29.1\%$) and 60 ($\geq 3.75 = 38.4\%$ vs. $\leq 2.75 = 22.3\%$) after AI, and were also less likely ($P = 0.02$) to lose their pregnancy ($\geq 3.00 = 14.0\%$ vs. $\leq 2.75 = 23.3\%$) in the first 60 d of gestation. Cows inseminated during the hot season were less likely ($P < 0.001$) to become pregnant at the first AI evaluated on Days 32 (hot = 24.3% vs. cool = 47.7%) and 60 (hot = 19.6% vs. cool = 40.0%) after insemination, but pregnancy loss did not differ with season (hot = 19.2% vs. cool = 16.0%, $P = 0.32$).

Reproductive Performance During the Entire Lactation

Of the 331 1TAI cows not pregnant to the first AI, only 9 (2.7%) became pregnant by NS within the next 21 d after the first insemination. This low pregnancy did not improve the proportion of pregnant cows by study Day 21 (Table 3-1) compared with cows in 3TAI that did not have the opportunity to be re-inseminated until study Day 42. In fact, after two potential estrous cycles, by study Day 42, the proportion of pregnant cows was greater ($P < 0.01$) for 3TAI than 1TAI (ARR = 1.24; 95% CI = 1.09-1.42). A similar response was observed on study Day 84, when cows in 3TAI had the opportunity to receive their third AI and had a 20% greater ($P < 0.01$) risk of being pregnant than cows in 1TAI (ARR = 1.20; 95% CI = 1.09-1.33). When the entire breeding period was analyzed, the rate of pregnancy was greater ($P = 0.04$) for 3TAI than 1TAI (Figure 3-2). In fact, 3TAI cows had fewer median and mean days to pregnancy (Table 3-2). No interactions between treatment and parity, BCS, or season were observed for interval to pregnancy.

Because of lack of parallelism of survival curves between 1TAI and 3TAI in the first 84 d of breeding (average of 162 d postpartum; Figure 3-2) and interaction between

treatment and day postpartum, therefore, lack of proportional hazard, data also were analyzed separately for before and after study Day 84. For the analyses before study Day 84, nonpregnant cows by study Day 84 were censored. For analysis after study Day 84, only cows that became pregnant or censored after that were included. For the 725 cows (336 in 1TAI and 389 in 3TAI) that became pregnant or were censored before study Day 84, the hazard of pregnancy was 26% greater ($P < 0.01$) for 3TAI than 1TAI (AHR = 1.26; 95% CI = 1.07-1.47). However, for the remaining 325 cows (197 in 1TAI and 128 in 3TAI) that became pregnant or were censored after study Day 84, the hazard of pregnancy did not differ ($P = 0.30$) between 3TAI and 1TAI (AHR = 0.87; 95% CI = 0.66-1.14). Therefore, the increased hazard of pregnancy for 3TAI cows was primarily the result of faster pregnancy attained after during the period of timed AI, before study Day 84. Pregnancy rate was also greater ($P = 0.01$) for 3TAI than 1TAI when analyzed based on 21-d cycles (Table 3-1). Interestingly, the 17% increased relative risk (ARR = 1.17; 95% CI = 1.04-1.31) of becoming pregnant over time for 3TAI compared with 1TAI was similar to the 15% increased hazard observed from the survival analysis.

Despite improvements in the rate of pregnancy with 3TAI, the proportion of cows diagnosed pregnant on Day 60 of gestation was not different between 1TAI and 3 TAI at the end of the study (Table 3-1). Treatment altered the proportion of cows becoming pregnant to AI and NS. At the end of the study 30.8% of 1TAI cows and 64.8% of 3TAI cows became pregnant to AI, whereas 49.9 and 16.3% of the 1TAI and 3TAI cows became pregnant to NS, respectively.

In addition to treatment, parity, BCS and season also affected the rate of pregnancy. Primiparous cows had greater ($P < 0.01$) rate of pregnancy than multiparous cow, which resulted in fewer median and mean days to pregnancy (Table 3-2). Similarly, cows with $BCS \geq 3.00$ and those that received the first AI during the cool season had faster ($P < 0.01$) rate of pregnancy and fewer median and mean days nonpregnant (Table 3-2). Similarly, the 21-d cycle pregnancy rate was greater ($P < 0.01$) in primiparous than multiparous ($ARR = 1.27$; 95% CI = 1.05-1.54), in cows with $BCS \geq 3.00$ than those with $BCS \leq 2.75$ ($ARR = 1.51$; 95% CI 1.34-1.71), and for cows in the cool than hot season ($ARR = 1.75$; 95% CI = 1.55-1.99).

Discussion

A major objective of this study was to determine if the incorporation of timed AI in cows not observed for detection of estrus after breeding should be restricted to one or more services because of the long re-insemination interval resulting from this program (Lima et al., 2009). In the current study, increasing the number of timed AI before introduction to NS improved reproductive performance of dairy cows by increasing the rate of pregnancy by 15% and by reducing median and mean days nonpregnant in 9 and 10 d, respectively. Furthermore, when considering a 21-d cycle, the pregnancy rate of cows in the 3TAI treatment was 17% greater than that of 1TAI.

The modernization of the dairy industry in many countries has been marked by consolidation of farms resulting in increased herd size, housing of cows on concrete floor with smaller area per cow, increased milk production per cow, and less time allotted to individual observation of cows for estrus (Senger, 1994; Roellofsa et al., 2010). These changes in management and the increase in production have often been cited as impediments for estrous expression and high estrous detection (Senger, 1994;

Lopez et al., 2004; Roellofsa et al., 2010). Because of low detection of estrus in high-producing dairy cows, timed AI has become an integral component of the reproductive management of many herds that use AI (Caraviello et al., 2006; NAHMS, 2009). Likewise, low detection of estrus has often been described as a reason to use NS either as the sole breeding program or for breeding of cows with advanced lactation (NAHMS, 2002; NAHMS, 2009). When the sole use of timed AI was compared with NS, minor differences were observed in reproductive performance of dairy cows in the first 7 months postpartum (Lima et al., 2009). Because of the long re-insemination interval in cows subjected only to timed AI, and more opportunities for breeding in NS cows, NS cows had a slight decrease in time to pregnancy of 5 fewer days (Lima et al., 2009). Despite this minor difference, pregnancies from cows exposed only to NS were generally more expensive than that of cows exposed only to timed AI (Lima et al., 2010). Nonetheless, retrospective analysis of herds that used both AI and NS observed greater pregnancy rates for cows in herds that kept cows in the AI groups longer than those that moved them sooner to NS groups (Overton and Sisco, 2005). In fact, the authors suggested that in herds that practice a combination of AI and NS, reproductive performance might be improved by allowing cows more opportunities for AI before moving them into clean-up bullpens (Overton and Sisco, 2005). The current study clearly demonstrated that in herds using both AI and NS, increasing the opportunities for AI improved pregnancy rate. This is noteworthy as cows subjected to AI in the current study had a 42-d interval between inseminations. Nevertheless, despite this long re-insemination interval, the 21-d cycle pregnancy rate was greater for 3TAI than 1TAI. Furthermore, the results of 21-cycle pregnancy rate obtained for cows in 3TAI were far

superior than those often cited from observational studies across many herds in North America (De Vries et al., 2005; Leblanc, 2010).

As expected, the proportion of pregnant cows at the first AI and the pregnancy loss between 32 and 60 d of gestation did not differ between treatments. Approximately 16.8% of the cows lost their pregnancy. Santos et al. (2009) reviewed data on pregnancy losses in dairy cattle and concluded that approximately 12.8% of the pregnant cows lose their pregnancy between 30 and 45 d of gestation, with studies reporting from 3.2% in grazing cows in Ireland to as much as 42.7% in high-producing cows exposed to heat stress. This extensive pregnancy loss in high-producing dairy cows is multifactorial, but the exact underlying mechanisms remain unknown (Santos et al., 2009).

Exposure to NS starting 7 d after the first AI in 1TAI cows was expected to increase the proportion of pregnant cows in the first 21 and 42 d of breeding because of the delay in rebreeding nonpregnant cows in 3TAI. Nonetheless, cows in 3TAI had similar probability of pregnancy on Day 21, but a 24 and 20% increased risk of becoming pregnant by Days 42 and 84 of breeding, respectively. Only an additional 9 cows became pregnant in the first 21 d after the first AI in 1TAI. Also, only 57 cows in 1TAI compared with 93 cows in 3TAI became pregnant in the first 42 d after the initial insemination. The low number of cows becoming pregnant between Days 7 and 21 in the 1TAI treatment may be attributed to several factors, such as a reduced percentage of cows returning to estrus before Day 21, low estrous expression of cows housed on concrete, inability of bulls to detect cows in estrus when expression of estrus is compromised by lactation or footing, and low fertility of the bulls. Similarly, the lower

number of pregnancies between Days 7 and 42 for 1TAI compared with 3TAI might be the result of a combination of low expression/detection of estrus and low fertility of bulls. Bulls in the current study underwent a breeding soundness evaluation before being used for breeding and were managed with alternate periods of 2 weeks of breeding and 2 weeks of rest. This approach to bull management was chosen to minimize the risk of subfertile bulls impairing reproductive performance of cows exposed to NS in both treatments. In general, the management used for bulls in the current study and by others (Lima et al., 2009) is more intensive than that of most dairy producers relying on NS as a component of the breeding program (Champagne et al., 2002).

Despite the advantages of increasing the number of AI in 3TAI compared with 1TAI, the proportion of pregnant cows at the end of the study did not differ between treatments. This was expected as management changes that impact rate of pregnancy might not necessarily alter the final proportion of pregnant cows when the period of observation is long enough such that cows have multiple opportunities for rebreeding. The experimental period in the current study was 231 d of breeding, which allowed cows in 1TAI a total of 11 opportunities for breeding based on the standard 21-d duration of the estrous cycle. Nevertheless, the benefits of 3TAI in increasing the rate of pregnancy and reducing days to pregnancy were observed during the first 84 d in the study, when 3TAI cows received their third insemination. The two additional pre-determined AI performed in the 3TAI treatment resulted in 64.8% of cows pregnant to AI, whereas only 30.8% of the 1TAI cows became pregnant to AI.

The enhanced reproductive performance for the 3TAI treatment may be attributed to a greater submission to breeding in the first 84 d after the first AI, but also

to a potential benefit to increased fertility to a breeding. High-producing dairy cows have reduced expression of estrus associated with high milk production (Lopez et al., 2004), although production has not been associated with reduced pregnancy per AI or risk of pregnancy loss (Santos et al., 2009). Furthermore, exposure of cows and bulls to concrete might limit mounting activity and further impair estrous expression (Senger, 1994, Roelofsa et al., 2010). The resynchronization program used starting on Day 32 after the previous AI and incorporating supplemental progesterone likely optimized fertility when cows are subjected to timed AI (Dewey et al. 2010; Bisinotto et al., 2010a). Incorporating a progesterone insert during resynchronization with the Ovsynch protocol improved pregnancy per AI similar to presynchronizing the estrous cycle with GnRH 7 d before resynchronization (Dewey et al., 2010). When cows are subjected to NS, no hormonal manipulation is possible as it is unknown if a cow without a detectable pregnancy has been recently bred by a bull. Furthermore, cows with ovarian problems such as follicular cysts or low concentrations of progesterone during the final stages of follicle development would be less likely to manifest estrus. This would likely reduce submission to bull breeding and potentially compromise fertility if bred.

In this study parity, BCS, and season influenced all measures of reproductive performance evaluated and their impacts were similar for cows in 1TAI and 3TAI. Primiparous cows had greater pregnancy per AI at the first service, greater rate of pregnancy and fewer median days to pregnancy than multiparous, corroborating findings from previous studies (Lima et al., 2009, Santos et al., 2009). In lactating dairy cows, a BCS usually above 3.00 (one to five scale) at first AI is a critical indicator of fertility and those with improved degree of fatness usually have marked increases in

pregnancy per AI and reduced risk of pregnancy loss (Santos et al., 2009). Finally, season when cows received the first AI influenced reproduction during the entire lactation. Depression in fertility of dairy cows exposed to heat stress has been shown in cows exposed to AI following spontaneous estrus, timed AI, or NS (Lima et al., 2009; De Vries et al., 2005). Because heat stress not only depresses pregnancy per AI, but also reduces estrous behavior of cows (Roelofsa et al., 2010) and affects semen quality (Kastelic et al., 1997) and libido in bulls collectively, these detrimental effects would alternatively extend the benefit of increased number of timed AI to favorably influence fertility during the warm season. The lack of interaction between treatment and season suggests that the improved reproduction in 3TAI was observed in cows exposed to the hot and cool seasons. Others had also observed a lack of interaction between method of breeding (NS or AI) and season of year (Lima et al., 2009; De Vries et al., 2005).

Conclusion

In spite of the long re-insemination interval for second and third AI, cows receiving 3TAI became pregnant at a faster rate than cows receiving a single timed AI before introduction to natural service. The improved reproductive performance of 3TAI cows resulted in 15% greater hazard of pregnancy, 17% greater risk of pregnancy, and 9 fewer days nonpregnant than 1TAI cows. The faster pregnancy rate was likely a combination of increased breeding of nonpregnant cows associated with improved probability of pregnancy to a breeding, which improved reproduction during the first 84 d in the study. Therefore, in herds in which detection of estrus is not carried out, a combination of AI and natural service is used, bulls are managed to optimize their fertility, and the resynchronized timed AI is implemented using the Ovsynch protocol

with a CIDR insert, it is advantageous to allow cows multiple inseminations before bull exposure for natural service to optimize pregnancy rate.

Results from this study indicate that in herds in which detection of estrus is not carried out, and a combination of AI and natural service is used, cows should receive at least three timed AI before bull exposure for natural service.

Table 3-1. Effect of number of timed AI before exposure to natural service on reproduction of dairy cows not observed for detection of estrus

	Treatment ¹		AOR (95% CI) ²	P
	1TAI	3TAI		
	% (n/n)			
First AI				
Pregnant Day 32	37.9 (202/533)	39.3 (203/517)	1.07 (0.82-1.39)	0.60
Pregnant Day 60	30.8 (164/533)	33.5 (173/517)	1.15 (0.87-1.51)	0.30
Pregnancy loss	18.8 (38/202)	14.8 (30/203)	0.75 (0.44-1.27)	0.27
Pregnant ³				
Study Day 21	32.5 (173/533)	33.5 (173/517)	1.06 (0.81-1.39)	0.67
Study Day 42	41.5 (221/533)	51.5 (266/517)	1.58 (1.22-2.05)	< 0.01
Study Day 84	54.0 (288/533)	64.8 (335/517)	1.63 (1.27-2.15)	< 0.01
Study Day 231	80.7 (430/533)	81.1 (419/517)	1.05 (0.77-1.43)	0.75
21-d cycle pregnancy rate				
Pregnant Day 32	22.2 (486/2190)	26.6 (483/1818)	1.31 (1.09-1.59)	< 0.01
Pregnant Day 60	19.6 (429/2190)	23.1 (419/1818)	1.27 (1.06-1.53)	0.01

¹1TAI = cows received timed AI for first insemination and were subjected to breeding by natural service 7 d later; 3TAI = cows received up to three timed AI and were subjected to breeding by natural service 7 d after the third insemination.

² AOR = adjusted odds ratio (1TAI was the reference for comparison); CI = confidence interval.

³ Based on pregnancy evaluation on Day 60 after breeding.

Table 3-2. Factors affecting the hazard of pregnancy of dairy cows not observed for detection of estrus and subjected to 1 or 3 timed AI before exposure to natural service

Item	Days to pregnancy			
	Median (95% CI) ¹	Mean ± SEM	Adjusted HR ² (95% CI)	P
Treatment ³				
1TAI	142 (130-150)	165.3 ± 3.9	---	
3TAI	123 (121-144)	155.6 ± 3.7	1.15 (1.00-1.31)	0.04
Parity				
Multiparous	145 (130-160)	166.3 ± 2.9	---	
Primiparous	81 (80-89)	115.7 ± 5.1	1.44 (1.16-1.78)	< 0.01
Body condition				
≤ 2.75	161 (148-172)	180.6 ± 4.6	---	
≥ 3.00	121 (118-123)	147.6 ± 3.2	1.59 (1.38-1.84)	< 0.01
Season				
Hot	186 (169-194)	193.2 ± 4.2	---	
Cool	117 (111-120)	137.5 ± 3.1	1.77 (1.53-2.05)	< 0.01

¹CI = confidence interval.

²HR = hazard ratio.

³1TAI = cows received timed AI for first insemination and were subjected to breeding by natural service 7 d later; 3TAI = cows received up to three timed AI and were subjected to breeding by natural service 7 d after the third insemination.

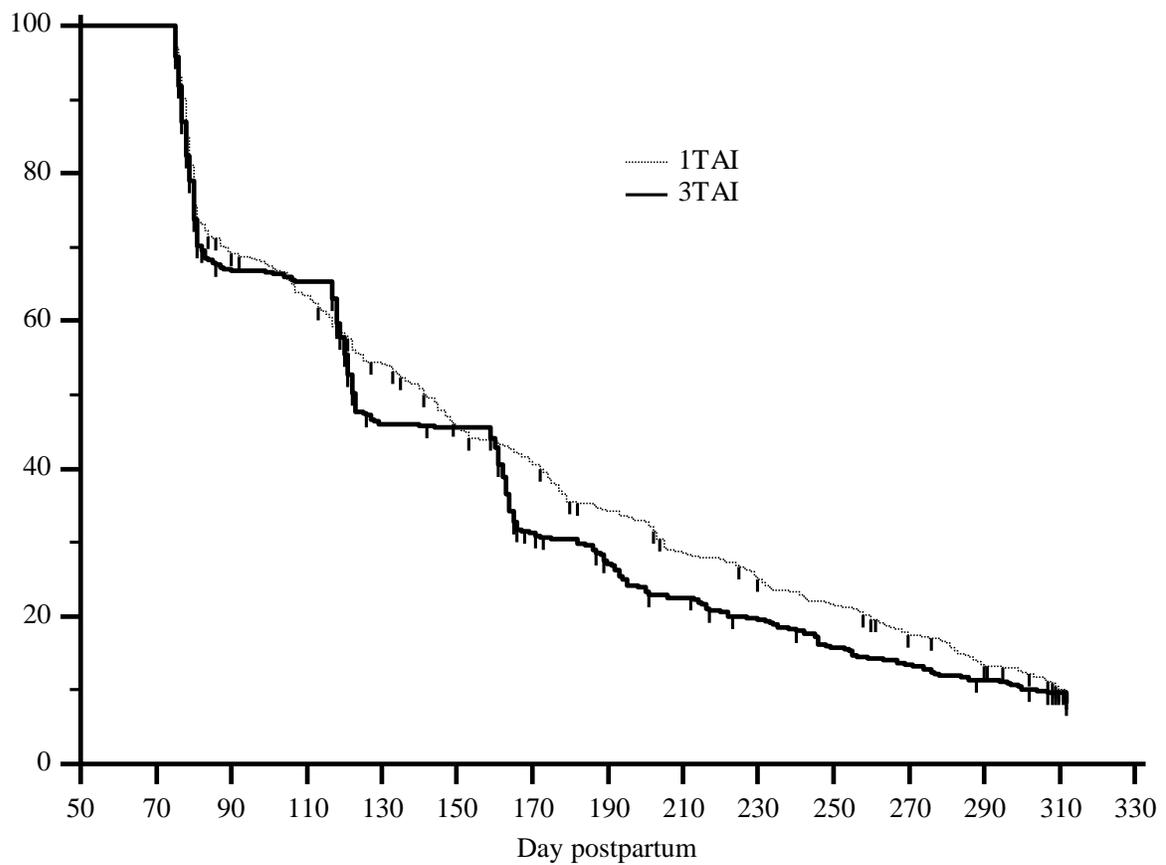


Figure 3-2. Kaplan-Meier survival curves for proportion of nonpregnant cows according to treatment. 1TAI (dashed line) = cows received timed AI for first insemination and were subjected to breeding by natural service 7 d later; 3TAI (continuous line) = cows received up to three timed AI and were subjected to breeding by natural service 7 d after the third insemination.

CHAPTER 4

EFFECTS OF GNRH AT INITIATION OF THE 5-D TIMED AI PROGRAM AND TIMING OF INDUCTION OF OVULATION RELATIVE TO AI ON OVARIAN DYNAMICS AND FERTILITY OF DAIRY HEIFERS

Two experiments evaluated the effects of the first GnRH injection of the 5-d timed AI program on ovarian responses and pregnancy per artificial insemination (P/AI), and the effect of timing of the final GnRH to induce ovulation relative to AI on P/AI. In experiment 1, 605 Holstein heifers were synchronized for their second insemination and assigned randomly to receive GnRH on study d 0 (n = 298) or to remain as untreated controls (n = 307). Ovaries were scanned on study d 0 and 5. All heifers received a controlled internal drug-release (CIDR) insert containing progesterone on d 0, a single injection of PGF_{2α} and removal of the CIDR on d 5, and GnRH concurrent with timed AI on d 8. Blood was analyzed for progesterone at AI. Pregnancy was diagnosed on d 32 and 60 after AI. Ovulation on study d 0 was greater for GnRH than control (35.4 vs. 10.6%). Presence of new corpus luteum (CL) at PGF_{2α} injection was greater for GnRH than control (43.1 vs. 20.8%), although the proportion of heifers with a CL at PGF_{2α} did not differ between treatments and averaged 87.1%. Progesterone on the day of AI was greater for GnRH than control (0.50 ± 0.07 vs. 0.28 ± 0.07 ng/mL). The proportion of heifers at AI with progesterone < 0.5 ng/mL was less for GnRH than control (73.8 vs. 88.2%). Proportion of heifers in estrus at AI did not differ between treatments and averaged 66.8%. Pregnancy per AI was not affected by treatment at days 32 or 60 (GnRH = 52.5 and 49.8% vs. control = 54.1 and 50.0%), and pregnancy loss averaged 6.0%. Responses to GnRH were not influenced by ovarian status on study d 0. In experiment 2, 1,295 heifers were synchronized for their first insemination and assigned randomly to receive a CIDR on d 0, PGF_{2α} and removal of the CIDR on d 5, and either

GnRH 56 h after PGF_{2α} and AI 16 h later (OVS56, n = 644) or GnRH concurrent with AI 72 h after PGF_{2α} (COS72; n = 651). Estrus at AI was greater for COS72 than OVS56 (61.4 vs. 47.5). Treatment did not affect P/AI on d 32 in heifers displaying signs of estrus at AI, but COS72 improved (P = 0.05) P/AI compared with OVS56 (55.0 vs. 47.6%) in those not in estrus at AI. Similarly, P/AI on d 60 did not differ between treatments for heifers displaying estrus, but COS72 tended (P = 0.07) to improve P/AI compared with OVS56 (53.0 vs. 44.7%) in those not in estrus at AI. Administration of GnRH on the first day of the 5-d timed AI program resulted in low ovulation rate and no improvement in P/AI when heifers received a single PGF_{2α} injection 5 d later. Moreover, extending the proestrus by delaying the final GnRH from 56 to 72 h concurrent with AI benefited fertility of dairy heifers that did not display signs of estrus at insemination following the 5-d timed AI protocol.

Introductory Remarks

The use of timed AI programs in dairy heifers is low compared with that for lactating dairy cows (NAHMS, 2009). Programs to synchronize ovulation of dairy heifers based on GnRH and PGF_{2α} resulted in low pregnancy per AI (P/AI) compared with insemination performed after detection of estrus (Schmitt et al., 1996, Pursley et al., 1997 and Rivera et al., 2004). The depressed P/AI for most timed AI programs based on GnRH and PGF_{2α} and the perception by dairy producers that heifers become pregnant easily without the need for intervention justifies the low use of ovulation synchronization protocols for management of reproduction in heifers.

Recently, a 5-d timed AI protocol investigated by Rabaglino et al. (2010a) resulted in P/AI ranging from 52.2 to 61% in dairy heifers in the first two inseminations, which resembled the reproductive performance obtained to AI after detection of estrus

(Kuhn et al., 2006). In fact, additional work by the same investigators evaluating anti-luteolytic strategies with 325 heifers synchronized with the 5-d timed AI program observed P/AI of 59.5% on d 45 after insemination (Rabaglino et al., 2010b). Therefore, it is possible to achieve acceptable P/AI in dairy heifers following synchronized ovulation with the 5-d timed AI protocol.

The program is comprised of an injection of GnRH and insertion of a controlled internal drug-release (CIDR) intravaginal device containing progesterone, followed 5 d later by CIDR removal and an injection of PGF_{2α}, and AI concurrent with a second GnRH injection 72 h after PGF_{2α} (Rabaglino et al., 2010a). Only 23% of the heifers had multiple corpora lutea (CL) 5 d after the injection of the GnRH (Rabaglino et al., 2010a), suggesting that ovulation to the initial GnRH was probably low. In fact, heifers receiving a single injection of PGF_{2α} 5 d after GnRH had similar luteolysis and P/AI to those receiving 2 injections given 12 h apart (Rabaglino et al., 2010a). The same was not true when lactating dairy cows were subjected to a similar program with a 5-d interval between GnRH and PGF_{2α} (Santos et al., 2010a). Therefore, the low incidence of ovulation induced by the first GnRH combined with more rapid turnover of follicles in heifers (Sirois and Fortune, 1988) might result in little benefit from the initial GnRH in the 5-d timed AI program in dairy heifers.

Altering the timing of the final GnRH to induce ovulation relative to AI in the Ovsynch protocol influences P/AI in lactating dairy cows. Brusveen et al. (2008) reported that GnRH administered 56 h after PGF_{2α} increased P/AI compared with GnRH given concurrent with timed AI at 72 h. In a series of experiments with beef cows subjected to the 5-d timed AI program, extending the proestrus from 60 to 72 h was

beneficial to fertility (Bridges et al., 2008). In dairy cows subjected to the 5-d timed AI program, P/AI did not differ when the final GnRH was administered either 16 h before or concurrent with AI at 72 h after PGF_{2α} (Bisinotto et al., 2010a). Although inducing ovulation 16 h before AI benefits fertility of dairy cows in the standard 7-d timed AI Ovsynch program, it is unclear if a similar benefit would occur in dairy heifers when follicle dominance is reduced such as in the 5-d timed AI.

The hypotheses of the current study were that the first GnRH would result in low ovulation rate, thereby having little or no impact on fertility of dairy heifers subjected to the 5-d timed AI protocol. A second hypothesis was that administration of the final GnRH concurrent with AI at 72 after PGF_{2α} would result in similar P/AI as that when GnRH is administered 16 h before AI and AI is performed 72 h after PGF_{2α}. Two experiments with heifers inseminated following the 5-d timed AI protocol were designed to test our hypotheses. The first experiment evaluated the effect of the first GnRH injection on ovarian responses and P/AI, whereas the second experiment evaluated the effect of timing of the final GnRH to induce ovulation relative to AI on P/AI.

Materials and Methods

The University of Florida Institute of Food and Agricultural Sciences Animal Research Committee approved all procedures in this study.

Experiment 1

Heifers, Diets, and Housing

Six-hundred and five nulliparous nonpregnant Holstein heifers on d 32 after the first insemination were synchronized to receive second AI. Heifers were from a commercial dairy farm in north central Florida. Heifers averaged 15.3 ± 1.7 mo of age, and were enrolled in the study in the months of December of 2009 and March of 2010.

Heifers were managed on pasture, with access to portable shades and trees, and fed a TMR once daily that met or exceeded the nutritional requirements of Holstein heifers weighing 360 kg and gaining 0.8 kg/d (NRC, 2001). The diet was based on a mixture of lactating cow ration orts, Bermuda grass silage, wet brewer's grain, and a mineral and vitamin supplement. For implementation of synchronization protocols, insemination, blood collection, and pregnancy examination, heifers were handled in an open-sided barn with self-locking stanchions.

Experimental Design and Treatments

Nonpregnant heifers on d 32 after the first AI were blocked according to age and, within each block, allocated randomly to receive 100 µg of GnRH (gonadorelin hydrochloride; Factrel, Pfizer Animal Health, New York, NY) administered i.m. on study d 0 (GnRH = 298) or to remain as untreated controls (control = 307). All heifers received a CIDR (Eazi-Breed CIDR Cattle Insert, Pfizer Animal Health) containing 1.38 g of progesterone on study d 0. On study d 5, the CIDR was removed and heifers received an i.m. injection of 25 mg of PGF_{2α} (dinoprost tromethamine; Lutalyse sterile solution, Pfizer Animal Health). On study d 8, an injection of GnRH was administered concurrently with timed AI (Figure 4-1). Beginning on the day of PGF_{2α} administration, tailheads were painted daily with chalk, and removal of chalk was used as an indication of estrus. Heifers were inseminated by 5 technicians and semen from 5 Holstein and 6 Jersey sires were used. Technicians and sires were balanced between treatments and later used in the statistical analyses. Heifers were classified according to their age as < 15 mo or ≥ 15 mo of age.

Ultrasonography of Ovaries

Ovaries of all heifers were scanned using a 5-MHz ultrasound unit (Easi-Scan, BCF Systems, Livingston, UK) on study d 0 and ovarian maps were drawn with the presence and location of CL and follicles ≥ 10 mm. On study d 5, the ovaries of 473 heifers were scanned and presence and location of CL were recorded.

Blood Sampling and Analysis of Progesterone in Plasma

Blood was sampled from 312 of the 473 heifers evaluated for ovulation to the first GnRH. Blood was sampled on study d 8 by puncture of the median coccygeal vein or artery using evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K₂ EDTA for plasma separation. Samples were placed immediately in ice and kept refrigerated until transported to the laboratory. Blood tubes were centrifuged at 2,000 x g for 15 min, and plasma frozen at -20 °C until analysis. Concentration of progesterone in plasma was analyzed in all samples by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.05 ng/mL calculated at 2 SD below the mean counts per min at maximum binding. Samples were analyzed in a single assay. Two known plasma samples containing 1.5 ng/mL and 2.5 ng/mL of progesterone were included in the assay several times to calculate the intra-assay CV, and they were 2.5% for the sample with 1.5 ng/mL and 2.9% for the sample with 2.5 ng/mL.

Evaluation of Ovulation and Progesterone at AI

Ovulation on study d 0 was considered when the heifer had a follicle ≥ 10 mm on d 0 and a new CL was observed on study d 5. Heifers with follicles < 10 mm on study d 0, but with a new CL on study d 5 were considered to have a new CL, but ovulated before study d 0. The proportion of heifers with a visible CL by ultrasound on the day of

PGF_{2α} that had low progesterone at AI was calculated. Three different cut-off values for plasma concentrations of progesterone were used, < 1.0 ng/mL, < 0.50 ng/mL, and < 0.25 ng/mL. These values were chosen because traditionally 1 ng/mL has been used to indicate CL regression, and values < 0.50 ng/mL have been implicated as cut-off values that best predict P/AI (Rabaglino et al., 2010a; Santos et al., 2010a).

Pregnancy Diagnoses and Evaluation of P/AI and Pregnancy Loss

Pregnancy was diagnosed 32 d after AI by transrectal ultrasound. The presence of an embryo with a heartbeat was the criterion used to determine pregnancy. Heifers diagnosed pregnant were re-examined by transrectal palpation of uterine contents 28 d later, at 60 d of gestation to reconfirm pregnancy and to identify pregnancy loss.

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

Experiment 2

Heifers, Diets, and Housing

A total of 1,295 nulliparous Holstein, Jersey and crossbreed Holstein-Jersey heifers (15.5 ± 2.6 mo of age) located in two farms in north central Florida were enrolled in the study between January and March of 2010. Heifers in both locations were managed on pastures and fed as described in experiment 1. Heifers were moved to an open-sided barn with self-locking stations in farm 1 or to a palpation rail in farm 2 for hormonal treatments, insemination, and pregnancy diagnoses.

Experimental Design and Treatments

Within farm, nulliparous heifers were blocked by breed and age and, within each block, allocated randomly to one of 2 treatments for the first AI. All heifers received a CIDR on study d 0. On study d 5, the CIDR was removed and heifers received an i.m. injection of PGF_{2α}. Heifers in the 5-d timed AI program denominated **OVS56** (n = 644) received an injection of GnRH at 56 h after the PGF_{2α} and timed AI was performed 16 h later. Heifers in the 5-d timed AI program denominated **COS72** (n = 651) received an injection of GnRH at 72 h after the PGF_{2α}, concurrent with AI. Therefore, in both treatments heifers were inseminated at 72 h after CIDR removal and PGF_{2α}, but in OVS56, induction of ovulation was 16 h before AI (Figure 4-2). Beginning on the day of PGF_{2α} administration, tailheads were painted daily with chalk, and removal of chalk was used as an indication of estrus. The same nine technicians inseminated heifers in both farms, and 3 Holstein and 4 Jersey sires were used. Technicians and sires were balanced between treatments and later used in the statistical analyses. Heifers were classified according to age, e.g., < 13 mo, between 13 and 15 mo, or ≥ 15 mo of age.

Pregnancy Diagnoses and Evaluation of Pregnancy Outcomes

Pregnancy diagnoses and calculation of P/AI and pregnancy loss were exactly as described for experiment 1.

Statistical Analysis

Sample sizes were calculated for both studies to allow for sufficient experimental units to detect a difference of 8 percentage units in experiment 1 and 6 percentage units in experiment 2 ($\alpha = 0.05$; $\beta = 0.20$). These differences were based on the expected P/AI of 53% for the second insemination at experiment 1 and 58% for experiment 2. We anticipated that P/AI for the second AI (experiment 1) would range from 45% to 60%.

Similarly, it was anticipated that P/AI for the first AI (experiment 2) would range from 50 to 62% based on previous studies with the 5-d timed AI protocol (Rabaglino et al., 2010a; 2010b) and pregnancy results at the study farms. Under these assumptions, a total of 300 experimental units per treatment would be necessary for experiment 1 and 550 experimental units per treatment in experiment 2.

In both experiments, binary responses were analyzed by logistic regression using the LOGISTIC procedure of SAS version 9.2 (SAS/STAT, SAS Institute Inc., Cary, NC, USA). Backward stepwise logistic regression models were used and variables were continuously removed from the models by the Wald statistic criterion when $P > 0.10$. In experiment 1, the models for ovarian responses to treatments, proportion of heifers with low progesterone at AI, and estrus at AI included the effects of treatment (GnRH vs. control), age of the heifer, ovarian status on study d 0 (presence or absence of CL), and interaction between treatment and ovarian status. The models for P/AI and pregnancy loss included the effects of treatment, age of the heifer, ovarian status on study d 0, sire, technician, and interaction between treatment and ovarian status. In experiment 2, the model for detection of estrus included the effects of treatment (OVS56 vs. COS72), farm, breed of the heifer, and age of the heifer. The models for P/AI and pregnancy loss included the effects of treatment, farm, breed of the heifer, age of the heifer, sire, technician, and display of signs of estrus at AI. In all analyses in both experiments, treatment was forced in the final model.

In experiment 1, concentration of progesterone at AI was analyzed by ANOVA using the GLM procedure of SAS, and the model included the effects of treatment, age of the heifer, and ovarian status on study d 0.

Results

Experiment 1

A CL visible by ultrasound was observed in 88.5% of the heifers on study d 0, indicating that the majority of the heifers were cycling. Ovulation on study d 0 and presence of a new CL at the injection of PGF_{2α} were both greater ($P < 0.01$) for GnRH than control heifers (Table 4-1). Ovulation on study d 0 was greater ($P < 0.01$) for heifers without a CL than those with CL, and this was observed in both, GnRH (59.7 vs. 26.9%) and control (32.5 vs. 6.1%) heifers. Although ovulation rate increased with GnRH, the proportion of heifers with a visible CL on study d 5 did not differ between treatments and averaged 87.1%. Treatment with GnRH influenced ($P < 0.03$) the proportion of heifers with low progesterone at AI, and the effect was observed when the progesterone cut-off was either 0.50 or 0.25 ng/mL. This difference resulted in GnRH heifers having greater ($P < 0.01$) concentration of progesterone at AI than control heifers.

Detection of estrus at AI did not differ between treatments and averaged 67.4% (Table 4-2). Treatment with GnRH on study d 0 did not affect P/AI on either d 32 or 60 after insemination. The response to treatment was not influenced by the ovarian status on study d 0. For instance, P/AI on d 32 for heifers with CL on study d 0 were 53.3 and 54.5% for GnRH and control, respectively. For heifers without a CL on study d 0, P/AI were 48.6 and 42.3% for GnRH and control, respectively. Similarly, pregnancy loss between 32 and 60 d of gestation did not differ between treatments and averaged 6.0%.

Experiment 2

As expected, the detection of heifers in estrus on the day before timed AI was similar between treatments (Table 4-3). However, when GnRH was given 16 h before AI

in OVS56, it decreased ($P < 0.001$) the proportion of heifers in estrus on the day of timed AI. Heifers in estrus at timed AI had greater ($P < 0.001$) P/AI than those not detected in estrus (62.0 vs. 50.8%). An interaction ($P = 0.05$) between treatment and detection of signs of estrus at AI was observed for pregnancy on d 32. For heifers in estrus, treatment did not affect P/AI (COS72 = 60.5% vs. OVS56 = 64.1%), but for those not displaying estrus at AI, COS72 tended ($P = 0.07$) to increase P/AI than OVS56 (55.0 vs. 47.6%). On d 60 after AI, heifers receiving COS72 had greater ($P = 0.05$) P/AI than those receiving OVS56, and this effect was observed primarily because for heifers not detected in estrus, those in the COS72 group had greater ($P < 0.05$) P/AI than heifers in the OVS56 (53.0 vs. 44.7%). For heifers detected in estrus, P/AI was not influenced by treatment (COS72 = 57.3 vs. OVS56 = 59.2%). Pregnancy loss between 32 and 60 d of gestation did not differ between treatments and averaged 5.8%.

Discussion

Optimization of the 5-d timed AI program to synchronize ovulation of dairy heifers allows producers to incorporate timed insemination when needed with acceptable fertility. Earlier work with the Ovsynch program in dairy heifers resulted in low P/AI (Pursley et al., 1997), and it was suggested that timed AI should not be used in dairy heifers. The 5-d timed AI program initially described by Bridges et al. (2008) and investigated for dairy heifers by Rabaglino et al. (2010a; 2010b) resulted in P/AI that resemble those of heifers inseminated following estrus and usually better than results previously obtained with heifers subjected to the standard Ovsynch protocol and some of its variations (Pursley et al., 1997; Rivera et al., 2004). In fact, results from Rabaglino et al. (2010a; 2010b) and those from the current experiments are close to the 57% P/AI reported for Holstein heifers in the US (Kuhn et al., 2006).

An interesting aspect of the 5-d timed AI program evaluated by Rabaglino et al. (2010a) was the low incidence of heifers with multiple CL 5 d after the injection of GnRH, thereby suggesting poor ovulatory response. In lactating dairy cows subjected to ovulation synchronization programs such as Ovsynch and Cosynch, ovulation to the first GnRH is variable according to day of the cycle when it is administered (Vasconcelos et al., 1999). It is optimized when GnRH is administered on day 6 of the estrous cycle (Bello et al., 2006). In dairy heifers, ovulation to the first GnRH usually is less than that observed for lactating dairy cows, even when the estrous cycle is presynchronized (Stevenson et al., 2008). When given at random stages of the estrous cycle, GnRH resulted in only 35.4% ovulation in experiment 1, and only 26.9% of the heifers with a CL ovulated in response to administration of GnRH. Because a large proportion of heifers had CL on study d 0, the low ovulatory response to GnRH did not influence the proportion of heifers with visible luteal tissue by ultrasound on study d 5. Nevertheless, treatment with GnRH reduced the proportion of heifers with progesterone concentrations < 0.50 ng/mL. The traditional cut-off for luteolysis commonly cited in the literature has been 1 ng/mL, but on the day of insemination, P/AI is optimized when progesterone concentrations are usually < 0.50 ng/mL. In fact, in many cases with both dairy heifers and lactating dairy cows, the optimized cut-off value for progesterone to predict pregnancy was < 0.30 ng/mL (Rabaglino et al., 2010a; Santos et al. 2010). Therefore, although ovulation rate was low, it was sufficient to compromise the proportion of heifers with low progesterone at AI when a single injection of PGF_{2α} is administered 5 d later. In lactating dairy and beef cows, compromised luteolysis can reduce P/AI in the 5-d timed AI program, thereby requiring 2 sequential treatments with

PGF_{2α} (Kasimanickam et al., 2009; Santos et al., 2010a). However, in dairy heifers, an additional treatment with PGF_{2α} did not further improve P/AI (Rabaglino et al., 2010a).

In the current study, administration of GnRH on study d 0 did not benefit P/AI of dairy heifers, and this response was observed regardless of the presence or absence of a CL on study d 0. In lactating dairy cows, ovulation to the initial GnRH of the timed AI protocol is critical to improve P/AI (Vasconcelos et al., 1999; Bello et al., 2006; Santos et al., 2010a), but the same has not been observed in dairy heifers (Stevenson et al., 2008). Stevenson et al. (2008) administered GnRH 6 d before the initiation of the timed AI protocol to presynchronize the estrous cycle of dairy heifers. Although they were able to increase ovulation to the first GnRH of the timed AI program in presynchronized heifers, ovulation did not influence P/AI or pregnancy loss. Therefore, it is possible that in dairy heifers, typically having 3 waves of follicle development (Sirois and Fortune, 1988), ovulation and recruitment of a new wave has less impact on fertility in timed AI protocols because of the typically shorter period of ovulatory follicle dominance than in lactating cows. Also, it is possible that the benefit of GnRH inducing ovulation was mitigated by the single injection of PGF_{2α} that resulted in a smaller proportion of cows with progesterone < 0.5 ng/mL at AI. Nevertheless, when a single injection of PGF_{2α} is used, the results of this study indicate that the initial GnRH is not required to optimize P/AI in dairy heifers subjected to the 5-d timed AI protocol.

Because of the lack of benefit from GnRH on fertility of dairy heifers, experiment 2 was designed to evaluate whether induction of ovulation 16 h before AI would improve P/AI of dairy heifers in the 5-d timed AI protocol without the GnRH on study d 0. Administering GnRH to induce ovulation concurrent with AI, in general, was beneficial to

P/AI of dairy heifers compared with GnRH 16 h before AI. However, the benefit was observed only in heifers that did not display signs of estrus on the day of AI. Bisinotto et al. (2010) observed that for lactating dairy cows subjected to the 5-d timed AI program, induction of ovulation before AI was not beneficial to fertility. When cows undergo timed AI protocols with 7 d between the first GnRH and PGF_{2α}, administration of the final GnRH 16 h before AI benefits P/AI (Brusveen et al., 2008). This is thought to be mediated by improved synchrony between sufficient numbers of spermatozoa capable of fertilization in the oviduct and the presence of a viable oocyte (Saacke, 2008). In fact, results from Brusveen et al. (2008) agree with those of Dransfield et al. (1998) in which the highest P/AI was achieved when insemination was performed 4 to 16 h after the onset of estrus.

When cows are subjected to the 5-d timed AI program, the period of ovulatory follicle development is reduced by approximately 2 d compared with conventional programs (Santos et al., 2010a). This reduction results in ovulatory follicles of smaller diameter, reduced concentration of estradiol in plasma, and a smaller proportion of cows in estrus at AI compared with cows in the conventional 7-d program (Santos et al., 2010a). In dairy heifers, these parameters have not been fully characterized. In experiment 2, delaying the administration of the GnRH to 72 h increased the proportion of heifers in estrus at AI, which is a sign of increased exposure to endogenous estradiol. In a series of experiments with beef cows subjected to the 5-d program, extending proestrus from 60 to 72 h was beneficial to fertility (Bridges et al., 2008). It would be expected that induction of ovulation 16 h before AI might be beneficial to fertility of dairy heifers because of the potentially better synchrony between the moment of ovulation

and availability of capacitated spermatozoa in the oviduct for fertilization of the oocyte (Saacke, 2008). Nevertheless, 45% of the heifers were in estrus in the afternoon before the scheduled AI. Furthermore, when heifers were subjected to COS72, more than 61% were in estrus at the moment of timed AI. Heifers in estrus receiving COS72 likely already had a spontaneous LH surge when insemination was performed, which would diminish the benefit of administering GnRH 16 h before AI. The fact that delaying the administration of GnRH to the moment of AI improved P/AI of heifers not in estrus suggests that the additional period of proestrus was beneficial to fertility. This might have been mediated by additional exposure to estradiol and additional growth of the ovulatory follicle. The additional proestrus might be particularly important in a program of reduced period of follicle dominance to allow for sufficient pre-ovulatory follicle growth and production of estradiol as suggested by Bisinotto et al. (2010). Thus, these results suggest that the prolonged proestrus in COS72 benefit fertility of dairy heifers in the 5-d program counterbalancing the potentially better synchrony between ovulation and insemination obtained by the OVS56.

Conclusion

Fertility of dairy heifers subjected to a 5-d timed AI protocol was not affected by administration of the first GnRH on study d 0. The lack of benefit is attributed to the low ovulation rate to the initial GnRH and the reduced proportion of heifers with low progesterone at AI when receiving a single PGF_{2α} treatment 5 d later. Timing of induction of ovulation with GnRH relative to AI influenced P/AI of heifers not displaying estrus at AI, and it was usually better when heifers received the ovulatory stimulus concurrent with AI at 72 h after PGF_{2α} than 16 h before timed AI. Therefore, when heifers are subjected to the 5-d timed AI program with a single treatment of PGF_{2α}, it is

suggested that the initial GnRH is not necessary and the period of proestrus should be 72 h with administration of GnRH to induce ovulation concurrent with timed AI.

Table 4-1. Ovarian responses of heifers treated with or without GnRH at the initiation of the 5-d timed AI protocol

	Treatment ¹		AOR ² (95% CI)	P
	GnRH	Control		
	% (n/n)			
Study d 0				
Heifers with follicle ≥ 10 mm	90.4 (216/239)	88.6 (210/237)	1.21 (0.67-2.17)	0.53
CL on study d 0	74.1 (177/239)	83.1 (197/237)	0.57 (0.36-0.89)	0.01
Ovulation on study d 0				
All heifers	35.4 (84/237)	10.6 (25/236)	4.49 (2.68-7.51)	< 0.01
Heifers with follicles ≥ 10 mm	39.3 (84/214)	12.0 (25/209)	4.82 (2.80-8.30)	<0.01
New CL at PGF _{2α}	43.1 (102/237)	20.8 (49/236)	3.13(1.91-5.13)	< 0.01
CL at PGF _{2α}	88.2 (209/237)	86.0 (203/236)	1.30 (0.75-2.24)	0.35
Proportion of heifers according to progesterone at AI				
< 1 ng/mL	90.1 (127/141)	94.9 (129/136)	0.51 (0.20-1.30)	0.16
< 0.50 ng/mL	73.8 (104/141)	88.2 (120/136)	0.34 (0.18-0.66)	< 0.01
< 0.25 ng/mL	41.1 (58/141)	52.9 (72/136)	0.57 (0.35-2.93)	0.03
Progesterone at AI, ng/mL	0.50 ± 0.07	0.28 ± 0.07	---	< 0.01

¹ GnRH = d 0 GnRH and CIDR, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and timed AI; Control = d 0 CIDR, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and timed AI.

² AOR = adjusted odds ratio. Control is the reference for comparison.

Table 4-2. Effect of the first GnRH injection of the 5-d timed AI protocol on fertility responses of dairy heifers - Experiment 1

	Treatment ¹		AOR ² (95% CI)	P
	GnRH	Control		
	% (n/n)			
Estrus at timed AI ³	69.2 (206/298)	64.5 (198/307)	1.23 (0.88-1.73)	0.23
Pregnant ⁴				
Day 32	52.5 (155/295)	54.1 (165/305)	0.94 (0.68-1.30)	0.70
Day 60	49.8 (147/295)	50.0 (150/300)	0.99 (0.72-1.37)	0.97
Pregnancy loss ⁵	5.8 (9/155)	6.2 (10/160)	0.93 (0.37-2.34)	0.87

¹ GnRH = d 0 GnRH and CIDR, d 5 PGF_{2α} and removal of the CIDR, d 8 GnRH and timed AI; Control = d 0 CIDR, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and timed AI.

² AOR = adjusted odds ratio. Control is the reference for comparison.

³ Evaluated based on removal of tail chalk on the day of timed AI.

⁴ Three GnRH and 2 control heifers left the study before pregnancy diagnosis on d 32.

⁵ Five control heifers left the study before reconfirmation of pregnancy on d 60 after AI.

Table 4-3. Effect of time of administration of the final GnRH of the 5-d timed AI protocol relative to insemination on fertility responses of dairy heifers - Experiment 2

	Treatment ¹		AOR ² (95% CI)	<i>P</i>
	OVS56	COS72		
	% (n/n)			
Estrus³				
Day before timed AI	46.7 (301/644)	43.2 (281/651)	1.16 (0.93-1.45)	0.19
Day of timed AI	47.5 (306/644)	61.4 (400/651)	0.52 (0.45-0.70)	< 0.001
Pregnant				
Day 32	55.4 (357/644)	58.4 (380/651)	0.75 (0.42-1.08)	0.08
Day 60	51.6 (332/644)	55.6 (362/651)	0.72 (0.39-1.04)	0.05
Pregnancy loss	7.0 (25/357)	4.7 (18/380)	1.52 (0.81-2.83)	0.19

¹ COS72 = d 0 CIDR, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and timed AI; OVS56 = d 0 GnRH and CIDR, d 5 PGF_{2α} and removal of CIDR, d 7.3 GnRH, d 8 timed AI.

² AOR = adjusted odds ratio. COS72 is the reference for comparison.

³ Evaluated based on removal of tail chalk on the day before and day of timed AI.

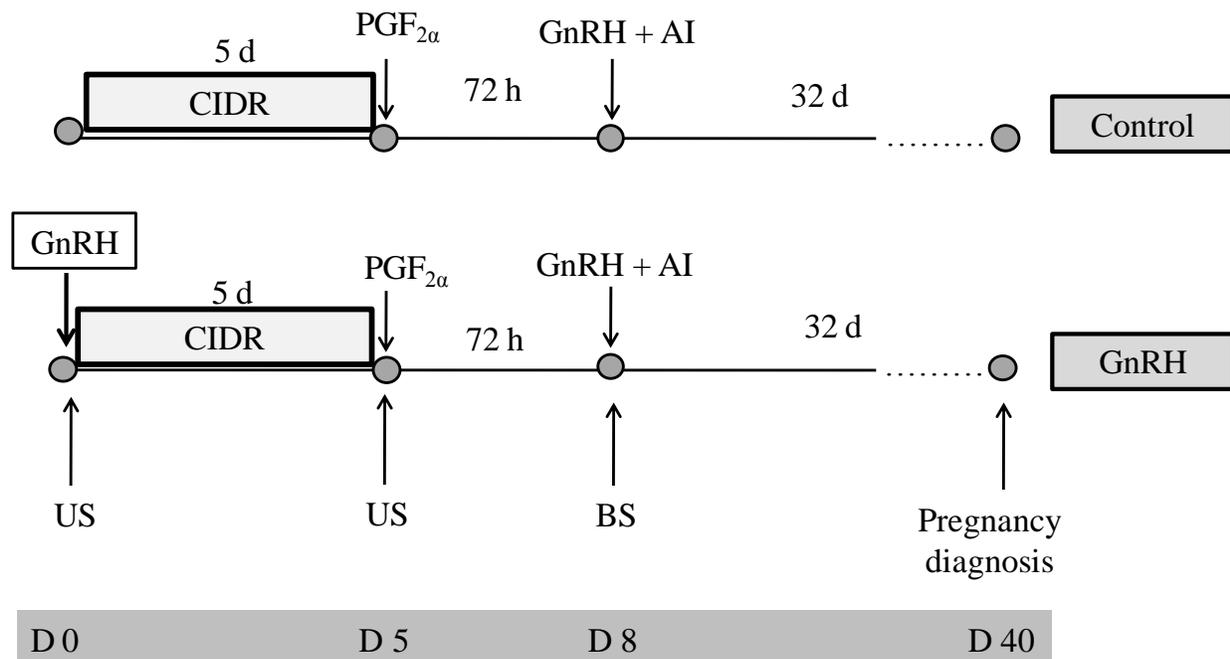


Figure 4-1. Diagram of activities in experiment 1. BS = blood samples for analysis of progesterone concentration; CIDR = controlled internal drug-release containing 1.38 g of progesterone; GnRH = injection of 100 µg of gonadorelin hydrochloride; PGF_{2α} = injection of 25 mg of dinoprost as tromethamine salt; US = ultrasonography of the ovaries.

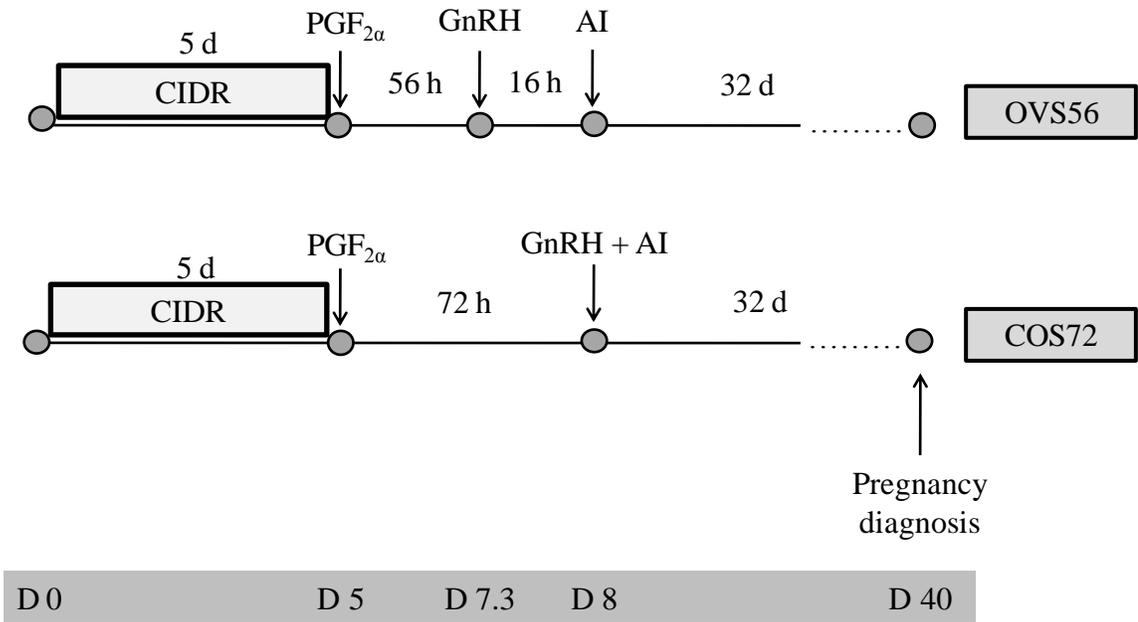


Figure 4-2. Diagram of activities in experiment 2. CIDR = controlled internal drug-release containing 1.38 g of progesterone; GnRH = injection of 100 μ g of gonadorelin hydrochloride; PGF_{2 α} = injection of 25 mg of dinoprost as tromethamine salt.

CHAPTER 5

HORMONAL MANIPULATIONS IN THE 5-D TIMED AI PROTOCOL TO OPTIMIZE ESTROUS CYCLE SYNCHRONY AND FERTILITY IN DAIRY HEIFERS

Objectives were to determine the effects of GnRH at the initiation of the 5-d timed artificial insemination (AI) program combined with two injections of PGF_{2α} on ovarian responses and pregnancy per AI (P/AI) in dairy heifers, and the role of progesterone concentrations on LH release and ovulation in response to GnRH. In study 1, heifers received a controlled internal drug release (CIDR) insert containing 1.38 g of progesterone on d 0, an injection of 25 mg of PGF_{2α} and CIDR removal on d 5, and an injection of 100 µg GnRH concurrently with AI on d 8. Heifers were assigned to receive no additional treatment (control, n = 559) or an injection of GnRH on d 0 and a second injection of PGF_{2α} on d 6 (G2P, n = 547). In study 2, all heifers were treated as described for control in study 1, and were allocated to receive no additional treatment (control = 723), an injection of PGF_{2α} on d 6 (NG2P = 703), or an injection of GnRH on d 0 and an injection of PGF_{2α} on d 6 (G2P = 718). In study 3, heifers received a CIDR on d 7 after ovulation and were assigned randomly to a low progesterone (LP; n = 6) treatment in which two injections of 25 mg of PGF_{2α} each were administered 12 h apart, on d 7 and 7.5 after ovulation, or to a high progesterone (HP, n = 12) in which no PGF_{2α} was administered. On d 8, heifers received 100 µg of GnRH and blood was sampled at every 15 min from -30 to 180 min relative to the GnRH for assessment of LH concentrations. Additionally, 94 heifers were assigned to LP or HP and ovulation in response to GnRH was evaluated. In study 1, P/AI was greater for G2P than for control on d 32 (59.4 vs. 53.5%) and 60 after AI (56.6 vs. 51.3%). In study 2, administration of GnRH on d 0 increased the proportion of heifers with a new corpus luteum on d 5 (control = 21.9 vs. NG2P = 20.1 vs. G2P = 34.4%). Administration of a second PGF_{2α}

increased the proportion of heifers with progesterone < 0.5 ng/mL at AI (control = 83.1 vs. NG2P = 93.0 and G2P = 87.2%). Pregnancy per AI was greater for G2P than for control and NG2P on d 32 (control = 52.9 vs. NG2P = 55.0 vs. G2P = 61.7%) and 60 (control = 49.0 vs. NG2P = 51.6 vs. G2P = 59.1%). In study 3, HP attenuated LH release and reduced ovulation (19.0 vs. 48.4%) in response to GnRH compared with LP. Combining GnRH and two doses of PGF_{2α} in the 5-d timed AI protocol improved follicle turnover, luteolysis, and P/AI in heifers. Elevated concentrations of progesterone suppressed LH release and are linked with the low ovulatory response to the initial GnRH treatment of the protocol.

Introductory Remarks

Reproductive efficiency in dairy heifers affects age at first calving which has major impacts on rearing costs and subsequent productive life (Gabler et al., 2000; Ettema and Santos, 2004). Most dairy operations in the United States use AI after observed estrus to manage reproduction in heifers (NAHMS, 2009). Nevertheless, advances in protocols for synchronization of the estrous cycle have supported the use of timed AI as an alternative method to control reproduction and improve economics when detection of estrus is less than 70% (Ribeiro et al. 2012a). Recent studies have consistently reported pregnancy per AI (P/AI) ranging from 50 to 60% in dairy heifers subjected to the 5-d timed AI program (Rabaglino et al., 2010; Lima et al., 2011), which are comparable to those observed in heifers inseminated at detected estrus (Kuhn et al., 2006). Further optimization of such programs to either simplify or improve fertility will likely increase acceptance by dairy producers.

Ovulation in response to the initial GnRH injection in timed AI programs enhances synchrony of estrous cycle, shortens follicle dominance, and improves

embryo quality and P/AI (Vasconcelos et al., 1999; Chebel et al., 2006; Cerri et al., 2009a). Nevertheless, only 15 to 35% of heifers ovulate when treated with GnRH at random stages of the estrous cycle (Stevenson et al., 2008; Lima et al., 2011). In addition, heifers that ovulate in response to the initial GnRH will have a newly formed corpus luteum (CL), which is generally refractory to a single treatment with PGF_{2α} on d 5 of the cycle (Rowson et al., 1972; Henricks et al., 1974). Eliminating the first GnRH reduced ovulation at the beginning of the synchronization protocol, but increased the proportion of heifers that underwent luteolysis at AI when a single PGF_{2α} injection was used (Lima et al. 2011). Because the benefits associated with follicle turnover were offset by a less effective CL regression, P/AI did not differ between heifers that received or not GnRH at the initiation of the timed AI program (Lima et al. 2011). These results indicate that the initial GnRH is not necessary when a single PGF_{2α} is used, which simplifies and reduce costs associated with the synchronization protocol.

Results from lactating dairy cows subjected to the 5-d timed AI program indicate that the use of two injections of PGF_{2α} administered 24 h apart improved CL regression and P/AI (Santos et al., 2010a), particularly when ovulation to initial GnRH was high (Ribeiro et al., 2012b). Shorter intervals between PGF_{2α} treatments, ranging from 7 to 8 h, have been shown to increase P/AI compared with a single injection in beef cows (Kasimanickam et al., 2009), although preliminary results in dairy heifers did not confirm such benefit (Rabaglino et al., 2010). Therefore, it is reasonable to speculate that the combination of the initial GnRH and the administration of PGF_{2α} on d 5 and 6 of the protocol will improve follicle turnover and luteal regression, which are expected to increase P/AI.

The differences in catabolism of steroid hormones (Sangsrivong et al., 2002) explain the nearly 1.5 ng/mL greater progesterone concentration in heifers than lactating cows during mid-diestrus (Sartori et al., 2004). In fact, the increase in progesterone concentrations with a controlled internal drug-release (CIDR) is expected to be greater in nonlactating (Zuluaga and Williams, 2008) than in lactating cows (Cerri et al., 2009b). Progesterone affects LH secretion, which might compromise ovulatory response to GnRH treatment, which might partially explain the low ovulatory response to GnRH in dairy heifers. Results from beef heifers support this idea (Colazo et al., 2008; Dias et al., 2010).

It was hypothesized that a combination of GnRH at the initiation of the 5-d timed AI and injections of PGF_{2α} on d 5 and 6 of the protocol improves the synchrony of the estrous cycle and fertility in dairy heifers. Furthermore, it was hypothesized that elevated concentrations of progesterone compromise the release of LH and ovulation in response to a GnRH injection in dairy heifers. Study 1 was designed to compare a simplified 5-d timed AI protocol with a protocol that is expected to optimize P/AI by inducing ovulation and optimizing regression of newly formed CL. Study 2 was designed to evaluate the effects of GnRH at the initiation of 5-d timed AI program combined with two injections of PGF_{2α} on ovarian responses and fertility. Finally, the objectives of study 3 were to assess LH release and ovulation in response to GnRH in dairy heifers with low or high concentrations of progesterone in plasma.

In heifers that receive GnRH in the beginning of 5-d timed AI program might require multiple PGF_{2α} to optimize luteolysis and P/AI. Results from lactating dairy cows subjected to the 5-d timed AI program indicate that the use of two injections of PGF_{2α}

administered 24 h apart improved CL regression and P/AI (Santos et al., 2010a), particularly when ovulation to initial GnRH is high and more cows present a newly formed CL (Ribeiro et al., 2012b). Shorter intervals between PGF_{2α} treatments, ranging from 7 to 8 h, have been shown to increase P/AI compared with a single injection in beef cows (Kasimanickam et al., 2009), although preliminary results in dairy heifers did not confirm such benefit (Rabaglino et al., 2010). Therefore, it is reasonable to speculate that the combination of the initial GnRH and the administration of PGF_{2α} on d 5 and 6 of the protocol will improve follicle turnover and luteal regression, which is expected to result in increased P/AI in dairy heifers.

Presumably because of less catabolism of steroid hormones in heifers than lactating cows because of differences in splanchnic blood flow (Sangsrivong et al., 2002); progesterone concentrations in the plasma of dairy heifers are nearly 1.5 ng/mL greater than those in lactating cows during mid diestrus (Sartori et al., 2004). Because of reduced catabolism, the increase in progesterone concentrations with a CIDR is expected to be greater in nonlactating (Zuluaga and Williams, 2008) than in lactating cows (Cerri et al., 2009b). Progesterone affects LH secretion, which might compromise ovulatory response to GnRH treatment, which might partially explain the low ovulatory response to GnRH in dairy heifers. In fact, results from beef heifers support the idea that progesterone compromise LH release and impair ovulation following an injection of GnRH (Colazo et al., 2008; Dias et al., 2010).

It was hypothesized that a combination of GnRH at the initiation of the 5-d timed AI and injections of PGF_{2α} on d 5 and 6 of the protocol improve the synchrony of the estrous cycle and fertility in dairy heifers. Furthermore, it was hypothesized that

elevated concentrations of progesterone compromise the release of LH and ovulation in response to a GnRH injection in dairy heifers. Study 1 was designed to compare a simplified 5-d timed AI protocol with a protocol that is expected to optimize P/AI by inducing ovulation and optimizing regression of newly formed CL. Study 2 was designed to evaluate the effects of GnRH at the initiation of 5-d timed AI program combined with two injections of PGF2 α on ovarian responses and fertility. Finally, the objectives of study 3 were to assess LH release and ovulation in response to GnRH in dairy heifers with low or high concentrations of progesterone in plasma.

Materials and Methods

The University of Florida Institute of Food and Agricultural Sciences Animal Research Committee approved all procedures in the 3 studies reported.

Study 1

Heifers, Diets, and Housing

A total of 1,106 nulliparous Holstein and crossbred Holstein-Jersey heifers at an average (\pm SD) of 14.0 ± 2.1 mo of age from two farms in north central Florida were enrolled in the study between March and June, 2010. Crossbred heifers ($n = 231$) were located only in farm 2. Four-hundred and fifty-seven heifers received their first insemination, whereas the remaining 649 heifers were diagnosed non-pregnant on d 32 after insemination and resynchronized to receive their second AI. Heifers in both locations were managed on pasture with access to portable shades and trees. Heifers were fed a TMR once daily formulated to meet or exceed the nutritional requirements of Holstein heifers weighing 360 kg and gaining 0.8 kg/d (NRC, 2001). The diet consisted of a mixture of lactating cow ration orts, Bermuda grass silage, wet brewer's grain, minerals and vitamins supplement. For administration of hormonal treatments,

insemination, and pregnancy examination, heifers were moved to an open-sided barn with self-locking stations in farm 1 or to a palpation rail in farm 2.

Experimental Design and Treatments

All heifers received a CIDR containing 1.38 g of progesterone (Eazi-Breed CIDR Cattle Insert, Zoetis, Madison, NJ) on d 0, an i.m. injection of 25 mg of PGF_{2α} (dinoprost tromethamine; Lutalyse sterile solution, Zoetis, Madison, NJ) and CIDR removal on d 5, and an i.m. injection of 100 µg of GnRH (gonadorelin hydrochloride; Factrel, Zoetis, Madison, NJ) concurrently with AI on d 8. On d 0, heifers were blocked according to number of AI (first or second) and then age and, within each block, they were allocated randomly to receive no additional treatment (control, n = 559) or an injection of GnRH on d 0 and a second injection of PGF_{2α} on d 6 (G2P, n = 547; Figure 4-1). On d 5 and 6, heifers had their tailheads painted using paintsticks (All-Weather Paintstick; LA-CO Industries, Chicago, IL). Expression of estrus at AI was evaluated based on removal of the tail paint on d 8. Inseminations were performed by 11 technicians with semen from six Holstein and six Jersey sires.

Pregnancy Diagnosis and Calculation of Reproductive Outcomes

Pregnancy was diagnosed 32 d after AI by transrectal ultrasonography of the uterus using a portable ultrasound equipped with a 7.5 MHz transrectal probe (Easi-Scan, BCF Technologies, Rochester, MN). The visualization of an amniotic vesicle containing an embryo with heartbeat was used as the determinant of pregnancy. Pregnant heifers on d 32 were re-examined by transrectal palpation of uterine contents on d 60 after AI. Pregnancy per AI was calculated by dividing the number of heifers diagnosed pregnant on d 32 or 60 after AI by the number of heifers receiving AI. Pregnancy loss was calculated as the number of heifers that lost their pregnancy

between d 32 and 60 after AI divided by the number of heifers diagnosed pregnant on d 32 after AI.

Study 2

Heifers, Diets, and Housing

A total of 2,144 nulliparous Holstein heifers with an average (\pm SD) of 13.5 ± 1.0 mo of age from a single farm in north central Florida were enrolled in the study between July 2010 and April, 2011. A total of 1,723 heifers received their first insemination, whereas the remaining 421 heifers were diagnosed non-pregnant on d 32 after their first AI and resynchronized to receive the second AI. Heifers were managed and fed as described in study 1. For administration of hormonal treatments, ultrasonography examination, blood sampling, insemination, and pregnancy diagnosis, heifers were moved to an open-sided barn with self-locking stations.

Experimental Design and Treatments

All heifers received a CIDR containing 1.38 g of progesterone on d 0, an i.m. injection of 25 mg of PGF_{2 α} and CIDR removal on d 5, and an i.m. injection of 100 μ g of GnRH concurrently with AI on d 8. On d 0, heifers were blocked by number of AI (first or second) and then age and, within each block, they were allocated randomly to receive no additional treatment (control, n = 723), an additional injection of PGF_{2 α} on d 6 (NG2P, n = 703), or an injection of GnRH on d 0 and an additional injection of PGF_{2 α} on d 6 (G2P, n = 718; Figure 5-2). Estrus at AI was detected as described in study 1. Inseminations were performed by 10 technicians with semen from 10 Holstein and 3 Jersey sires.

Ultrasonography of Ovaries and Evaluation of Ovulatory Responses

Ovaries from a subset of 623 heifers were scanned on study d 0 and 5 and the presence and location of CL > 15 mm and follicles \geq 10 mm in diameter were recorded. Ovulation at the beginning of the timed AI program was considered when the heifer had a follicle \geq 10 mm on d 0 and a newly formed CL was observed in the same ovary on d 5. Heifers with follicles < 10 mm on study d 0 but with a new CL on study d 5 were considered to have a new CL, but ovulated before study d 0.

Blood Sampling and Analysis of Progesterone Concentrations

Blood was sampled from 610 of the same subset of 623 heifers evaluated for ovulation described previously. Blood samples were collected on d 8 by puncture of the median coccygeal vein or artery using evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K₂ EDTA for plasma separation. Samples were placed immediately on ice and kept refrigerated until arrival to the laboratory. Blood tubes were centrifuged at 2,000 x g for 15 min, and an aliquot of 2 mL of plasma was frozen at -20 °C until analysis. Concentration of progesterone in plasma was analyzed in all samples by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.05 ng/mL calculated at 2 SD below the mean counts per min at maximum binding. All samples were analyzed in a single assay. Two plasma samples with progesterone concentrations of 1.5 and 2.5 ng/mL were included throughout the sequence of samples in the assay for quality control. The intra-assay CV were 2.5 and 2.9% for the samples containing 1.5 and 2.5 ng/mL, respectively.

Three different cut-off values for plasma concentrations of progesterone at AI were used to determine luteolysis, progesterone < 1.0, < 0.50, and < 0.30 ng/mL. These

three values were selected based on the traditional threshold used to indicate CL regression (1 ng/mL), or based on concentration of progesterone at AI that have been used as cut-off values that best predicted P/AI (Rabaglino et al., 2010; Santos et al., 2010a).

Pregnancy Diagnosis and Calculation of Reproductive Outcomes

Pregnancy was diagnosed 32 and 60 d after AI as described in study 1. Similarly, P/AI and pregnancy loss were calculated as described for study 1. Of all 2,144 heifers, 26 did not have a pregnancy diagnosis performed (control = 12; NG2P = 7; G2P = 7) because they were moved to another farm before d 32 after AI.

Study 3

Experimental Design and Treatments

Holstein nulliparous heifers from the University of Florida Dairy Unit had their ovaries scanned and those having a CL \geq 15 mm and at least one follicle \geq 10 mm in diameter received an injection of PGF_{2 α} . Heifers had their tailheads painted and were observed daily for signs of estrus based on removal of the tail chalk. Heifers detected in estrus between 48 and 72 h after the injection of PGF_{2 α} had their ovaries scanned to map the ovarian follicles. Ovaries were scanned again 24 and 48 h after estrus and the disappearance of one or more follicles \geq 10 mm was considered ovulation, which was assumed to have occurred in the preceding day to the scanning when the dominant follicle was no longer visible by ultrasonography. Only heifers that ovulated within 48 h of detected estrus were included in the study. The day of ovulation was considered study d 0. Heifers were assigned randomly to either a low (LP; n = 6) or high progesterone (HP; n = 12) treatment (Figure 5-3). All heifers received a CIDR on d 7 after ovulation and those assigned to LP received two injections of PGF_{2 α} 12 h apart

beginning at CIDR insertion. On d 8, all heifers received an i.m. injection of 100 µg of GnRH (gonadorelin diacetate tetrahydrate; Cystorelin, Merial, Iselin, NJ). An additional group of 94 nulliparous Holstein heifers were randomly assigned to the same treatments in a ratio of 1 to 2, i.e. 31 LP and 63 HP. An injection of 100 µg of GnRH was administered on d 8 and ovaries were scanned on d 7, 8 and 12 to characterize ovulation in response to GnRH.

Blood Sampling and Analyses of LH and Progesterone Concentrations

The release of LH induced by the injection of 100 µg GnRH was evaluated in the 18 heifers subjected to LP and HP treatments. A 14 gauge x 14 cm indwelling catheter (Abbocath-T; Hospira Inc., Lake Forest, IL) was placed in the left jugular vein for the duration of blood sampling. Samples were collected at -30, -15, 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 240, and 300 min relative to the injection of GnRH. Before each sampling, approximately 8 mL of blood were drawn and discarded for cleansing of the catheter. Samples were then collected using a 10 mL syringe and transferred into evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) for subsequent serum separation. After sampling, the catheters were flushed with heparinized solution (30 USP heparin sodium; Sigma-Aldrich, Saint Louis, MO) to avoid clogging. Samples were immediately placed on ice remaining there for 30 min and then placed in room temperature for 30 min before centrifugation for serum separation. Tubes were centrifuged at 2,200 x g at 4 °C for 15 min for serum separation. Serum samples were frozen at -20 °C until later analysis.

Concentrations of LH in serum were determined by RIA as previously reported (McVey and Williams, 1991). Highly purified ovine LH (AFP-8614B, NIDDK-oLH-I-4; National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA)

was used as both the reference preparation and as iodinated tracer. The primary antiserum used was produced in rabbits immunized against ovine LH (AFP-192279, NIDK-anti-oLH-1; National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA). This antiserum displays similar cross-reactivity between highly purified preparations of ovine (NIDDK – I-2) and bovine (AFP11743B) LH and does not cross-react with other pituitary hormones. Sensitivity of the assay was 0.1 ng/mL and the intra- and inter-assay CV averaged 11.9 and 10.4%, respectively.

An additional blood sample was collected immediately before the injection of GnRH and transferred to evacuated tubes (Becton Dickinson, Franklin Lakes, NJ) containing K₂ EDTA for plasma separation. Plasma was separated, stored, and assayed for progesterone concentration as described in study 1. Concentrations of progesterone in plasma were analyzed in the same assay described in study 2 with intra-assay CV of 2.5 and 2.9% for known samples containing 1.5 and 2.5 ng/mL, respectively.

Statistical Analysis

Power analyses were performed to calculate sample sizes in all three studies using Minitab 16 (Minitab Inc., State College PA, USA). Sample sizes were calculated for studies 1 and 2 to allow sufficient experimental units to detect a difference of 6 percentage units in P/AI between treatments ($\alpha = 0.05$; $\beta = 0.20$; two-tailed test). The expected P/AI for first and second AI combined was of 58% for G2P and 52% for the remaining treatments based on previous studies (Rabaglino et al., 2010; Lima et al., 2011). Under these assumptions, a minimum of 540 and 636 experimental units per treatment were deemed necessary in studies 1, which had two treatments, and 2, which had three treatments, respectively. Because of potential attrition, additional heifers were added to all treatments in both studies. In study 3, the sample size was calculated to

allow sufficient experimental units to detect a difference of 30 percentage units in ovulatory response to GnRH ($\alpha = 0.05$; $\beta = 0.20$). The ovulatory response was anticipated to increase from 20% in HP to 50% in LP based on previous studies with the 5-d timed AI protocol (Lima et al., 2011).

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) fitting a binary distribution. Treatment was forced in the final models, but covariates and the interaction between treatment and covariates were sequentially removed from the model if $P > 0.10$.

In study 1, the model for detection of estrus included the effects of treatment, breed, farm, number of AI, age of heifer (< 13 mo vs. 13 to 15 mo vs. > 15 mo), and the interactions of treatment and breed, treatment and farm, and treatment and number of AI. The models for P/AI and pregnancy loss included the effects of treatment, breed, farm, number of AI, age of heifer, sire, AI technician, and the interactions of treatment and breed, treatment and farm, and treatment and number of AI. In study 2, the models for analyses of ovarian responses to treatments, proportion of heifers with progesterone at AI below one of three cut-off points (< 1.0, < 0.50, and < 0.30 ng/mL), and proportion of heifers in estrus at AI included the effects of treatment, age of heifer (< 13 mo vs. 13 to 14 mo vs. > 14 mo), presence of CL on d 0, and interaction between treatment and presence of CL on d 0. The models for P/AI and pregnancy loss included the effects of treatment, number of AI, age of heifer, sire, AI technician, and interaction between treatment and AI number.

Concentrations of progesterone at AI in study 2 were analyzed by ANOVA using the GLM procedure of SAS. The model included the effects of treatment, age group, and presence of CL on study d 0. In study 3, concentrations of LH were analyzed by ANOVA for repeated measurements using the MIXED procedure of SAS. The values for LH concentration at -30, -15 and 0 min was averaged for each individual heifer and used as a covariate. The model included the effects of treatment, time, and the interaction between treatment and time, with heifer nested within treatment as the random term for test of effects of treatment. The covariance structure that resulted in the smallest Akaike's information criterion was selected for the model.

Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.

Results

Study 1

The proportion of heifers detected in estrus on the day of timed AI did not differ between treatments and averaged 76.8% (Table 5-1). A greater ($P < 0.001$) proportion of heifers were detected in estrus at AI in farm 1 compared with farm 2 (81.5 vs. 62.1%). There were no interactions between treatment and other independent variables for detection of estrus at AI.

Pregnancy per AI on d 32 was greater ($P = 0.04$) for G2P than for control heifers (Table 5-1). Similarly, the proportion of pregnant heifers on d 60 tended ($P = 0.06$) to be greater in G2P than in control. Although P/AI was greater ($P = 0.02$) in farm 1 compared with farm 2 on d 32 (57.3 vs. 54.0%) and 60 after AI (54.4 vs. 52.7%), no interaction between treatment and farm was observed for P/AI. Pregnancy loss was not affected by treatment or by the interactions between treatment and other independent variables.

Study 2

A CL visible by ultrasonography was observed in 80.4% of the heifers on d 0, indicating that the majority were cyclic. As anticipated, ovulation on d 0 and the presence of a new CL on d 5 were greater ($P \leq 0.01$) for G2P than for control or NG2P heifers (Table 5-2). Ovulation on study d 0 was greater ($P < 0.01$) for heifers without a CL ($n = 122$) than in those with a CL ($n = 501$) on d 0 (45.9 vs. 10.4%). Although ovulation at the initiation of the timed AI program was increased by the administration of GnRH, the proportion of heifers with a visible CL on study d 5 did not differ between treatments. The proportion of heifers with progesterone concentration at AI below the cutoffs of 0.30 and 0.50 ng/mL was less for control than NG2P and G2P (Table 5-2). A tendency ($P = 0.09$) for interaction between treatment and new CL was observed for luteolysis based on progesterone < 0.50 ng/mL on study d 8. This interaction was because treatments with 2 PGF_{2α} increased luteolysis in heifers with a new CL compared with control, but no difference was observed for those without a new CL. For controls, the proportions of heifers with progesterone < 0.50 ng/mL were 85.7 and 73.9% for those without and with a new CL; for NG2P, the same proportions were 93.7 and 90.2%, and for G2P they were 85.0 and 91.4%. There were not interactions between treatments and any other independent variables for any of the ovarian responses in study 2.

Treatment affected ($P = 0.02$) the proportion of heifers in estrus at AI (Table 5-3). Detection of estrus was greater ($P < 0.01$) for G2P than control, and tended ($P = 0.06$) to be greater for G2P than NG2P. No difference was observed between control and NG2P. Pregnancies per AI on d 32 and 60 after insemination were greater ($P < 0.01$) for G2P than control and NG2P. Heifers detected in estrus ($n = 1,612$) on the day of AI had

greater ($P < 0.001$) P/AI on d 32 (58.6 vs. 50.7%) and on d 60 (56.7 vs. 48.8%) than those not in estrus ($n = 532$). Heifers receiving their second insemination had greater ($P = 0.05$) P/AI on d 60 than heifers receiving their first insemination (57.6 vs. 52.2). Pregnancy loss tended ($P = 0.06$) to be less for G2P than controls (Table 5-3). Heifers receiving their first AI had greater ($P = 0.03$) pregnancy loss than heifers receiving their second AI (6.7 vs. 2.9%).

When P/AI were analyzed in the subset of 623 heifers with ovarian ultrasound and progesterone concentration at AI, an interaction ($P = 0.03$) between number of PGF_{2α} and new CL was observed for P/AI on d 32 and 60 after insemination. For heifers without a new CL, number of PGF_{2α} (control vs. NG2P + G2P) did not ($P = 0.36$) influence P/AI on d 32 (59.3 vs. 54.9%), but for those heifers with a new CL on d 5 of the timed AI protocol, administration of 2 doses of PGF_{2α} (NG2P + G2P) increased ($P < 0.05$) P/AI compared with control (62.8 vs. 45.7%). The same response was observed on d 60 after AI, in heifers without a new CL, number of PGF_{2α} did not ($P = 0.53$) influence P/AI (control = 54.9 vs. NG2P + G2P = 51.9%); however, in those with a new CL, administration of 2 doses of PGF_{2α} increased ($P < 0.05$) P/AI compared with control (61.1 vs. 43.5%).

Study 3

As expected, the concentration of progesterone on d 8 was less ($P = 0.01$) for LP than HP heifers (Table 5-4). The elevated concentration of progesterone in HP treatment reduced ($P = 0.04$) the mean concentration of LH in serum compared with LP heifers. A tendency ($P = 0.07$) of interaction between treatment and time relative GnRH injection was observed for LH concentrations. Serum concentrations of LH were less ($P < 0.05$) from 45 to 135 min after the injection of GnRH in HP compared with LP

treatment (Figure 5-4). Furthermore, HP treatment tended to reduce ($P = 0.09$) the peak concentration of LH in plasma compared with LP treatment. The interval from GnRH injection to peak of LH concentration did not differ between treatments. Finally, HP heifers had reduced ($P = 0.01$) incidence of ovulation in response to GnRH compared with LP heifers.

Discussion

The results of current study clearly support the concept that increased ovulation with administration of GnRH at the initiation of the protocol combined with improved luteolysis at insemination, by using two doses of $\text{PGF}_{2\alpha}$, are necessary to optimize P/AI in dairy heifers subjected to the 5-d timed AI program. These results are consistent with our hypothesis and clarify unanswered questions from findings of a previous study (Lima et al., 2011). Previously, administration of GnRH on d 0 of the 5-d timed AI protocol increased ovulation compared with no GnRH administration, but did not improve P/AI in heifers receiving a single dose of $\text{PGF}_{2\alpha}$ (Lima et al., 2011). This lack of benefit was suggested to be caused by the inability of a single injection of $\text{PGF}_{2\alpha}$ to fully regress newly formed CL to optimize fertility.

Study 1 was designed as a practical study to evaluate two breeding strategies for dairy heifers, one with minimum intervention without GnRH on d 0 and with a single $\text{PGF}_{2\alpha}$ on d 5, and another treatment expected to optimize P/AI by improving follicle turnover and CL regression. Although the study design does not permit identification of the mechanisms for improved fertility in heifers receiving G2P compared with controls, it is clear that a simplified version of the 5-d timed AI protocol, as implemented in control heifers, does not maximize P/AI. Based on results of study 1, it was unclear if benefits in P/AI in heifers were derived from the combined improved follicle turnover and luteolysis

or if just an improved luteolysis would be sufficient to improve P/AI. Therefore, study 2 was designed to investigate if a combined improved follicle turnover and luteolysis was necessary to maximize P/AI in dairy heifers.

Dairy cows that ovulate to the initial GnRH of the timed AI program have enhanced overall synchrony of the estrous cycle (Vasconcelos et al., 1999; Rutigliano et al., 2008), which shortens the period of follicle dominance and improves embryo quality (Cerri et al., 2009a). This follicle response is beneficial to fertility in dairy cows (Vasconcelos et al., 1999; Rutigliano et al., 2008; Santos et al., 2010a). For instance, the results of a previous study in dairy heifers suggested that follicle turnover was not as critical as it appears to be in dairy cows to improve fertility, when only one PGF_{2α} was used in the 5-d timed AI program (Lima et al., 2011). However, it is possible that the potential benefits of improved follicle turnover were offset by reduced luteolysis obtained with a single treatment with PGF_{2α}. In fact, the 5 d interval between GnRH and PGF_{2α} might limit adequate luteolysis when a newly formed CL is present.

The refractoriness of the early CL with fewer than 5 d of development to a single PGF_{2α} treatment has been reported (Rowson et al., 1972; Henricks et al. 1974), and one solution to overcome this problem is the use of multiple PGF_{2α} treatments (Santos et al., 2010a; Ribeiro et al., 2012b). Although multiple doses of PGF_{2α} have been shown to successfully induce luteolysis even in cows with CL having less than 5 d (Beal et al., 1980), the use of two PGF_{2α} injections did not always improve fertility (Cruppe et al., 2010; Rabaglino et al., 2010). One possibility to explain the lack of benefit to two doses of PGF_{2α} in the 5-d timed AI program is a poor ovulatory response to GnRH when the protocol is initiated, so only a small proportion of animals have newly formed CL. The

mechanism of refractoriness of the early CL to PGF_{2α} has not been elucidated completely. Tsai and Wiltbank (1998) suggested that early CL, in spite of having functional PGF_{2α} receptors, is incapable of inducing intra-luteal synthesis of PGF_{2α} via prostaglandin-endoperoxidase synthase 2, increasing expression of monocyte chemoattractant protein 1, and inhibiting progesterone production through StAR. Additionally, Miyamoto et al. (2009) suggested a site restricted action of PGF_{2α} depending on the stage of the estrous cycle. In the mid-cycle CL (d 8 to 12), PGF_{2α} induces an acute increase in blood flow in the periphery of the CL concurrent with expression of endothelial nitric oxide synthase, but the same phenomenon is not observed in the early-cycle CL (d 4). Moreover, Atli et al. (2012) reported that although the initial pulse of PGF_{2α} upregulates mRNA expression of many pathways related to luteolysis, the second and later pulses of PGF_{2α} are actually responsible for a distinct pattern of gene expression that results in luteolysis. Therefore, either multiple doses of PGF_{2α} are needed to fully regress the newly formed CL, or the fact that the second dose of PGF_{2α} was administered on d 6 after the GnRH might have allowed some CL to become more mature and responsive to the luteolytic effects of prostaglandin. It is important to indicate that when no GnRH was administered, as in control and NG2P, the second dose of PGF_{2α} did not improve P/AI despite the increase in luteolysis. These results reinforce the need to combine follicle turnover with adequate CL regression to optimize fertility in the 5-d timed AI protocol for dairy heifers.

Low ovulatory response to GnRH in dairy heifers has been demonstrated by others (Stevenson et al., 2008; Lima et al., 2011). One of the factors that might affect ovulation to GnRH is the diameter of the dominant follicle (Sartori et al., 2001), which is

known to be related to the expression of LH receptors on granulosa cells (Xu et al., 1995). Granulosa cells acquired LH receptors at the time of follicle deviation, approximately 3 d after the emergence of a follicular wave, when the dominant follicle achieves 8.5 mm in diameter. However, most heifers in previous studies had follicles of at least 10 mm in diameter when GnRH was administered (Martinez et al., 1999; Lima et al., 2011). Another possibility is that approximately 50% of the heifers have 3 waves of follicle development (Bisinotto and Santos, 2012), which limits the period of follicle dominance and the opportunity to have a dominant follicle responsive to a GnRH/LH surge to induce ovulation. Interestingly, dairy heifers without a CL had greater ovulation to GnRH than heifers with a CL in a previous study (Lima et al., 2011). This finding suggests that high progesterone concentration might be another impediment to ovulation, particularly when heifers are in mid luteal phase and receive a CIDR as in the 5-d timed AI protocol. The negative effects of progesterone on LH release in response to GnRH have been demonstrated in beef heifers, mature beef cows, and dairy cows (Colazo et al., 2008; Dias et al., 2010; Giordano et al., 2012). The results of the current study confirm the hypothesis of negative effects of progesterone on LH release and ovulation in dairy heifers treated with GnRH. Therefore, the present study clarifies that induction of follicle turnover with GnRH in dairy heifers subjected to the 5-d timed AI protocol is inhibited by elevated concentrations of progesterone, which attenuates LH release.

Conclusion

Increased follicle turnover at initiation of 5-d timed AI program by using GnRH combined with two doses of PGF_{2α} administered on d 5 and 6 to optimize luteolysis was a successful strategy to optimize P/AI in dairy heifers. Results of the current study

demonstrate similar concepts of previous work with lactating dairy cows reinforcing the need for synchronization protocols to incorporate physiological principles to optimize fertility in dairy heifers. Follicle turnover by inducing ovulation with GnRH, although low in dairy heifers, was beneficial to fertility. However, the benefit of GnRH to optimize fertility requires two doses of PGF_{2α} administered 24 h apart to increase regression of a newly formed CL. The P/AI of approximately 60% obtained in the current study supports the use of the 5-d timed AI protocol as an alternative breeding program for reproductive management of heifers when detection of estrus is not used. Finally, it was demonstrated that high concentrations of progesterone when GnRH was administered suppressed the LH release and impaired ovulation. Further research is needed to determine if additional increase in ovulation to the initial GnRH of the 5-d timed AI protocol can further improve fertility in dairy heifers.

Table 5-1. Effect of the initial GnRH and two doses of PGF_{2α}, on fertility responses of dairy heifers subjected to the 5-d timed AI program (Study 1)

	Treatment ¹		AOR (95% CI) ²	<i>P</i>
	Control	G2P		
	----- % (n/n) -----			
Estrus at AI ³	76.7 (429/559)	76.9 (421/547)	1.03 (0.77-1.38)	0.83
Pregnant				
Day 32	53.5 (299/559)	59.4 (325/547)	1.28 (1.01-1.63)	0.04
Day 60	51.3 (287/559)	56.6 (309/546)	1.24 (0.98-1.58)	0.07
Pregnancy loss ⁴	4.0 (12/299)	4.6 (15/324)	1.15 (0.53-2.52)	0.72

¹ Control = d 0 CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and timed AI. G2P = d 0 GnRH and CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 6 PGF_{2α}, d 8 GnRH and timed AI.

² AOR = adjusted odds ratio, CI = confidence interval. Control is the reference for comparison.

³ Evaluated based on removal of tail paint on the d of AI.

⁴ Calculated as the number of heifers that lost their pregnancies between d 32 and 60 after AI divided by the number of heifers pregnant on d 32. One pregnant heifer from G2P left the study before reconfirmation of pregnancy on d 60.

Table 5-2. Effect of the initial GnRH and two doses of PGF_{2α}, on ovarian responses in dairy heifers subjected to the 5-d timed AI program (Study 2)

	Treatment ¹			<i>P</i>
	Control	NG2P	G2P	
Study d 0	----- % (n/n) -----			
Follicle ≥ 10 mm	95.2 (200/210)	95.1 (194/204)	91.0 (190/209)	0.76
Presence of CL	81.0 (170/210)	82.3 (168/204)	78.0 (163/209)	0.83
Ovulation ²	13.8 (29/210) ^b	11.8 (24/204) ^b	26.3 (55/209) ^a	0.001
Study d 5				
Presence of CL	88.6 (186/210)	91.2 (186/204)	88.5 (185/209)	0.60
Presence of a new CL ³	21.9 (46/210) ^b	20.1 (41/204) ^b	34.4 (72/209) ^a	0.01
Progesterone on study d 8				
< 1.0 ng/mL ⁴	95.1 (196/206)	97.0 (192/198)	97.5 (197/202)	0.39
< 0.5 ng/mL	83.0 (171/206) ^b	92.9 (184/198) ^{ac}	87.1 (176/202) ^d	0.01
< 0.3 ng/mL	62.6 (129/206) ^{bd}	74.7 (148/198) ^a	70.8 (143/202) ^c	0.02

¹ Control = d 0 CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and AI; NG2P = d 0 CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 6 PGF_{2α}, d 8 GnRH and AI; G2P = d 0 GnRH and CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 6 PGF_{2α}, d 8 GnRH and AI.

² Proportion of heifers with a follicle ≥ 10 mm on d 0 and a new CL on d 5 in the same location.

³ Proportion of heifers with a new CL on d 5 independent of the presence of follicles ≥ 10 mm on d 0.

^{a,b} Different superscripts within the same row differ (*P* ≤ 0.05).

^{c,d} Different superscripts within the same row tend to differ (*P* = 0.07).

Table 5-3. Effect of the initial GnRH and two doses of PGF_{2α}, on fertility responses in dairy heifers subjected to the 5-d timed AI program (Study 2)

	Treatment ¹			<i>P</i>
	Control	NG2P	G2P	
	----- % (n/n) -----			
Estrus at AI ²	72.1 (513/711) ^b	74.3 (517/696) ^b	78.6 (559/711) ^a	0.01
Pregnant ³				
Day 32	52.8 (376/711) ^b	55.0 (383/696) ^b	61.7 (439/711) ^a	0.002
Day 60	48.9 (348/711) ^b	51.6 (359/696) ^b	59.1 (420/711) ^a	0.001
Pregnancy loss ⁴	7.4 (28/376) ^B	6.3 (24/383) ^{AB}	4.3 (19/711) ^A	0.15

¹ Control = d 0 CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 8 GnRH and AI; NG2P = d 0 CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 6 PGF_{2α}, d 8 GnRH and AI; G2P = d 0 GnRH and CIDR insertion, d 5 PGF_{2α} and removal of CIDR, d 6 PGF_{2α}, d 8 GnRH and AI.

² Evaluated based on removal of tail paint on the d of AI.

³ Twelve control heifers, 7 NG2P heifers, and 7 G2P heifers did not have a pregnancy diagnosis performed.

⁴ Calculated as the number of heifers that lost their pregnancies between d 32 and 60 after AI divided by the number of heifers pregnant on d 32.

^{a,b} Different superscripts within the same row differ ($P \leq 0.05$).

^{A,B} Different superscripts within the same row tended to differ ($P \leq 0.10$).

Table 5-4. Effects of concentration of progesterone in plasma on LH release and ovulation in response to GnRH in dairy heifers (Study 3)

	Treatment ¹		<i>P</i>
	LP	HP	
Progesterone, ng/mL ²	3.14 ± 0.88	7.35 ± 0.67	0.001
LH response to GnRH, ng/mL			
Mean	16.3 ± 2.3	10.1 ± 1.8	0.04
Peak	31.3 ± 2.3	19.2 ± 4.1	0.09
Minutes after GnRH to peak	81.4 ± 14.4	76.3 ± 11.1	0.78
Ovulation response to GnRH, % (n/n) ³	48.4 (15/31)	19.0 (12/63)	0.001

¹ LP = low progesterone heifers received a CIDR insert and two PGF_{2α} injections 12 h apart on d 7 after ovulation and were challenged with GnRH on d 8; HP = high progesterone heifers received a CIDR insert and were challenged with GnRH on d 8.

² Progesterone and LH concentrations were evaluated in 18 heifers (6 LP and 12 HP).

³ Ovulation was evaluated in 94 heifers.

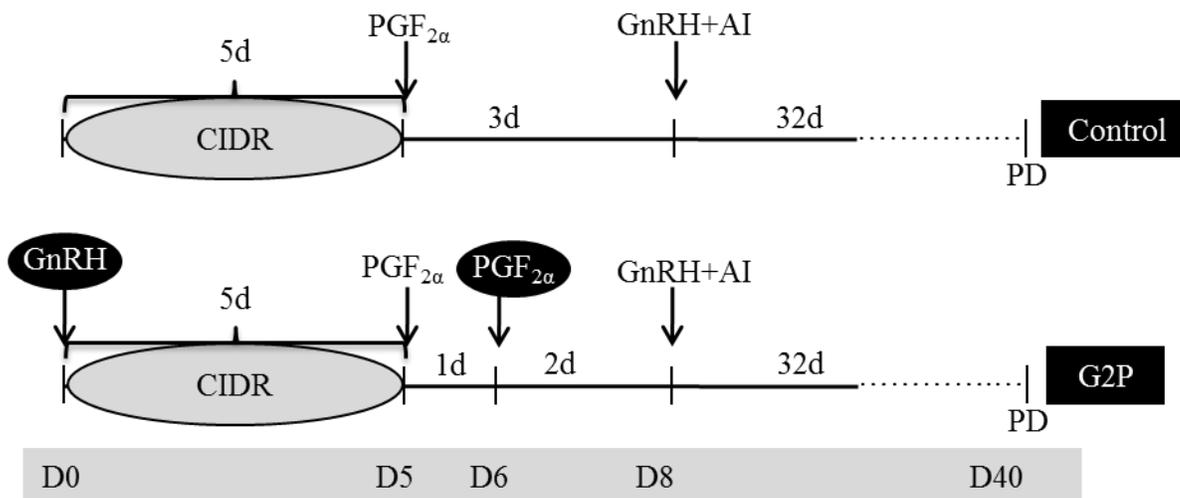


Figure 5-1. Diagram of activities in study 1. All heifers received a CIDR on d 0, an injection of PGF_{2α} and CIDR removal on d 5, and an injection of GnRH concurrently with AI on d 8. Control = no additional treatment (n = 559); G2P = additional injection of GnRH on d 0 and a second injection of PGF_{2α} on d 6 (n = 547). AI = artificial insemination; CIDR = controlled internal drug-release device containing 1.38 g of progesterone; PD = pregnancy diagnosis.

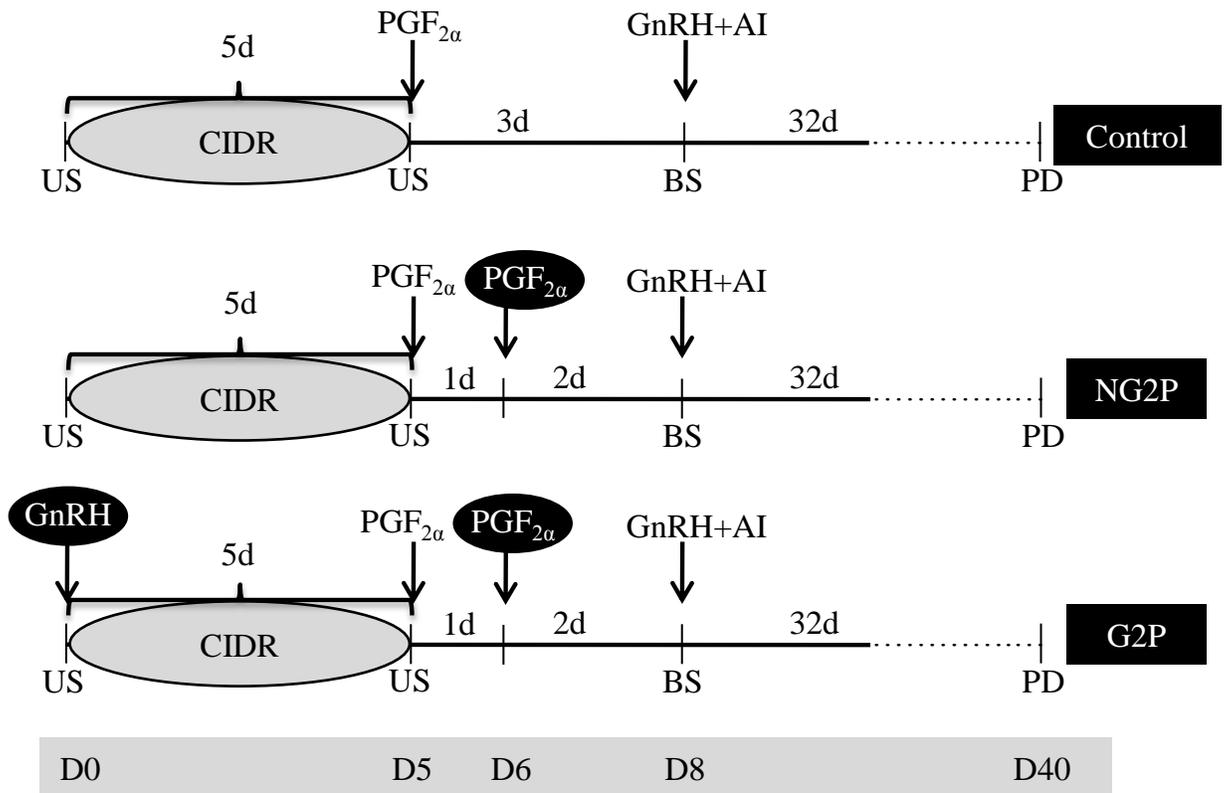


Figure 5-2. Diagram of activities in study 2. All heifers received a CIDR on d 0, an injection of PGF_{2α} and CIDR removal on d 5, and an injection of GnRH concurrently with timed AI on d 8. Control = no additional treatment (control = 711); NG2P = a second injection of PGF_{2α} on d 6 (n = 696); G2P = an injection of GnRH on d 0 and a second injection of PGF_{2α} on d 6 (n = 711). AI = artificial insemination; BS = blood sample for analysis of progesterone; CIDR = controlled internal drug-release device containing 1.38 g of progesterone; PD = pregnancy diagnosis.

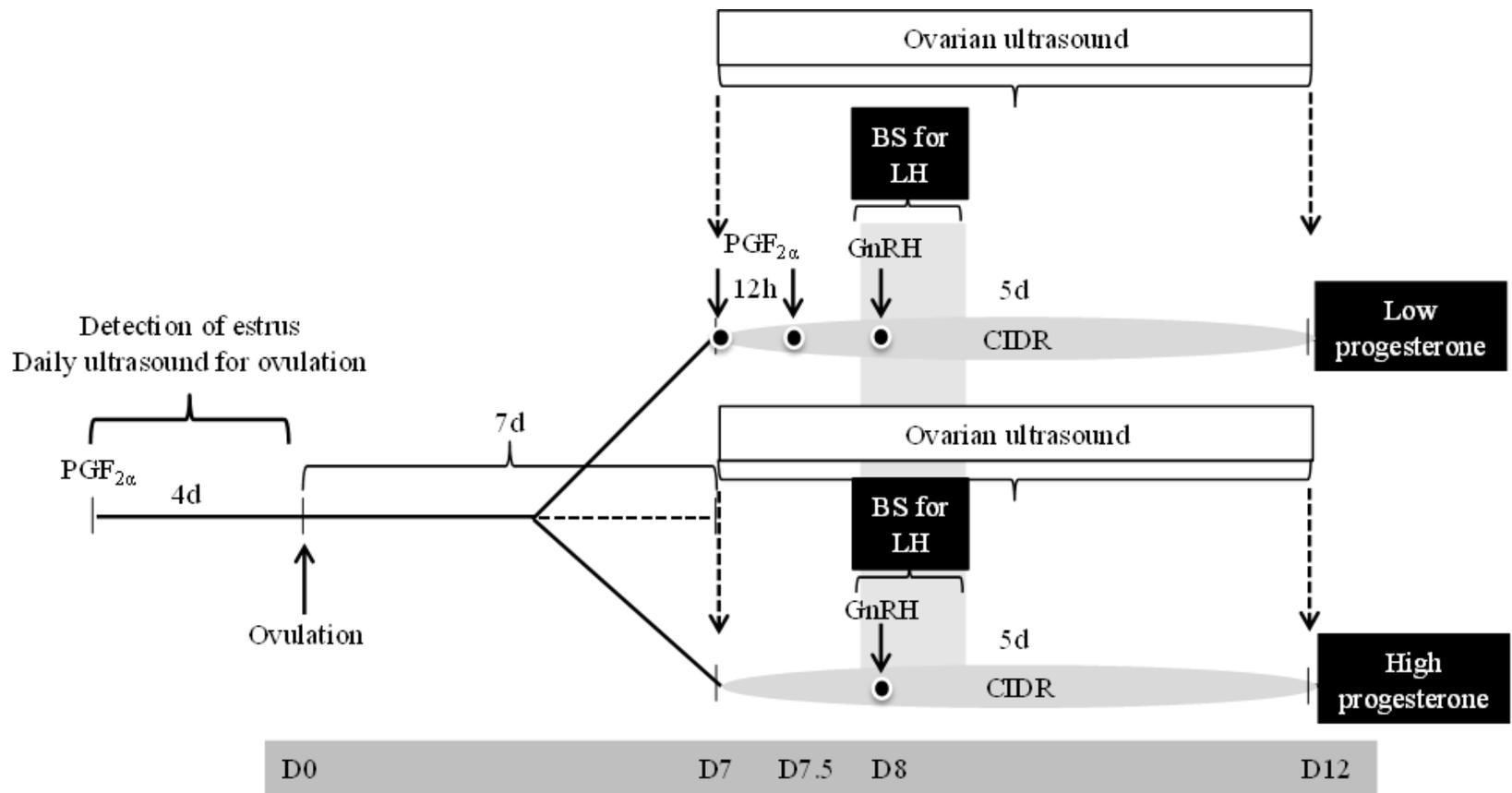


Figure 5-3. Diagram of activities in study 3. Heifers had their estrous cycle synchronized with PGF_{2α} and those in estrus were evaluated for ovulation (study d 0). Heifers received a CIDR insert on d 7 and were assigned to low progesterone (LP, n = 6) in which two injections of PGF_{2α} were administered 12 h apart on d 7.0 and 7.5, or high progesterone (HP, n = 12) in which no PGF_{2α} was administered. All heifers received 100 µg of GnRH on d 8 and blood was sampled every 15 min from -30 to 180 min and at 240 and 300 min relative to the GnRH injection for assessment of LH concentrations. BS = blood sample; CIDR = controlled internal drug-release containing 1.38 g of progesterone; GnRH = injection of 100 µg of gonadorelin hydrochloride; PGF_{2α} = injection of 25 mg of dinoprost as tromethamine salt.

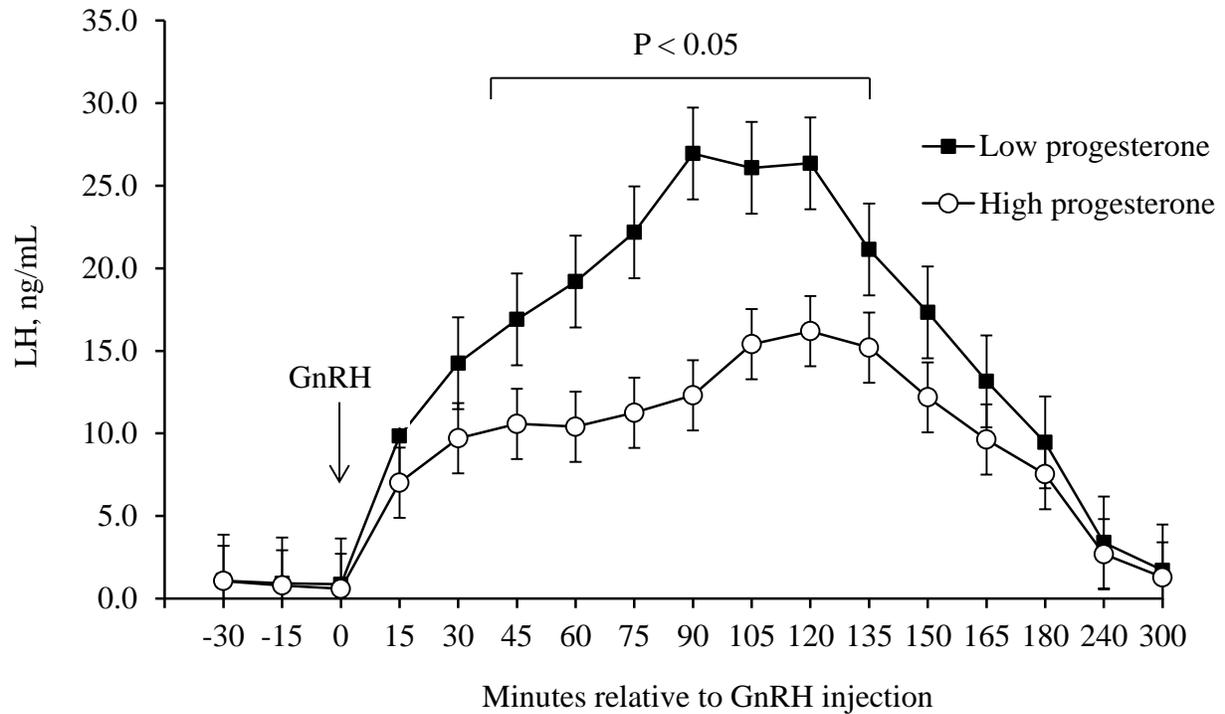


Figure 5-4. Effect of progesterone concentration on the LH release in response to GnRH. LH = luteinizing hormone; GnRH = injection of 100 µg of gonadorelin hydrochloride; LP (Low progesterone) = d 7 after ovulation CIDR and PGF_{2α}, d 7.5 after ovulation PGF_{2α}, d 8 after ovulation GnRH. HP (High progesterone) = d 7 after ovulation CIDR, d 8 after ovulation GnRH. Blood samples for LH were collected each 15 minutes from -30 before to 180 minutes after GnRH injection and at 4 and 5 hours after GnRH.

CHAPTER 6
EFFECTS OF ONE OR TWO TREATMENTS WITH PROSTAGLANDIN $F_{2\alpha}$ ON
SUBCLINICAL ENDOMETRITIS AND FERTILITY IN LACTATING DAIRY COWS
INSEMINATED BY TIMED AI

The objectives of the current study were to investigate the efficacy of $PGF_{2\alpha}$ as a therapy to reduce the prevalence of subclinical endometritis and improve pregnancy per artificial insemination (P/AI) in cows subjected to a timed AI program. A total of 1,342 lactating Holstein dairy cows were allocated randomly at 25 ± 3 d in milk (DIM) to remain as untreated controls (control, $n = 454$), receive a single $PGF_{2\alpha}$ treatment at 39 ± 3 DIM (1PGF, $n = 474$), or two treatments with $PGF_{2\alpha}$ at 25 ± 3 and 39 ± 3 DIM (2PGF, $n = 414$). All cows were enrolled in the double Ovsynch program at 48 ± 3 DIM, and were inseminated at 75 ± 3 DIM. A subset of 357 cows had uterine samples collected for cytological examination at 25 ± 3 , 32 ± 3 , and 46 ± 3 DIM to determine the percentage of polymorphonuclear leukocytes (PMNL). Subclinical endometritis was defined by the presence of $\geq 5\%$ of PMNL. Vaginal discharge score was evaluated at 25 ± 3 DIM and used to define the prevalence of purulent vaginal discharge. Body condition score was assessed at 25 ± 3 DIM. Pregnancy was diagnosed 32 d after AI and reconfirmed 28 d later. At 32 ± 3 DIM, the prevalence of subclinical endometritis was reduced by treatment with $PGF_{2\alpha}$ at 25 ± 3 DIM in 2PGF (control = 23.5% vs. 1PGF = 28.3% vs. 2PGF = 16.7%); however, this benefit disappeared at 46 ± 3 DIM and 14% of the cows remained with subclinical endometritis. One or two treatments with $PGF_{2\alpha}$ did not influence P/AI on d 32 or 60 after timed AI, and they averaged 39.9 and 35.2%. Similarly, treatment with $PGF_{2\alpha}$ had no effect on pregnancy loss between 32 and 60 d of gestation (11.9%). Cows diagnosed with both purulent vaginal discharge and subclinical endometritis had the lowest P/AI and the highest pregnancy loss compared

with those diagnosed with only one of the two diseases or with cows having no diagnosis of uterine diseases. Interestingly, subclinical endometritis depressed P/AI and increased pregnancy loss only when it persisted until 46 d in milk (DIM). On d 32 after AI, cows not diagnosed with subclinical endometritis and those that resolved subclinical endometritis by 46 DIM had greater P/AI than those that remained with subclinical endometritis at 46 DIM (45.4 vs. 40.0 vs. 25.0%). Similar to P/AI, cows not diagnosed with subclinical endometritis and those that resolved subclinical endometritis by 46 DIM had less pregnancy loss than those with subclinical endometritis at 46 DIM (9.6 vs. 13.5 vs. 43.9%). One or two treatments with PGF_{2α} before initiation of the timed AI program were unable to improve uterine health, P/AI, and maintenance of pregnancy in lactating dairy cows. Cows diagnosed with both purulent vaginal discharge and subclinical endometritis had the greatest depressions in measures of fertility at first AI, particularly when subclinical endometritis persisted in the early postpartum period.

Introductory Remarks

Uterine diseases are prevalent in dairy cows and they have been associated with reduced reproductive performance, which ultimately affects herd profitability (Gilbert et al., 2005; LeBlanc, 2008). Uterine diseases are often classified according to clinical presentation and defined based on their impacts on pregnancy per AI (**P/AI**) or time to pregnancy (Sheldon et al., 2006). Among them, clinical endometritis is defined as presence of inflammation in the reproductive tract visible by the type of vaginal discharge that typically contains pus and persists after 21 DIM (Leblanc et al., 2002a; Sheldon et al., 2006). More recently, clinical endometritis as diagnosed by presence of pus in the vagina was classified as purulent vaginal discharge (**PVD**) because of the

large proportion of cows without concurrent neutrophil infiltration in the endometrium (Dubuc et al., 2010). On the other hand, a large proportion of cows not diagnosed with any clinical signs of uterine disease have presence of inflammatory cells in the endometrium, and usually more than 5% PMNL in endometrial cytology reduce P/AI and extends the interval postpartum to pregnancy (Gilbert et al., 2005; Galvão et al., 2009a).

In the United States, no particular treatment is labeled for use in cows that have either PVD or subclinical endometritis, although use of intrauterine infusion of 500 mg of cephapirin as benzathine has demonstrated efficacy in reducing interval to pregnancy in cows with PVD (Leblanc et al., 2002b) or improving pregnancy at first AI in cows with subclinical endometritis (Kasimanickam et al., 2005). In those studies, cows were not subjected to standardized programs for first postpartum AI and many were inseminated following detection of estrus. When cows were subjected to a presynchronized timed AI program, use of intrauterine antibiotics did not benefit P/AI of dairy cows (Galvão et al., 2009b), even in those with previous diagnosis of PVD. An alternative therapy is the use of prostaglandin (PG) $F_{2\alpha}$ in an attempt to induce estrus and eliminate bacterial contamination that might be causing the inflammatory process in the endometrium. Use of $PGF_{2\alpha}$ in cows during diestrus results in luteolysis and induces cows to return to estrus, which has been suggested to enhance uterine immunity by removal of immunosuppressive effects of progesterone (Lewis, 2004). Kasimanickam et al. (2005) suggested that the improvements in P/AI caused by $PGF_{2\alpha}$ in postpartum cows were caused by inducing estrus and concurrent opening of the cervix and myometrium contractions that might enhance mechanical cleansing of the endometrium.

When PGF_{2α} is administered in early lactation, it is possible that the benefits to fertility might not be related to enhancing uterine health, but confounded with effects of presynchronizing the estrous cycle before timed AI programs (Moreira et al., 2001; Galvão et al., 2007). It is known that the stage of the estrous cycle when cows initiate timed AI protocols based on GnRH is critical for fertility (Vasconcelos et al., 1999), and treatment with PGF_{2α} 11 to 12 d before the initiation of the timed AI increased P/AI (Moreira et al., 2001; Galvão et al., 2007). In fact, in most studies evaluating PGF_{2α} as therapy for treatment of uterine diseases and subsequent impacts on fertility, uterine health was not evaluated after treatment to justify the increase in P/AI (Leblanc et al., 2002b; Kasimanickam et al., 2005). In some cases, when uterine health was evaluated after PGF_{2α} treatment, P/AI at first AI improved, but the benefits were not linked to a reduction in the prevalence of subclinical endometritis in treated cows (Galvão et al., 2009a).

Timed AI programs are commonly used for reproductive management of dairy herds for first and resynchronized inseminations to mitigate the negative impacts of poor estrous detection in lactating dairy cows (Caraviello et al., 2006). An alternative presynchronization treatment, in which stage of the estrous cycle is synchronized in cyclic and anovular cows, is called Double Ovsynch (Souza et al., 2008). When PGF_{2α} is administered before the Double Ovsynch protocol, the effects on uterine health or measures of fertility are not expected to be mediated by altering the stage of the estrous cycle when cows are subjected to the timed AI protocol. The goal of the current study was to demonstrate an improvement in P/AI in dairy cows with the systematic use of

PGF_{2α} by enhancing uterine health based on the reduction in the prevalence of subclinical endometritis.

The hypotheses of the current study was that treatment with PGF_{2α} would reduce the prevalence of subclinical endometritis and improve first-service P/AI in cows subjected to a presynchronized timed AI program. Therefore, the objectives were to investigate the efficacy of systematic use of one or two treatments with PGF_{2α} preceding a presynchronized timed AI protocol on the prevalence of subclinical endometritis and P/AI in lactating dairy cows.

Materials and Methods

The University of Florida Institute of Food and Agricultural Sciences Animal Research Committee approved all procedures in this study.

Animals, Housing, and Feeding

A total of 1,342 lactating Holstein cows from a commercial dairy farm located in north central Florida were used in this study. Cows enrolled in the study calved from August 2009 to July 2010. Cows were housed in free-stall barns equipped with fans and sprinklers for forced evaporative cooling. Cows from all treatments were kept together in the same pens throughout the entire period of the study. Lactating cow diets were formulated using the CPM-Dairy cattle ration analyzer (Cornell-Pen-Miner Ver. 3.0.8) to meet or exceed the nutrient requirements established by NRC (2001) for lactating Holstein cows weighing 650 kg, consuming 24 kg of DM, and producing 45 kg/d of milk containing 3.5% fat and 3.1% true protein during the first 80 d of lactation. The first insemination for cows in the study occurred between November 2009 and October 2010.

Reproductive Management

All cows in the study had ovulation synchronized for first postpartum AI with the double Ovsynch program starting on 48 ± 3 DIM as depicted in Figure 3-1 (Souza et al., 2008). A total of 4 technicians and 18 sires were distributed randomly for all treatments. At 32 d after the first postpartum timed AI cows were diagnosed for pregnancy by ultrasonographic examination of the uterus and its contents. 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 28 d later, at 60 d of gestation, to reconfirm pregnancy status and to identify pregnancy loss.

Treatments and Body Condition Scoring

Weekly cohorts of cows at 25 ± 3 DIM were blocked by parity and, within each block, allocated randomly to remain untreated (control, $n = 454$), or receive a single i.m. injection of 25 mg $\text{PGF}_{2\alpha}$ (dinoprost tromethamine; Lutalyse sterile solution, Zoetis, Madison, NJ, USA) treatment at 39 ± 3 DIM (1PGF, $n = 474$), or two treatments with $\text{PGF}_{2\alpha}$ at 25 ± 3 and 39 ± 3 DIM (2PGF, $n = 414$), as depicted in Figure 6-1.

The body condition of all cows was assessed at 25 ± 3 DIM using a 1 (emaciated) to 5 (obese) scale according to Ferguson et al. (1994) as depicted in the Elanco BCS chart (Elanco, 2009).

Evaluation of Uterine Health

Samples of vaginal discharge and uterine endometrial cytology were collected from a subset of 357 cows (control = 115; 1PGF = 125; and 2PGF = 117). All samples were collected by the investigators who were blinded to treatments. Vaginal discharge retrieved using the Metricheck (Metricheck, Simcro, New Zealand) at 25 ± 3 DIM was used as a criterion to determine PVD (Dubuc et al., 2010), formerly known and

classified as clinical endometritis (Sheldon et al., 2006). Briefly, vaginal discharge was scored as: 1 = clear or translucent mucus; 2 = mucus containing flecks of white or off-white pus; 3 = discharge containing 50% or less white or off-white mucopurulent material; 4 = discharge containing more than 50% purulent material, usually white or yellow; and 5 = bloody, purulent and fetid discharge. Cows with score > 2 were classified as having PVD.

Uterine cytology samples were collected on d 25 ± 3 , 32 ± 3 and 46 ± 3 postpartum using the cytobrush technique (Kasimanickam et al., 2005) with the stainless steel gun protected by a one-way plastic tube protector (Continental plastics, Delaval, WI). The DIM at sampling were selected to be able to evaluate the effects of treatments with $\text{PGF}_{2\alpha}$ before cows were enrolled in the Double Ovsynch protocol at 48 DIM. The evaluations on d 32 and 46 postpartum were to maintain the same interval of 7 d between each $\text{PGF}_{2\alpha}$ treatment and the endometrial cytology.

After collecting the endometrial cytology, the cytobrush was rolled onto a slide and air dried immediately. The slides were transported to the laboratory and stained using diff-quick stain kit (IMEB, San Marcos, CA). Three technicians not aware of treatments read the slides. Two-hundred cells were counted in each slide using a microscope at 400 x magnifications to determine the proportion of PMNL relative to the total of PMNL, mononuclear, and endometrial cells counted. Cows with a proportion of $\text{PMNL} \geq 5\%$ were classified as having subclinical endometritis (Gilbert et al., 2005).

Statistical Analysis

The sample size was calculated using Minitab 15 (Minitab Inc., State College, PA) for a two-tailed test ($\alpha = 0.05$; $\beta = 0.80$). It was assumed that subclinical

endometritis would affect 30% of the cows and that treatment with PGF_{2α} would reduce the prevalence of subclinical endometritis by 12 percentage units. Under those assumptions, 120 cows/treatment were needed to evaluate the effects of treatment on the prevalence of subclinical endometritis. For P/AI in all cows, the sample size was calculated based on an increase in at least 6 percentage units. Others have demonstrated that administration of PGF_{2α} to postpartum cows inseminated on estrus or following a combination of detected estrus and timed AI had increments in pregnancy at first postpartum AI of 12 to 15 percentage units (Kasimanickam et al., 2005; Galvão et al. 2009a). A maximum of 433 cows/treatment was calculated to allow for detection of statistical effect when the difference between treatments was of at least 6 percent units in P/AI.

Binary data such as prevalence of subclinical endometritis and P/AI were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC, USA) and fitting a binary distribution. The models included the effects of treatment (control vs. 1PGF vs. 2PGF), parity (primiparous vs. multiparous), BCS categorized as ≤ 2.75 or > 2.75 , and season of breeding classified as cool, when AI occurred from October 1 to May 14 or hot, when AI occurred from May 15 to September 30. For subclinical endometritis, the prevalence on d 25 postpartum was used as covariate. For P/AI, technician and sire were also included in the statistical models. Treatment was forced in the final models. Covariates and interactions between treatment and covariates were maintained in the statistical models if $P < 0.10$. Orthogonal contrasts were performed to determine the impact of PGF_{2α} (control vs. 1PGF + 2PGF), and number of PGF_{2α} treatments (1PGF vs. 2PGF).

Two additional multivariable analyses were performed with the subset of cows in which uterine health was evaluated to model P/AI and pregnancy loss. In the first model, cows were classified based on uterine health as no uterine disease, when no PVD or subclinical endometritis was diagnosed any time in the first 46 DIM, or as having PVD only, subclinical endometritis only, or both PVD and subclinical endometritis. A cow was considered positive for subclinical endometritis if it was present at least once in the evaluations at 25, 32, and 46 DIM. The models included the effects of treatment (control vs. 1PGF vs. 2PGF) and uterine health (no uterine disease vs. PVD only vs. subclinical endometritis only vs. PVD and subclinical endometritis). Orthogonal comparisons were performed to evaluate the effect of uterine health (no uterine disease vs. all others), the differential effect of PVD compared with subclinical endometritis (PVD only vs. subclinical endometritis only), and the additive effect of PVD and subclinical endometritis (PVD only + subclinical endometritis only vs. both PVD and subclinical endometritis). In the second model, cows were classified only based on subclinical endometritis as never being diagnosed with subclinical endometritis, having resolved subclinical endometritis when diagnosed on d 25 and/or 32, but negative on d 46 postpartum, and those that persisted with subclinical endometritis based on diagnosis on d 46 postpartum. The models for P/AI and pregnancy loss included the effects of treatment (control vs. 1PGF vs. 2PGF) and subclinical endometritis (no subclinical endometritis vs. subclinical endometritis that resolved by d 46 vs. subclinical endometritis on d 46). Orthogonal comparisons were performed to evaluate the effect of subclinical endometritis (no subclinical endometritis vs. resolved + persistent), and the effect of persistent subclinical endometritis (resolved vs. persistent).

Adjusted proportions for binary data were generated by back-transforming the estimates using the *ilink* function of SAS. Proportions are displayed for binary data, whereas LSM and SEM are displayed for continuous data. Differences with $P \leq 0.05$ were considered significant, whereas those with $0.05 < P \leq 0.10$ were considered tendency to differ.

Results

Cows in the three treatments had similar lactation number (2.93 ± 0.05), DIM at enrolment in the study (24.7 ± 0.05), DIM at AI (81.0 ± 0.2), vaginal discharge score on d 25 postpartum (2.08 ± 0.06), and percentage of PMNL in endometrial cytology on d 25 postpartum (6.98 ± 0.67); however, BCS at enrollment was greater ($P = 0.01$) for control and 2PGF than 1PGF cows (control = 2.96 ± 0.02 vs. 1PGF = 2.91 ± 0.02 vs. 2PGF = 2.98 ± 0.02).

Effects of $\text{PGF}_{2\alpha}$ Treatments on the Prevalence of Subclinical Endometritis

The prevalence of PVD on d 25 postpartum tended ($P = 0.10$) to be greater for cows in the 2PGF than those receiving 1PGF, whereas control cows had intermediate prevalence that did not differ from the other two groups (Figure 6-2). Nevertheless, the prevalence of subclinical endometritis in cows on d 25 postpartum, before treatments were applied, did not differ among treatments and averaged 29.5%. Treatment with $\text{PGF}_{2\alpha}$ at 25 ± 3 DIM in 2PGF reduced ($P < 0.05$) the prevalence of subclinical endometritis on d 32 postpartum compared with control and 1PGF cows (Figure 6-2). However, there was no difference in the prevalence of subclinical endometritis at 46 DIM. Cows with PVD on d 25 postpartum had greater ($P < 0.001$) prevalence of subclinical endometritis than those without PVD on d 25 (47.3 vs. 17.8%) and 32 (40.3 vs. 16.8%) postpartum, but the same association was not observed on d 46 postpartum

(PVD = 17.4 vs. no PVD = 12.1%; $P = 0.20$). Additionally, no association was observed between parity, BCS and season of AI with the prevalence of subclinical endometritis.

Effects of PGF_{2α} on Pregnancy per AI and Pregnancy Loss

Treatments with 1PGF or 2PGF failed to increase P/AI on d 32 and 60 after insemination in cows bred exclusively after subjected to the double Ovsynch timed AI program (Table 6-1). Overall, 39.9 and 35.2% of the cows were pregnant on d 32 and 60 after AI, respectively. Pregnancy loss between 32 and 60 d after AI affected 11.9% of the pregnant cows and treatment with either 1PGF or 2PGF had no influence on maintenance of pregnancy in the first 60 d of gestation.

Parity and season of AI affected ($P < 0.01$) P/AI on d 32 and 60 after timed insemination. Primiparous cows had greater ($P = 0.01$) P/AI than multiparous cows on d 32 (42.5 vs. 35.0%) and 60 after AI (37.1 vs. 30.5%). Cows inseminated during the cool season had greater ($P < 0.001$) P/AI than those inseminated during the hot season on d 32 (47.1 vs. 30.8%) and 60 after insemination (41.7 vs. 26.6%). None of the other covariates evaluated influenced pregnancy loss between 32 and 60 d of gestation.

Associations Among PVD and/or Subclinical Endometritis with Measures of Fertility

The negative impacts of uterine diseases on P/AI and maintenance of pregnancy were only observed when cows were diagnosed with both PVD and subclinical endometritis (Table 6-2). On d 32 and 60 after insemination, cows not diagnosed with uterine diseases had greater ($P < 0.05$) P/AI than those diagnosed with both PVD and subclinical endometritis. However, P/AI did not differ statistically among cows not diagnosed with uterine diseases and those diagnosed with only PVD or subclinical endometritis. Interestingly, cows diagnosed with both diseases had lower ($P = 0.03$)

P/AI on d 60 than those diagnosed with only either PVD or subclinical endometritis. Similar to P/AI, pregnancy loss increased ($P = 0.05$) only when cows were diagnosed with both PVD and subclinical endometritis.

Subclinical endometritis depressed P/AI and increased pregnancy loss in dairy cows, but these negative effects were only observed when the disease persisted until 46 DIM (Table 6-3). Of the 50 cows categorized as persistent subclinical endometritis (diagnosis at 46 DIM), 19 had the first diagnosis with $\geq 5\%$ PMNL at 46 DIM, and the remaining 31 had a previous diagnosis. Cows with no diagnosis of subclinical endometritis had similar P/AI on d 32 or 60 after insemination compared with cows diagnosed with subclinical endometritis that resolved by 46 DIM. Nevertheless, those cows in which subclinical endometritis persisted until 46 DIM, immediately before enrollment on the Double Ovsynch protocol, tended ($P = 0.08$) to have lower P/AI on d 32 and had lower P/AI ($P = 0.01$) on d 60 after insemination because of greater ($P = 0.04$) pregnancy loss than cows that resolved subclinical endometritis by 46 DIM.

Discussion

Treatments with one or two doses of PGF_{2 α} before enrollment in a timed AI protocol had minor impacts on the prevalence of subclinical endometritis and did not improve P/AI or reduce pregnancy loss in lactating Holstein cows. The design of the current experiment allowed the evaluation of the effects of 1 or 2 doses of PGF_{2 α} on measures of fertility in dairy cows while excluding the confounding effect of presynchronization of the estrous cycle when cows are subjected to timed AI protocols (Moreira et al., 2001; Galvão et al., 2007), or inducing earlier insemination because of estrus. The current study was designed to evaluate the effects of systematic use of PGF_{2 α} on the prevalence of subclinical endometritis and P/AI, but it was not our aim to

assess PGF_{2α} as a direct therapy for cows with PVD or only those diagnosed with subclinical endometritis.

The strategic use of PGF_{2α} early postpartum, when PVD and subclinical endometritis are prevalent (Gilbert et al., 2005), was initially thought to reduce the prevalence of subclinical endometritis, which could improve P/AI in cows inseminated exclusively by timed AI. The suggested mechanism of PGF_{2α} action in cyclic cows involves induction of luteolysis and return to estrus leading to opening of the cervix and myometrium contractions that might improve mechanical cleansing of the uterus by eliminating bacteria and the products that attract PMNL. However, PGF_{2α} may have effects on the uterus beyond induction of luteolysis and estrus in cyclic cows. In human uterine tissue *in vitro*, PGF_{2α} has been shown to induce myometrium contractions (Senior et al., 1992). Ulug et al. (2001) demonstrated that PGF_{2α} stimulated the release of pro-matrix metalloproteinase-2 and pro-matrix metalloproteinase-9 from uterine tissue explants that are involved with breakdown of extracellular matrix mainly by degrading collagen type IV, which might aid myometrium contraction and uterine involution independent of cyclic status. Administration of exogenous PGF_{2α} early postpartum promoted uterine involution in cows (Lindell and Kindahl, 1983). In the current study, reduction in the prevalence of subclinical endometritis only occurred at 32 DIM in cows receiving PGF_{2α} on d 25 postpartum, when the proportion of estrous cyclic cows and luteolytic response to PGF_{2α} are typically low (Galvão et al., 2010). The positive effect of PGF_{2α} on subclinical endometritis was no longer apparent by 46 DIM, probably because of the observed high spontaneous cure documented in control cows. In fact, a single treatment with PGF_{2α} at 39 DIM resulted in no benefit in reducing the prevalence of

subclinical endometritis compared with control cows. Therefore, the initial benefits of PGF_{2α} on uterine health of earlier postpartum cows were offset by spontaneous resolution of uterine inflammatory process 2 weeks later. Interestingly, PGF_{2α} as administered was unable to reduce the prevalence of subclinical endometritis at 46 DIM, which was observed to have marked effects on P/AI and maintenance of pregnancy at first postpartum AI. One of the potential limitations of the study is that estrous cyclic status and presence of CL on the days when PGF_{2α} was administered was unknown, so it is possible that a high prevalence of anovular cows would have limited the benefit of PGF_{2α} on improving uterine health through induction of luteolysis. Nevertheless, Dubuc et al. (2011) observed that administration of PGF_{2α} on d 35 and 49 postpartum did not improve P/AI at first service or reduced time to pregnancy in estrous cyclic cows.

The effects of PGF_{2α} on uterine health and fertility have been evaluated by others in cows subjected to varying reproductive management at first AI (Kasimanickam et al., 2005; Galvão et al., 2009a; Dubuc et al., 2011). In agreement with our findings, administration of PGF_{2α} given 14 d apart with the last treatment at 49 DIM (Galvão et al. 2009a; Dubuc et al. 2011) did not reduce the prevalence of subclinical endometritis in dairy cows.

Kasimanickam et al. (2005) observed that cows with subclinical endometritis treated with PGF_{2α} had increased P/AI at first AI and pregnancy rate compared with untreated controls. The benefits of PGF_{2α} on fertility were similar between treatment with PGF_{2α} or with intrauterine administration of cephalosporin suggesting an effect on the uterine microbiota and improved uterine health, although these responses were not verified. The lack of reduction in the prevalence of subclinical endometritis at 46 DIM

with PGF_{2α} treatments supports the fact that PGF_{2α} did not improve P/AI or reduce pregnancy loss in cows bred exclusively by timed AI. The double Ovsynch timed AI protocol presynchronizes the estrous cycle of dairy cows (Ribeiro et al., 2012c), thereby eliminating a potential effect of prior presynchronization with PGF_{2α} at improving response to the timed AI program. On the other hand, one cannot completely exclude the possibility that, by imposing the double Ovsynch protocol for first AI, the benefits of PGF_{2α} might have been reduced as cows receive additional hormonal interventions for synchronization of ovulation that might have effects on the uterus through induction sequential estruses and ovulations.

Cows with subclinical endometritis had reduced P/AI which corroborates with previous reports (Kasimanickam et al., 2005; Galvão et al., 2009a; Dubuc et al., 2011) that identified a negative association between PVD and/or subclinical endometritis and fertility. In the current study cows that persisted with subclinical endometritis at 46 DIM had a remarkable reduction in P/AI in comparison with healthy cows or cows that resolved subclinical endometritis by 46 DIM. It is clear that the prevalence of subclinical endometritis decreases with day postpartum, but those that persisted until enrollment in the timed AI protocol suffered reductions in P/AI and had increased risk of pregnancy loss. It is unknown if the prevalence of subclinical endometritis reduced even further after 46 DIM. With the exception of Galvão et al. (2009a) that observed a tendency for increased pregnancy loss in cows with PVD, none of the previous studies reported an association between PVD or subclinical endometritis and increased risk of pregnancy loss (Kasimanickam et al., 2005; Gilbert et al., 2005; Dubuc et al., 2011). In some studies, pregnancy was evaluated only once, so data for pregnancy loss were not

available (Kasimanickam et al., 2005; Gilbert et al., 2005). It is unclear the exact mechanism by which subclinical endometritis decreases P/AI and increases pregnancy loss. Products of endometrial inflammation compromised early embryo development *in vitro* (Hill and Gilbert, 2008). Soto et al. (2003) suggested that mediators of the inflammatory cascade, including cytokines can impair early embryo development and might be part of the mechanism by which fertility is depressed in cows suffering from inflammatory diseases in early lactation.

There is mounting evidence that cows with subclinical endometritis have altered embryo quality and endometrial function. Inflammation in the endometrium has been shown to reduce fertilization in single ovulating postpartum dairy cows (Cerri et al., 2009c). Dairy cows with no detectable PMNL in endometrial cytology had increased number of transferable embryos when subjected to superstimulation compared with cows with presence of PMNL in endometrial cytology (Drillich et al., 2012). Cows diagnosed with subclinical endometritis have altered endometrial and embryonic gene expression that might explain the reduced fertility (Hoelker et al., 2012). Endometrium from cows diagnosed with subclinical endometritis had an altered pattern of expression of genes involved in cell adhesion and immune modulation, which was then linked to changes in d 7 embryo gene expression. The changes in endometrial gene expression might be induced by altered number of immune cells present in the tissue. Nevertheless, embryos from cows with subclinical endometritis had altered pattern of gene expression involving pathways in cell cycle and apoptosis, which might explain a reduction in P/AI or even increased risk of pregnancy loss (Hoelker et al., 2012). Whether subclinical endometritis per se is the causative agent of changes in

endometrial and embryonic gene expression or that cows that develop subclinical endometritis have underlying factors that also cause changes in the transcriptome remain to be elucidated.

It is noteworthy that the combination of PVD and subclinical endometritis led to additive negative effects on P/AI and pregnancy loss in dairy cows when compared with PVD and subclinical endometritis alone. Dubuc et al. (2011) reported a decline in first service P/AI in cows suffering from both PVD and subclinical endometritis when compared with cows diagnosed with only subclinical endometritis. In the same study, cows with both PVD and subclinical endometritis had longer interval to pregnancy compared with counterparts diagnosed with only one of the two problems. Similar to our findings, Dubuc et al. (2011) also showed that cows that persist with uterine disease before the end of the voluntary waiting period are those that suffer the greatest negative consequences to interval to pregnancy. In the current study, it was observed that 14% of the cows had subclinical endometritis by 46 DIM, and these cows suffered the most losses in fertility at first postpartum AI. Similar to subclinical endometritis, Dubuc et al. (2011) observed that PVD can also persist in some cows. According to their data, 42% of the cows diagnosed with PVD and subclinical endometritis on d 35 postpartum persisted with PVD at 56 DIM. The persistence of PVD was not evaluated in the current study. The mechanisms by which some cows are unable to eliminate inflammation from the uterus are not completely elucidated; however, previous studies suggest that endometrial inflammation is regulated by immune response rather than pathogen load (Herath et al., 2009). It is possible that cows with inadequate immune function are those with longer duration of the endometrial inflammatory process that compromises fertility.

Conclusions

Treatment with one or two injections of PGF_{2α} in early lactation, before cows were subjected to a presynchronized timed AI protocol, was unable to improve uterine health and measures of fertility in lactating dairy cows. Subclinical endometritis impaired P/AI and maintenance of pregnancy in lactating dairy cows, particularly when associated with PVD, and the negative effect of subclinical endometritis was observed when the inflammatory process persisted until 46 DIM. Interestingly, when both PVD and subclinical endometritis were associated or when subclinical endometritis persisted by 46 DIM, pregnancy loss increased.

Table 6-1. Effect of one or two treatments of PGF_{2α} on pregnancy per AI and pregnancy loss of dairy cows subjected to a timed AI program

Item	Treatment ¹			P ²		
	Control	1PGF	2PGF	TRT	C1	C2
Pregnant % (n/n)					
d 32	38.1 (173/454)	40.7 (193/474)	41.1 (170/414)	0.58	0.32	0.72
d 60	33.7 (153/454)	36.7 (174/474)	35.0 (145/414)	0.70	0.43	0.78
Loss	11.6 (20/173)	9.8 (19/193)	14.7 (25/170)	0.36	0.87	0.16

¹ Control = no treatment with PGF_{2α} before enrollment in the timed AI protocol; 1PGF = a single treatment with PGF_{2α} on d 39 postpartum; 2PGF = treatment with PGF_{2α} on d 25 and 39 postpartum.

² TRT = effect of treatment; C1 = contrast for the effect of treatment with PGF_{2α} (control vs. 1PGF + 2PGF); C2 = contrast for the effect of number of treatments with PGF_{2α} (1PGF vs. 2PGF).

Table 6-2. Association between purulent vaginal discharge (PVD) and/or subclinical endometritis (SCE) with fertility of dairy cows at first postpartum insemination

	Category ¹				Contrasts ²		
	No disease	PVD only	SCE only	PVD and SCE	C1	C2	C3
Cows, n	156	22	105	74	---	---	---
Pregnant, %							
d 32	48.0 ^a	49.1 ^{a,b}	39.8 ^{a,b}	33.4 ^b	0.21	0.43	0.17
d 60	43.3 ^a	44.9 ^a	34.0 ^A	22.8 ^{b, B}	0.08	0.34	0.03
Loss, %	9.1 ^b	7.1 ^b	13.7 ^B	30.3 ^{a, A}	0.33	0.52	0.05

^{a,b,c} Different superscripts within a row differ ($P < 0.05$).

^{A,B} Different superscripts within a row tend to differ ($P < 0.10$).

¹ No disease = no diagnosis of PVD or SCE; PVD = vaginal discharge score > 2 on d 25 ± 3 postpartum. SCE = cows with uterine cytology containing $\geq 5\%$ PMNL in one or more of the days in which diagnosis was performed (25 or 32 or 46 ± 3 d postpartum).

² C1 = effect of uterine disease (no uterine disease vs. PVD only + SCE only + PVD and SCE); C2 = effect of PVD compared with SCE (PVD only vs. SCE only); C3 = additive effect of PVD and SCE (PVD only + SCE only vs. PVD and SCE).

Table 6-3. Association between subclinical endometritis (SCE) with fertility of dairy cows at first postpartum insemination postpartum insemination

	Category ¹			Contrasts ²	
	No SCE	Resolved SCE	Persistent SCE	C1	C2
Cows, n	178	129	50		
Pregnant, %					
d 32	45.4	40.0	25.0	0.03	0.08
d 60	40.5	34.3	13.7	< 0.01	0.01
Loss, %	9.6	13.5	43.9	0.03	0.04

¹Diagnosis of subclinical endometritis was based on uterine cytology containing $\geq 5\%$ PMNL. Resolved SCE = cows diagnosed with SCE on d 25 and/or 32 postpartum, but negative on d 46 postpartum. Persistent SCE = cows with presence of SCE on d 46 postpartum.

² C1 = effect of subclinical endometritis (No SCE vs. Resolved SCE + Persistent SCE); C2 = Effect of persistent SCE (Resolved SCE vs. Persistent SCE).

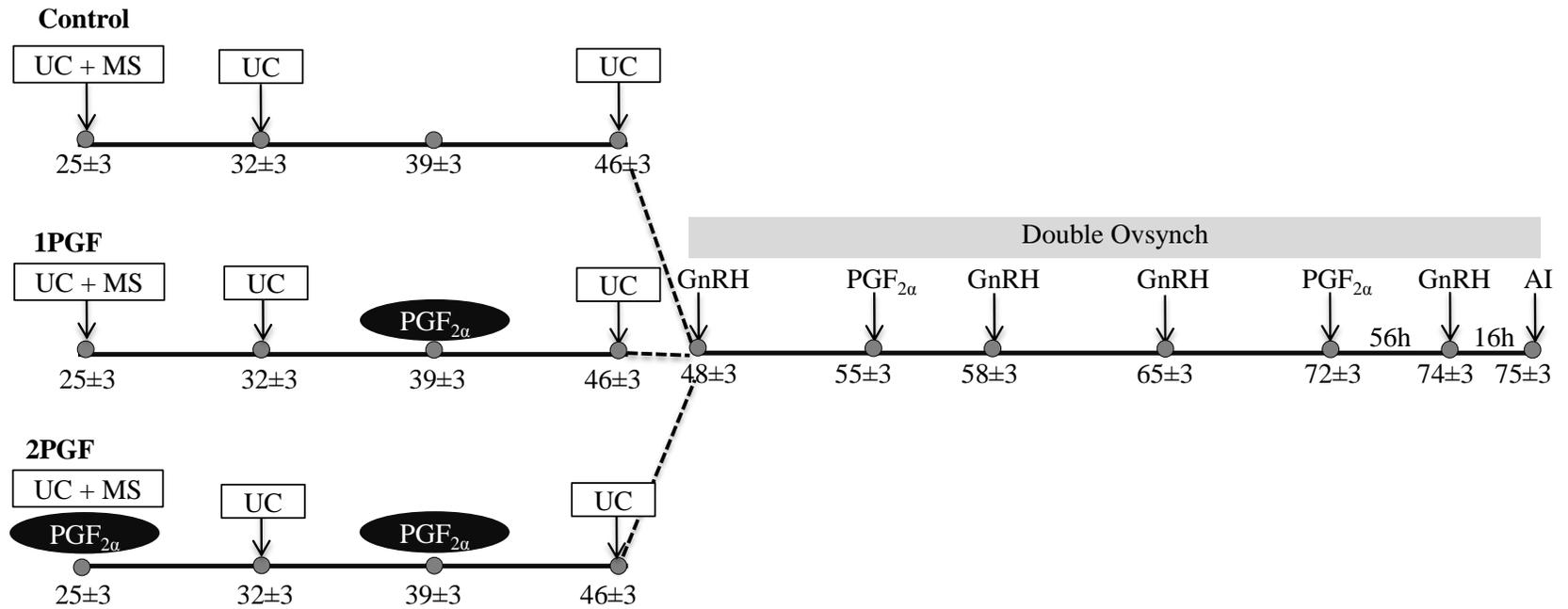


Figure 6-1. Diagram of treatments according to days in milk (± 3). Treatments were control, with no administration of PGF_{2α}, 1PGF in which cows received a single injection of PGF_{2α} on d 39 postpartum, and 2PGF in which cows received an injection of PGF_{2α} on d 25 and another on d 39 postpartum. All cows were inseminated at fixed time following the double Ovsynch protocol. MS = mucus score; UC = uterine cytology.

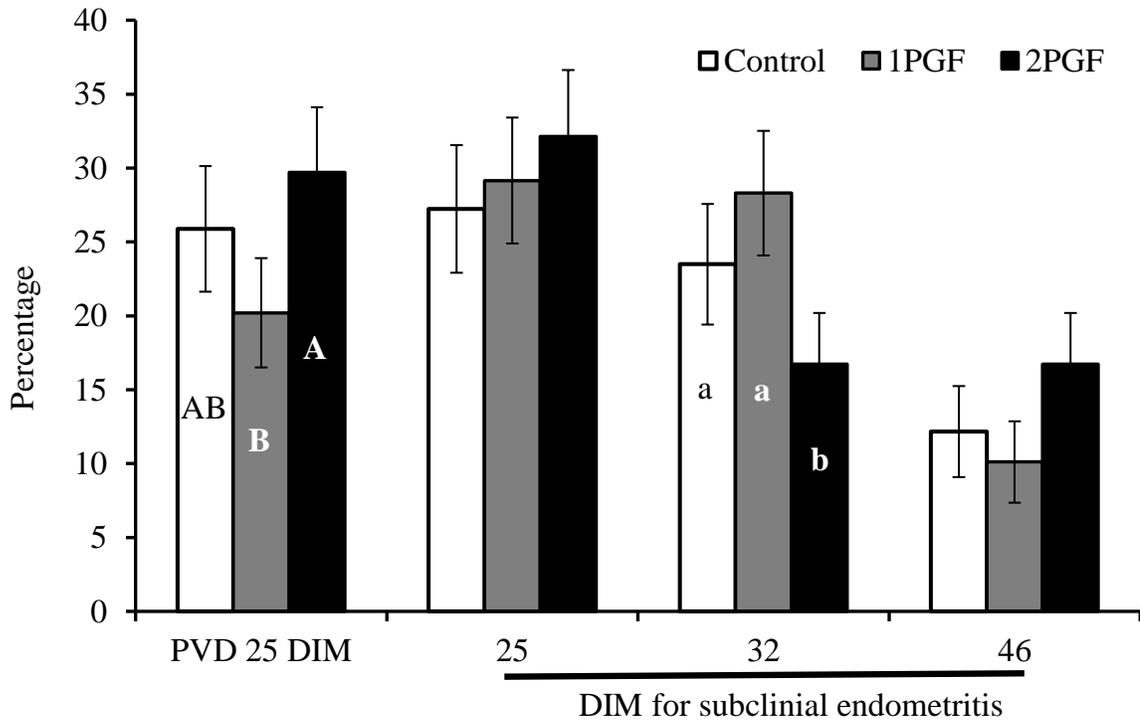


Figure 6-2. Effect of one or two treatments of $\text{PGF}_{2\alpha}$ on the prevalence of subclinical endometritis on d 32 and 46 postpartum in dairy cows. The analysis included the effect of one or two treatments of $\text{PGF}_{2\alpha}$ on the prevalence of subclinical endometritis on d 32 and 46 postpartum in dairy cows. Control = no treatment with $\text{PGF}_{2\alpha}$ before enrollment in the timed AI protocol; 1PGF = a single treatment with $\text{PGF}_{2\alpha}$ on d 39 postpartum; 2PGF = treatment with $\text{PGF}_{2\alpha}$ on d 25 and 39 postpartum. Purulent vaginal discharge (PVD) and subclinical endometritis on d 25 postpartum were the baseline prevalence at enrollment in the study. For subclinical endometritis on d 32 and 46, the analysis included the effects of treatment ($P = 0.98$), day postpartum ($P = 0.001$), and interaction between treatment and day postpartum ($P = 0.04$). ^{a,b} different superscripts among treatments denote statistical difference ($P < 0.05$). ^{A,B} different superscripts among treatments tended to differ ($P < 0.10$).

CHAPTER 7
EFFECTS OF INTRAUTERINE INFUSION OF *TRUEPERELLA* PYOGENES ON
ENDOMETRIAL mRNA EXPRESSION OF GENES ASSOCIATED WITH LUTEOLYSIS
AND CORPUS LUTEUM LIFESPAN IN DAIRY COWS

Objectives were to determine the effects of intrauterine infusion of *Trueperella pyogenes* on endometrial expression of genes affecting luteolysis and luteal lifespan. Estrous cycles were synchronized on 32 healthy Holstein cows. On d 4 after ovulation cows were allocated randomly to receive one of three treatments: TP (n=13), intrauterine infusion of 10 mL of saline solution containing 10^9 CFU/mL of *T. pyogenes*; TNF (n=9), intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control (n=10), intrauterine infusion of 10 mL of saline solution. Five cows per treatment had uterine biopsies collected at 6, 12 and 24 h after treatment to evaluate the endometrial expression of TNF α , interleukin 1 β (IL1B), IL6, IL8, prostaglandin E synthase (PGES), prostaglandin F synthase (PGFS) and oxytocin receptor (OXR). The remaining cows had ovaries scanned and blood samples collected for progesterone evaluation. Real time quantitative PCR (RT-PCR) was used to measure gene expression. Expressions of IL6, TNF α , PGES, PGFS and OXR genes were not affected by treatment, time or their interaction. Interleukin 1B gene expression was not different for treatment and time, but there was an interaction between treatment and time. Cows receiving TP had increased expression of IL1B than TNF cows at 24 h. Moreover, IL6 expression tended to be greater for TP than control at 12 h. Oxytocin receptor gene expression tended to be greater for TP and TNF than for control cows at 12 h. No difference in mean concentration of progesterone and CL size occurred among treatments. Although no differences in mean time for luteal lifespan were detected, the percentage of cows with early demise of the CL was greater for TP cows than control

(42.9% vs. 0). In conclusion, although intrauterine infusion of *T. pyogenes* led to early demise of CL in greater percentage of cows, the differences on endometrial expression of genes associated with inflammation and luteolysis were minor.

Introductory Remarks

Uterine diseases affect nearly half of the dairy cows after parturition leading to disruption of uterine and ovarian function which frequently results in hindered fertility, increased involuntary culling and remarkable economic losses for dairy producers (Sheldon et al., 2009). The economic losses caused by metritis alone are striking and it has been calculated at \$380 per affected cow due to reduced milk production, delayed conception, treatment and increased culling (Drillich et al., 2001). Thus, if we consider a conservative incidence rate of 20% for the 8.5 million dairy cows in US the annual cost of metritis alone is \$646 million, which does not include endometritis another presentation of uterine diseases with remarkable detrimental effects on fertility (Dubuc et al., 2011). Therefore, understanding the mechanism by which microbes subvert host innate immunity disrupting ovarian and uterine function is fundamental to developing preventatives to mitigate the negative impacts of uterine diseases.

Trueperella pyogenes is considered one of the most relevant pathogens involved in uterine diseases, especially endometritis. This is due to its relatively high prevalence in the environment, persistence in the uterus, severity of lesions on the endometrium, resistance to treatment, and synergistic action with gram-negative anaerobes (Ruder et al., 1981; Huszenicza et al., 1999; Mateus et al., 2002a, b; Williams et al., 2005,). However, the mechanism by which *T. pyogenes* affects the endometrium and reproductive events in dairy cows such as length of the estrous cycle and concentration

of ovarian steroids remain elusive (Williams et al., 2007; Kaneko and Kawakami, 2009; Kaneko et al., 2013).

Several studies in recent years have reported that intrauterine (IU) infusion of live *T. pyogenes* disrupts luteal development leading to early demise of the CL and ovulation of dominant follicle of first follicular wave (Kaneko and Kawakami, 2007; Kaneko and Kawakami, 2007; Kaneko et al., 2013). Cows receiving an intrauterine infusion of *T. pyogenes* on d 3 after ovulation had a peak of prostaglandin F metabolite (PGFM) 3 d later, followed by the regression of the newly formed CL and ovulation of the dominant follicle of the first follicular wave in approximately 50% of the time (Kaneko and Kawakami, 2008; Kaneko and Kawakami, 2009; Kaneko et al., 2013).

The mechanism by which *T. pyogenes* disrupts normal luteal function leading to short cycles is still unclear. Culture of endometrial cells with a bacteria free filtrate of *T. pyogenes* induces synthesis of PGF_{2α} (Miller et al., 2007). This bacterium possesses a number of virulence factors that may contribute to its pathogenic potential. One of the most important is a cholesterol-dependent cytolysin, pyolysin, which is a haemolysin cytolytic for macrophages (Jost and Bilington, 2005). A second important virulence factor is peptidoglycan, which is a pathogen associated molecular pattern that induces pro-inflammatory cytokines such as tumor necrosis factor α, interleukin 1β and interleukin 6 (Timmerman, et al., 1993; Stewart et al., 2003; Bromfield and Sheldon, 2011) that can stimulate endometrial synthesis of PGF_{2α} (Davidson et al., 1995; Hansen et al., 2004; Skarzynski et al., 2000). However, a possible stimulation of inflammatory mediators and its direct relationship with luteolytic cascade factors has never been investigated with an *in vivo* model of intrauterine induced infection with *T. pyogenes*.

Therefore, a study investigating the possible molecular mechanisms by which intrauterine infusion with live *T. pyogenes* leads to shortening of luteal phase in dairy cows is still needed. Our hypothesis is that intrauterine inoculation of live *T. pyogenes* in cows with a newly formed corpus luteum would increase endometrial expression of genes affecting the inflammation and the luteolytic cascade leading to an acute endometrial production of PGF_{2α} and early demise of the newly formed CL. The objectives of this study were to determine the effects of intrauterine infusion of *T. pyogenes* in dairy cows with newly formed CL on endometrial mRNA expression of genes affecting the luteolytic cascade, plasmatic concentration of progesterone, PGFM and CL lifespan

Materials and Methods

The University of Florida Institutional Animal Care and Use Committee approved the use of all animals procedures conducted in this study.

Animals, Housing, and Diets

The study was conducted between November of 2011 and March of 2012 in the University of Florida Dairy Unit (Hague, FL). Thirty-two lactating Holstein cows were enrolled in the study in two experiments. Cows were housed in freestall barns with sand-bedded stalls equipped with sprinklers and fans for forced evaporative cooling and ventilation. Cows were fed twice daily, immediately after the morning milking at 0830 h and again at 1230 h. Diets were mixed twice daily as base mixture containing corn silage, alfalfa hay and a base-concentrate mix, and the additional grain supplement. This base mixture contained 54% forage and was designed to meet the nutrient needs of a 650-kg cow consuming 23 kg of diet DM and producing 40.0 kg of milk with 3.5% fat and 3.0% true protein (CPM-Dairy ver. 3.0.10 software; www.cpm dairy.net).

Study Design and Treatments

At 21 ± 3 day postpartum, Holstein cows free of calving related disorders (dystocia, stillbirth, twins, retained placenta or metritis) had their estrous cycle synchronized with 100 μg of GnRH i.m. (2 mL Cystorelin®, Merial Ltd., Duluth, GA) followed 7 d later by one injection of PGF_{2 α} (25 mg of dinoprost tromethamine, Lutalyse®, Zoetis Animal Health, Madison, NJ) at 28 ± 3 days postpartum. Two days later, at day 30 ± 3 postpartum, a second dose of GnRH was given to induce ovulation, as depicted in Figure 4-1. Ovarian structures were scanned at days 21 ± 3 , 28 ± 3 , 30 ± 3 and 32 ± 3 to determine follicle turnover, luteolysis and ovulation.

Additionally, at day 30 ± 3 postpartum all cows had two uterine cytology samples collected for evaluation of subclinical endometritis and bacterial culture growth using the cytobrush technique as previously described with minor modifications (Kasimanickam et al., 2004). Briefly, for sample collection the vulva disinfected with alcohol and iodine wipes and dried with paper towel before having the cytology tool passed through the cervix. The autoclaved cytology tool was protected with a disposable sheath protector (Continental Plastic Corp., Delavan, WI) during vaginal transit. The first uterine cytology sample collected was placed directly into a transport media vial (BD Diagnostic Systems, Sparks, MD, USA) for safe transportation of the biological specimen from the farm to the microbiology laboratory of the Department of Animal Sciences where samples were streaked onto a blood agar plate and cultured aerobically for 48 hours. The culture plates were evaluated for microbial growth 48 hours later and questionable results were submitted to the College of Veterinary Medicine Diagnostic Laboratory at University of Florida for further evaluation. For the second uterine sample, the cytobrush was directly smeared onto the slide for cytologic evaluation of subclinical endometritis.

Briefly, the percentage of polymorphnuclear leukocytes (PMNL) was determined after a total of 200 cells were counted with the microscope using 400 times magnification and the presence of $\geq 10\%$ of PMNL was the criterion to determine occurrence of subclinical endometritis at this stage(Kasimanickam et al., 2004).

The criteria for inclusion of cows in the study were occurrence of ovulation in response to GnRH given on day 30 ± 3 postpartum, negative culture for microbial growth and $< 10\%$ PMNL in the uterine cytology examination.

The 32 eligible cows were allocated randomly (divided into experiment 1 and experiment 2.) to receive one of three treatments on day 4 after ovulation (35 ± 3 day postpartum): TP (n=13), intrauterine infusion of 10 mL of sterile saline solution containing 10^9 colony forming unit (CFU)/mL of *T. pyogenes*; TNF (n=9), intrauterine infusion of 10 mL sterile of saline solution containing 1 μg of tumor necrosis factor α (TNF α); and control (n=10), intrauterine infusion of 10 mL of saline solution. The *T. pyogenes* used in this study was isolated from a cow diagnosed with clinical metritis at the Dairy Unit, confirmed by PCR and the bacteria cultivated and prepared in batches of 10^9 CFU by the College of Veterinary Medicine Diagnostic Laboratory at University of Florida for intrauterine infusion.

The treatment TNF was added to provide a positive control to the study. The cytokine TNF α at a dose of 1 μg has been reported as a potent luteolytic agent capable of shortening the length of the estrous cycle when administered intravenously (Skarzynski et al., 2003b; Skarzynski et al., 2009). At the current study the treatment were given intrauterine to have a consistent site of application for all treatments

Experiment 1

A subset of 5 cows per treatment was enrolled at experiment 1 to collect endometrial sample for evaluation of mRNA expression of genes associated with the luteolytic cascade and to characterize tissue inflammation post intrauterine infusions in all the treatments. A sample for biopsy was not collected for all cows because we aimed to avoid any possible effects of the intervention and the potential inflammation generated for it on luteal lifespan and concentrations of progesterone and PGFM

Uterine Tissue Sample for Biopsy

Uterine tissue was collect by an uterine forceps instrument passed through the cervix and positioned in the mid portion of the uterine horn ipsilateral to the CL. The instrument was pressed against the uterine wall and a sample weighing between 100 and 250 mg was collected at 6, 12 and 24 hours after treatments administered on day 4 post ovulation for all cows. Two set of samples were collected at hour 6 post intrauterine infusion of treatments. The first set of samples at hour 6 and the samples collected at 12 and 24 hours post intrauterine infusion of treatments were immediately placed in a cryovial and snap-frozen in liquid nitrogen until arrival at that laboratory, when samples were stored at -80°C until RNA extraction and PCR analysis. The frozen samples were later evaluated to measure the gene expression of pro-inflammatory cytokines IL1B, IL6 and TNF α , the chemokine IL8 and the factors oxytocin receptor (OXR), prostaglandin E synthase (PGES) and prostaglandin F synthase (PGFS) gene expression.

The second set of samples collected at hour 6 post intrauterine infusions was immediately placed in a solution of 4% of paraformaldehyde (Sigma-Aldrich, Saint Louis, MO) and samples were fixed in paraffin and later sectioned at 4 μ m of thickness using an automatic rotary microtome. The sliced samples were stained with hematoxylin

and eosin (HE) stain to evaluate the development of inflammation on uterine tissue after intrauterine infusions. A modified scheme of grading was used (Snider et al.,2011). Briefly, grade 1 was characterized by normal endometrium or minimum mild focal inflammation or fibrosis. Grade 2 was characterized mild (2 A) to moderate (2 B) inflammation and or multifocal fibrosis with 1 to 4 layers of fibroblasts surrounding the fibrotic nest. Grade 3 was characterized as severe inflammation and/or diffuse fibrosis with 5 or more fibrotic nests per 5 mm linear field with or without occurrence of severe periglandular fibrosis and ectasia with disruption of gland epithelium.

RNA Extraction and Quantitative, Real-Time, Reverse Transcription PCR

The RNA from uterine tissues was extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) according to instructions provided by the manufacturer. Samples were purified (PureLink RNA Mini Kit, Invitrogen, Carlsbad, CA) and RNA concentrations were determined using NanoDrop Spectrophotometer 200 (Thermo Scientific, Rockford, IL).

Abundance of mRNA for IL1B, IL6, IL8, TNF α , PGES, PGFS and OXR were determined by qRT-PCR from uterine biopsy tissues collected 6, 12 and 24 hours after inoculation with *T. pyogenes*. RNA samples (50 ng/reaction, $A_{260/280} \geq 1.8$) were treated with RNase-free DNase I (Applied Biosystems, Foster City, CA) for 15 min at 37 °C, heat denatured (75 °C for 10 min), and then reverse transcribed using High Capacity cDNA Reverse Transcription Kit and random hexamers (Applied Biosystems, Foster City, CA). Primers specific for the selected transcripts were chosen and designed using basic local alignment search tool (BLAST, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and made on demand by Invitrogen (Invitrogen, Carlsbad, CA), as shown in Table 7-1.

Quantitative, reverse transcription PCR was completed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and the ABI 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). Amplification of each gene was obtained by running a mixture of 2.5 μ L of the cDNA product of each primer and 22.5 μ L SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The following cycling conditions were applied: initial activation/denaturation (60 °C for 2 min; 95 °C for 10 min); 40 cycles of 2 steps amplification protocol (95 °C for 15 s and 60 °C for 1 min) and for dissociation (55 to 95 °C). Each PCR was performed in triplicate, and the specificity of amplification was verified by melting curve analysis. A reaction lacking reverse transcriptase was included to verify the absence of genomic DNA contamination in reactions. Mitochondrial ribosomal protein S15 (*MRPS15*) was chosen as housekeeping gene because it was reported as the most stably expressed gene in uterine tissue (Wathes et al., 2009).

Experiment 2

For remaining 17 cows enrolled in the study, blood samples were collected and ovaries were scanned daily to evaluate plasmatic concentration of progesterone and luteal lifespan, respectively. Three (one for each treatment) of the 17 cows enrolled in the second experiment did not complete the study and were excluded from final analysis because two cows acquired severe mastitis and one cow suffered a severe leg injury. Therefore, the data presented in this section was from 14 cows with 3, 4 and 7 cows for control, TNF and TP treatments, respectively.

Blood Sampling, Analysis of Progesterone and PGFM Concentrations, and Ultrasound Scanning

Blood samples were collected from the coccygeal artery or vein using plasma K₂ EDTA vacutainer tubes daily starting at 35±3 days postpartum until 22 d after ovulation or two days after luteolysis was detected for evaluation of progesterone concentration on plasma. Samples were placed immediately on ice and kept refrigerated until arrival to the laboratory. Blood tubes were centrifuged at 2,000 x g for 15 min, and an aliquot of 2 mL of plasma was frozen at -20 °C until analysis. Concentration of progesterone in plasma was analyzed in all samples by RIA using a commercial kit (Coat-a-Count, Siemens Healthcare Diagnostics, Los Angeles, CA). The sensitivity of the assay was 0.05 ng/mL calculated at 2 SD below the mean counts per min at maximum binding. All samples were analyzed in a single assay. Two plasma samples with progesterone concentrations of 1.5 and 2.5 ng/mL were included throughout the sequence of samples in the assay for quality control. The intra-assay CVs were 3.9 and 6.7% for the samples containing 1.5 and 2.5 ng/mL, respectively.

Additional blood samples were collected from 48 to 120 hours each 12 hours intervals after intrauterine infusion with treatments to measure plasmatic concentration of PGFM following the procedures described above for progesterone. This time frame for collection of samples for PGFM was chosen to match the window on which recent studies identified a peak of PGFM as response to intrauterine infusion with *T. pyogenes* (Kaneko and Kawakami, 2008; Kaneko and Kawakami, 2008; Kaneko et al., 2013). The concentrations of PGFM were measured by an enzyme immunoassay as described previously (Ginther et al., 2010). The intra- and inter-assay CV was 4.59% and 5.92%, respectively.

Ovaries were scanned daily using a portable ultrasound equipped with a 5.0 MHz transrectal probe (Aloka SSD-500, Aloka Co. Ltd., Wallingford, CT) starting at 35±3 days postpartum until 22 d after ovulation or two days after luteolysis to monitor ovarian structures dynamics and to determine CL lifespan.

Statistical Analysis

All responses were analyzed using GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC). The models for all analyzes included the effects of treatment (control vs. TNF vs. TP), time and the interaction treatment by time. Continuous data were analyzed with models fitting a Gaussian or a Logarithmic distribution, and residuals were tested for normality. For repeated measures, models included the effects of time, interaction between treatment and time, and the random effect of cow nested within treatment. For endometrial mRNA expression a comparative method developed by Livak and Schmittgen, (2001) was applied to generate the data for presentation and samples from control cows at hour 6 post intrauterine infusions were used as reference for comparison. Relative expression values were obtained by raising the PCR amplification values to the power of delta-delta threshold cycle ($\Delta\Delta CT$) obtained from ΔCT least square mean differences of pairwise comparisons among treatment and reference groups (Yuan et al., 2006). The ΔCT values subjected to statistical analysis were generated by normalization of CT values from target genes with the mean of CT value from reference gene according to Vandesompele et al., (2002). Confidence interval for graphical representation of relative expression were generated from the lower and upper confidence limits obtained for ΔCT least square mean differences as described by Yuan et al., (2006).

Statistical significance was declared when P -value < 0.05 and tendency for P -value < 0.10 . Interactions were considered if P -value < 0.15 .

Results

Experiment 1

An interaction between treatment and time ($P = 0.05$) for IL1B (Figure 7-2). Cows treated with TP had an increased ($P = 0.03$) endometrial mRNA expression of IL1B in comparison with cows treated with TNF at 24 h post intrauterine infusions. The endometrial mRNA expression of IL6 (Figure 7-3) was not affected by treatment ($P = 0.57$), time ($P = 0.19$) or the interaction between treatment by time ($P = 0.34$). Nonetheless, mRNA expression of IL6 at hour 12 post intrauterine tended to be greater for TP cows ($P = 0.06$) than for control cows. The mRNA expression for the chemokine IL8 was not different among treatments ($P = 0.32$), time ($P = 0.16$) or interaction treatment by time ($P = 0.37$) as shown in Figure 5-4. For TNF once again no differences in mRNA expression were identified among treatments ($P = 0.80$), time ($P = 0.59$) or the interaction treatment by time ($P = 0.73$) as depicted in Figure 7-5. Likewise, the mRNA expression for PGES was not altered by treatments ($P = 0.11$) or time ($P = 0.68$), as shown in Figure 7-6. Additionally, there was not an interaction between treatment and time ($P = 0.46$) for mRNA expression of PGES. Moreover, endometrial mRNA expression for PGFS, the other luteolytic cascade transcript evaluated, did not differ among treatments ($P = 0.27$) and time ($P = 0.47$) as shown in Figure 7-7. Additionally, there was not an interaction between treatment and time ($P = 0.99$) for endometrial mRNA expression of PGFS. The endometrial mRNA expression OXR was not different among treatments ($P = 0.12$) or time ($P = 0.22$) as shown in Figure 7-8. Additionally, there was not an interaction between treatment and time ($P = 0.98$) for mRNA expression of OXR. However, mRNA expression

of OXR tended to be increased for TP ($P = 0.09$) and TNF ($P = 0.05$) in comparison with control cows at hour 12 post intrauterine infusions.

A histological evaluation of HE slides of uterine tissues was conducted revealed that all cows infused with *T. pyogenes* and TNF α had moderate (grade 2 B) to severe inflammation (grade 3) with transmigration of neutrophils into the uterine glands, separation of the epithelium from the stratum compactum and ectasia (dilation) of uterine glands with disruption of gland epithelium as shown in Figure 7-9B, 7-9C and 7-9D. On the other hand, only 1 of the 5 cows in control treatment had inflammation of the endometrium (grade 3) while the others were grade 1 Figure 7-9A.

Experiment 2

The criterion to determine occurrence of luteal regression was reduction on plasmatic concentration of progesterone below 1.0 ng/mL. The criterion to determine early luteolysis was reduction of progesterone concentration below 1.0 ng/mL before day 14 of the estrous cycle. The mean day of the estrous cycle for luteal regression were 17.3, 16.2 and 14.0 days for control, TNF and TP, respectively, and there were not different among treatments ($P = 0.38$). Although, no differences were observed among treatments for mean day of the estrous cycle for luteal regression the percentage of cows with early luteolysis was increased ($P = 0.001$) for TP cows in comparison with control and TNF and control being 42.9 %, 25.0 % and 0 % for control, TNF and TP treatments, respectively. A total of 3 cows infused with *T. pyogenes* had an earlier than usual luteolysis at days 10, 11 and 12 of the estrous cycle. Additionally, one cow from the TNF group also had an earlier than usual luteolysis at day 12 of estrous cycle. Moreover, one cow infused with *T. pyogenes* did not present luteolysis until day 22 of the estrous cycle. The mean concentration of progesterone were 3.84, 3.19 and 2.39

ng/mL for control, TNF and TP treatment, respectively, with no differences among treatments detected ($P = 0.33$). Additionally, no interaction between treatment and time were identified ($P = 0.60$) as shown in Figure 7-10. However, at day 12 of the estrous cycle the concentration of progesterone tended to decreased for TNF and TP treatments compared to the control treatment (Figure 7-10). The peak of plasmatic concentrations of progesterone were 9.59, 6.54 and 6.24 ng/mL for control, TNF and TP treatment, respectively, and no differences among treatments were observed ($P = 0.31$). Likewise, the day of the estrous cycle to achieve the peak of progesterone concentration was no different among treatments ($P = 0.85$) being 12.7, 12.2 and 11.3 days for control, TNF and TP treatments, respectively. Similarly, the size of corpus luteum also did not differ among treatments ($P = 0.22$). However an interaction between treatment and size of CL was present ($P < 0.01$), as depicted in Figure 7-11. The size of CL was increasing for control at days 16 and 17 of the estrous cycle, whereas TNF and TP had the CL reducing in size at the same days (Figure 7-11). In fact, CL size on days 16 and 17 of the estrous cycle was greater ($P < 0.05$) for control cows than for TNF and TP counterparts. At days 18 and 19 of the estrous cycle the size of the CL was smaller ($P < 0.05$) for TP cows than for TNF and control cows (Figure 7-11).

The mean concentration of PGFM was not different among treatments ($P = 0.66$) and no differences of time ($P = 0.58$) or an interaction treatment by time were found ($P = 0.19$) as shown in Figure 7-12.

Discussion

The current study was designed to test the hypothesis that uterine infusion with *T. pyogenes* could lead to acute inflammation eliciting the luteolytic cascade and early demise of the corpus luteum. *T. pyogenes* is one the most important bacterium

associated with uterine diseases (Sheldon et al. 2006), and previous studies with intrauterine infusion of *T. pyogenes* on d 3 after ovulation induced a peak of PGFM 3 d later, followed by the regression of the newly formed CL in approximately 50% of the cows (Kaneko and Kawakami, 2008, Kaneko and Kawakami, 2009, Kaneko et al., 2013). Although no difference in mean time of luteal regression occurred in this study, our data supports, although not following the same pattern, the findings of previous studies with three of seven cows infused with *T. pyogenes* having an early demise of the CL. Thus, it is reasonable to suggest that *T. pyogenes* disrupts luteal function and reduces lifespan of corpus luteum in at least part of the cows. It is unknown the reason why some cows respond with an early demise of CL, whereas some have normal estrous cycle lengths, and some have extended luteal cycles. Considering that only clinically healthy cows were included in the study and the exposure to the pathogen load was the same it is reasonable to speculate that individual immune competence and the individual subsequent host-pathogen interaction might be playing a role in the regulation of how cows respond to the infection of *T. pyogenes*. *In vitro* culture of endometrial cells in a bacteria free filtrate from *T. pyogenes* was able to induce synthesis of PGF_{2α} (Miller et al., 2007); however, it is not clear which molecular mechanism the *T. pyogenes* elicits to induce release of PGF_{2α}. Some evidence in the literature suggest that uterine inflammation induced by PAMPS molecules such as the peptidoglycan present in cell wall of *T. pyogenes* can induce release of pro-inflammatory cytokines such as TNF-α and IL-1β (Stewart et al., 2003; Timmerman, et al., 1993). These cytokines have been shown to be capable of directly stimulating

endometrial synthesis of PGF_{2α} (Davidson et al., 1995; Hansen et al., 2004; Skarzynski et al., 2000).

In the current study, cows received uterine infusion of *T. pyogenes* and uterine samples for biopsy were collected at critical times to evaluate this potential interplay between major cytokines shown to be altered by *T. pyogenes*, and critical factors of the luteolytic cascade that could potentially be altered by these mediators of the inflammatory response. Additionally, a group of cows received intrauterine infusion with a luteolytic dosage of TNF-α (1 µg) to attempt to have a positive control on the induction of luteolysis (Skarzynski et al., 2009). The histological evaluation at 6 h after intrauterine infusion clearly showed that an acute and severe inflammation was present in the uterine tissue of cows infused with *T. pyogenes* and TNF-α whereas control cows had normal to mild inflammation as a result of intrauterine infusion of sterile saline solution.

However, the evaluation of gene expression of pro-inflammatory cytokines, chemokines and luteolytic factors did not consistently support our hypothesis that inflammation was directly provoking stimulation of luteolytic cascade factors. Some minor differences in gene expression occurred in path expected with increased IL1B expression in cows infused with *T. pyogenes* when compared to TNF counterparts at hour 24 post intrauterine infusion; and a tendency for increased expression of IL6 in TP treatment in comparison with control counterparts at hour 12 post intrauterine infusions. Additionally, expression of *OXR* tended to be increased for TP and TNF treatments in comparison with control treatment at hour 12 post intrauterine infusions. The other inflammatory mediators and luteolytic factors were not affected by uterine infusion with *T. pyogenes*.

Given the degree of inflammation observed in cows that were infused with *T. pyogenes* and TNF- α , it was surprising not to observe a significant difference in the chemokine IL8, which is a bonafide neutrophil chemoattractant. Although no statistical differences were identified for *IL8*, the expression for cows infused with *T. pyogenes* was 4 to 8 times greater than in control cows suggesting that the lack of difference may be an artifact of the limitations of this study such as high variability in the individual response to *T. pyogenes*. Additionally, there is the possibility that the sample collected had other cells such as fibroblast, endothelial and muscle that may hamper the chance of identifying differences in expression of endometrial and immune cells such as previously demonstrated on *in vitro* studies (Jost and Bilington, 2005; Miller et al., 2007; Bromfield and Sheldon, 2011).

Hitherto remain elusive how *T. pyogenes* major virulence factor pyolysin elicit immune response, but evidence from some other similar pore-forming toxins (α -, β - and γ -hemolysin) suggest that pyolysin might activate the cytosolic nod like receptor NLRP3 inflammasome that activates caspase-1 that leads to cleavage of pro-inflammatory IL1 β and IL18 and subsequent release of these cytokines (Muñoz-Planillo et al., 2009; Embry et al. 2011). Therefore, it is reasonable to speculate that *T. pyogenes* potentially can induce similar mechanism, which could explain the molecular basis of induced migration of neutrophils after intrauterine infusion with *T. pyogenes* documented in Figure 7-9.

Uterine gene expression of *PGES*, *PGFS* and *OXR*, some of the major factors associated with the occurrence of luteolysis, were not remarkably altered by intrauterine infusion with *T. pyogenes* nor by intrauterine infusion of a luteolytic dosage of TNF- α . Although some minor effects on *OXR* expression were induced by TP and TNF, we

failed to observe the interplay between inflammation and luteolysis that we anticipated. Considering that some numerical trends also followed the path expected, we cannot exclude the possibility that the limitations of this study might have jeopardized the likelihood of identifying the expected response. Another unexpected outcome was that intrauterine infusion of luteolytic dosage of TNF- α was unable to influence uterine expression of *PGES* and *PGFS*, major luteolytic cascades factors known to be altered by this dose of TNF- α . These results may indicate that the uterus maybe resilient and does not allow enough amounts of TNF- α to reach the endometrial cells, blood stream and corpus luteum cells to mimic the effect of an intravenous a luteolytic dosages of TNF- α .

Collectively, the results of the current study indicate that the inflammation induced by *T. pyogenes* does not consistently alter mRNA expression of major molecules of the inflammatory responses and key factors of the luteolytic cascade. It suggests that an alternative factors not investigated under the scope of this study might be involved in the induction or early demise of CL. Additionally, there is the possibility that the limitations of the study hindered the ability to clearly show the anticipated response. On the other hand, the results of the current study support previous findings that intrauterine infusion of *T. pyogenes* may disrupt luteal function leading to early demise of the newly formed corpus luteum. Future studies should consider the investigation of the interplay of virulence factors of *T. pyogenes* with inflammation and luteolysis; and perhaps elaborate a model on which cows that responded to *T. pyogenes* with early demise of corpus luteum are compared to cows not responding to

T. pyogenes and normal cows to determine which factor might be regulating this different response

Conclusion

Intrauterine infusion with *T. pyogenes* disrupts luteal function and lead to early demise of CL at least in part of the cows. Although, *T. pyogenes* clearly induced inflammation we were unable to identify a consistent response in endometrial mRNA expression of mediators of the inflammation and luteolytic factors that could support a direct anticipated interplay between the bacterium-induced inflammation and factors triggering the early demise of the corpus luteum

Table 7-1. Primer reference and sequences for genes investigated by quantitative real-time PCR

Target gene*	NCBI Sequence	Primer	Primer sequence
<i>MRPS15</i>	NM_001192201.1	Forward	5' -AGATGACCCGCCCCCTTCCA-3'
		Reverse	5' -GGGAGCTGGTGTCTTCGGGT-3'
<i>TNFα</i>	NM_173966.2	Forward	5' -CCAGAGGGAAGAGTCCCCAG-3'
		Reverse	5' -TCGGCTACAACGTGGGCTAC-3'
<i>IL1B</i>	NM_174093.1	Forward	5' -ATTCTCTCCAGCCAACCTTCATT-3'
		Reverse	5' -TTCTCGTCACTGTAGTAAGCCATCA-3'
<i>IL6</i>	NM_173923.2	Forward	5' -TGAGTGTGAAAGCAGCAAGGA-3'
		Reverse	5' -TCGCCTGATTGAACCCAGAT-3'
<i>IL8</i>	NM_173925.2	Forward	5' -TGTGAAGCTGCAGTTCTGTCAA-3'
		Reverse	5' -TTTCACAGTGTGGCCGACTCT-3'
<i>PGES</i>	NM_174443.2	Forward	5' -ATCGTGACGGTCCGTCTCTAA-3'
		Reverse	5' -GCCCTTTGAGATTGTGACAGG-3'
<i>PGFS</i>	NM_001035367	Forward	5' -TGTGGTGCACGTATCACGACA-3'
		Reverse	5' -AATCACGTTGCCGTCCTCATC-3'
<i>OXR</i>	NM_174134.2	Forward	5' -GCACCTGAGCATAGCCGACC-3'
		Reverse	5' -GTGGCAAGGACGATGACGGG-3'

**MRPS15* = mitochondrial ribosomal protein S15; *TNF* = tumor necrosis factor α ; *IL1B* = interleukin 1 β ; *IL6* = interleukin 6; *IL8* = interleukin 8; *PGES* = prostaglandin E synthase; *PGFS* = prostaglandin F synthase; *OXR* = oxytocin receptor.

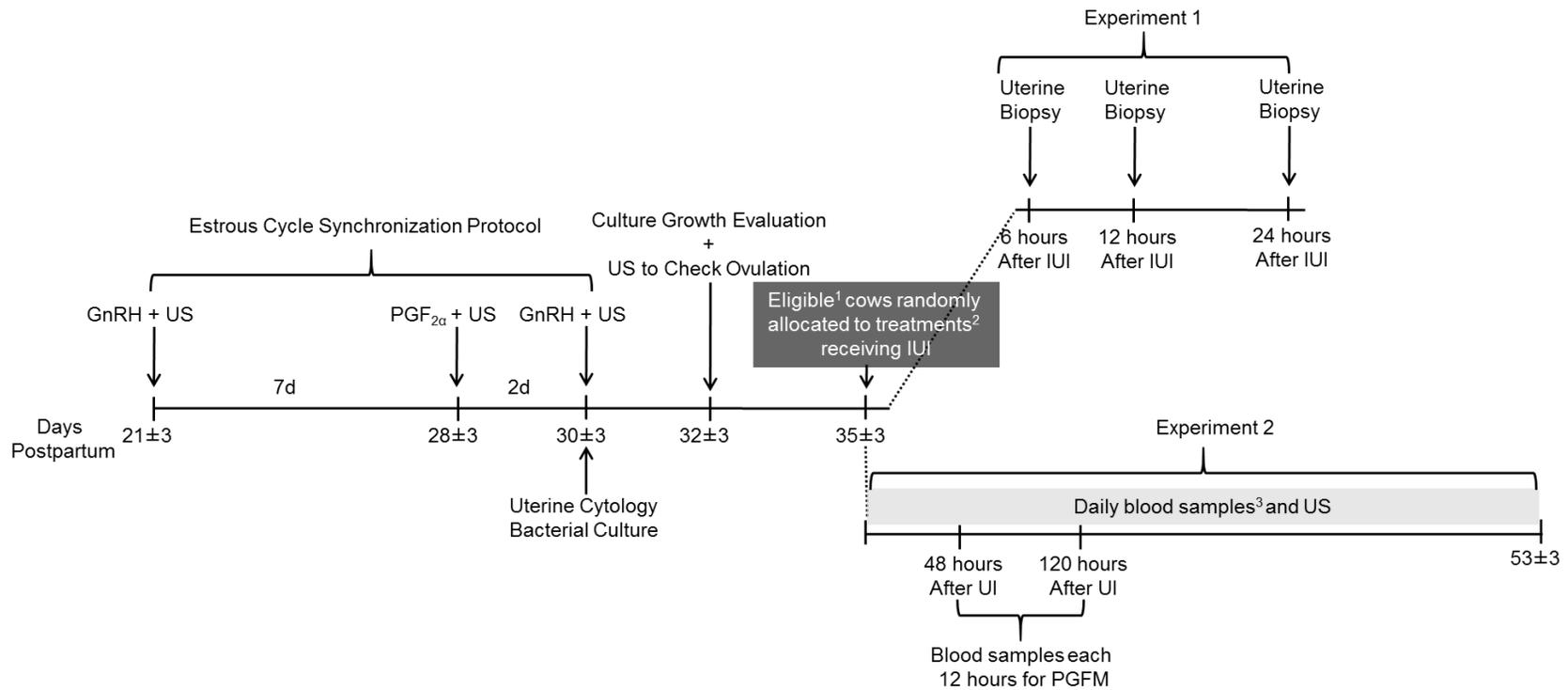


Figure 7-1. Diagram of experimental activities for experiments 1 and 2. ¹ Eligible cows ovulated to GnRH given on day 32 ± 3 postpartum and had no subclinical endometritis and bacterial growth. ² Treatments included intrauterine infusion (IUI) of 10 mL of sterile saline solution containing 10⁹ CFU/mL of *T. pyogenes* (TP); or IUI of 10 mL of sterile saline solution containing 1 µg of tumor necrosis factor α (TNF); or IUI of 10 mL of sterile saline solution (control). ³ Blood samples were collected daily from IUI until day 22 post ovulation or confirmation of luteolysis. US = ultrasonographic examination.

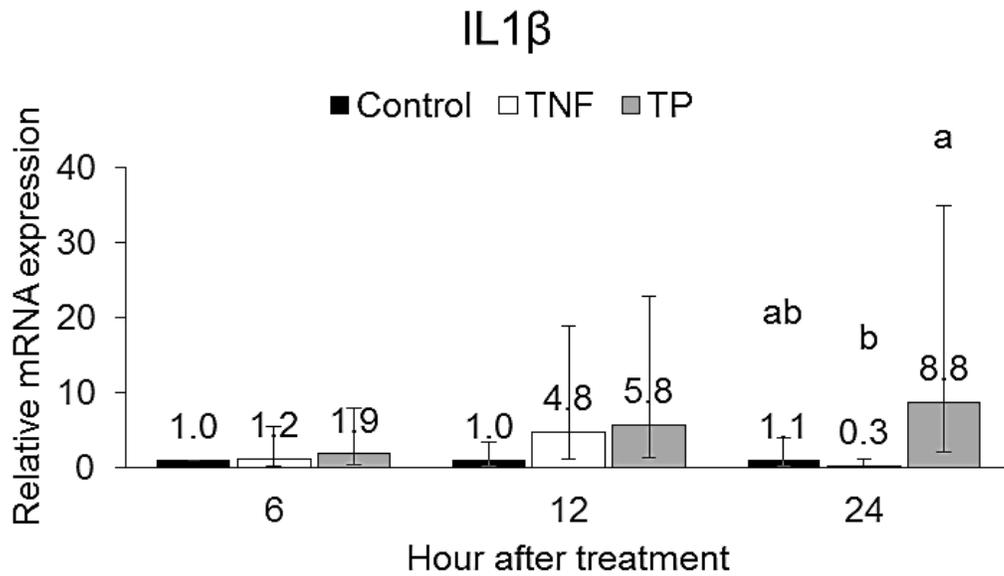


Figure 7-2. Relative endometrial mRNA expression of IL1 β according to treatments and time. Nonpregnant cows from the control group were used as reference for comparison. TP = intrauterine infusion of 10 mL of saline solution containing 10⁹ cfu/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution. Within same hour different letters means treatment difference (a, b; P \leq 0.05).

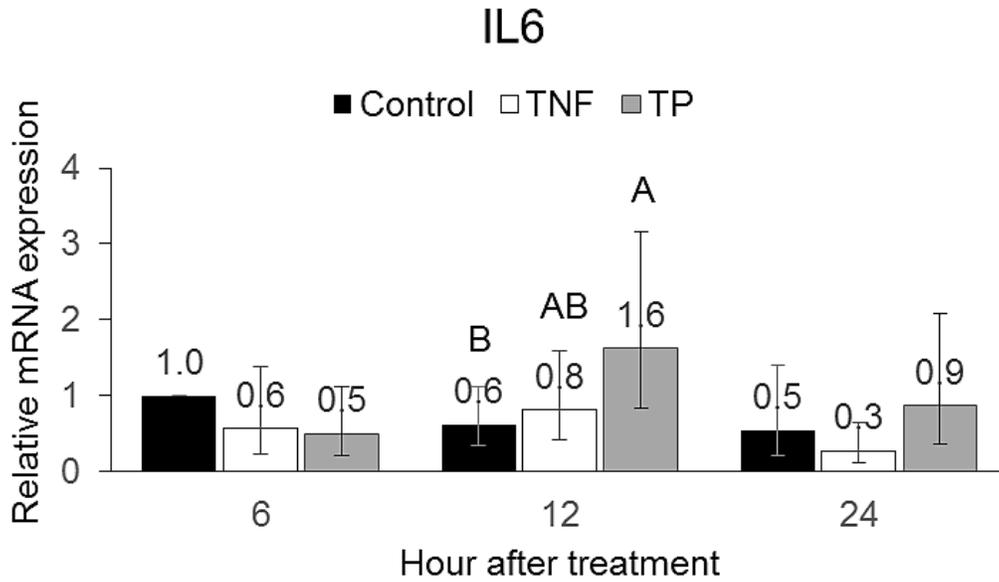


Figure 7-3. Relative endometrial mRNA expression of IL6 according to treatments and time. Nonpregnant cows from the control group were used as reference for comparison. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 CFU/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution. Within same hour after treatment different capital letters means tendency to be different (^{A, B}; $P < 0.10$).

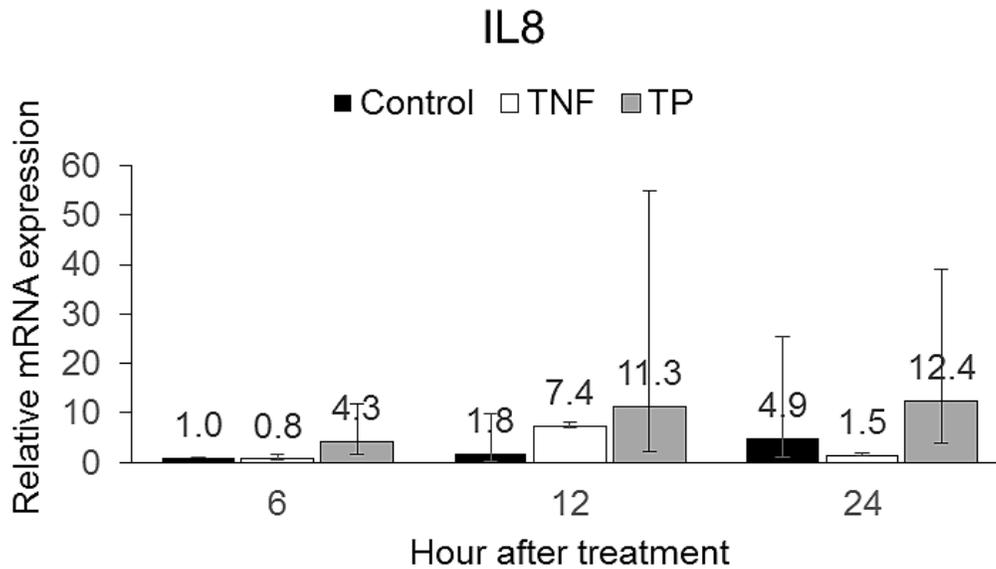


Figure 7-4. Relative endometrial mRNA expression of IL8 according to treatments and time. Nonpregnant cows from the control group were used as reference for comparison. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 cfu/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution.

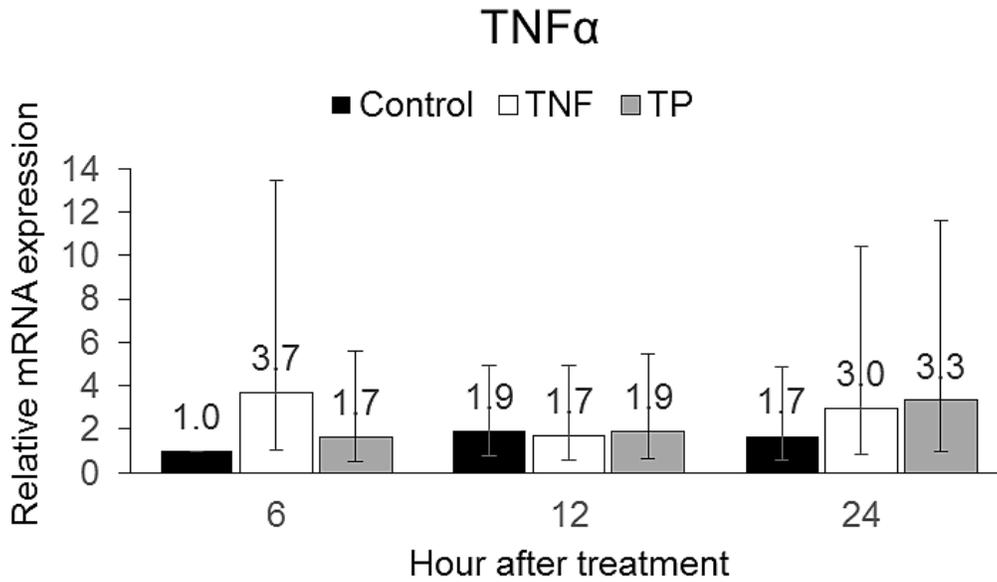


Figure 7-5. Relative endometrial mRNA expression of TNF α according to treatments and time. Nonpregnant cows from the control group were used as reference for comparison. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 cfu/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution.

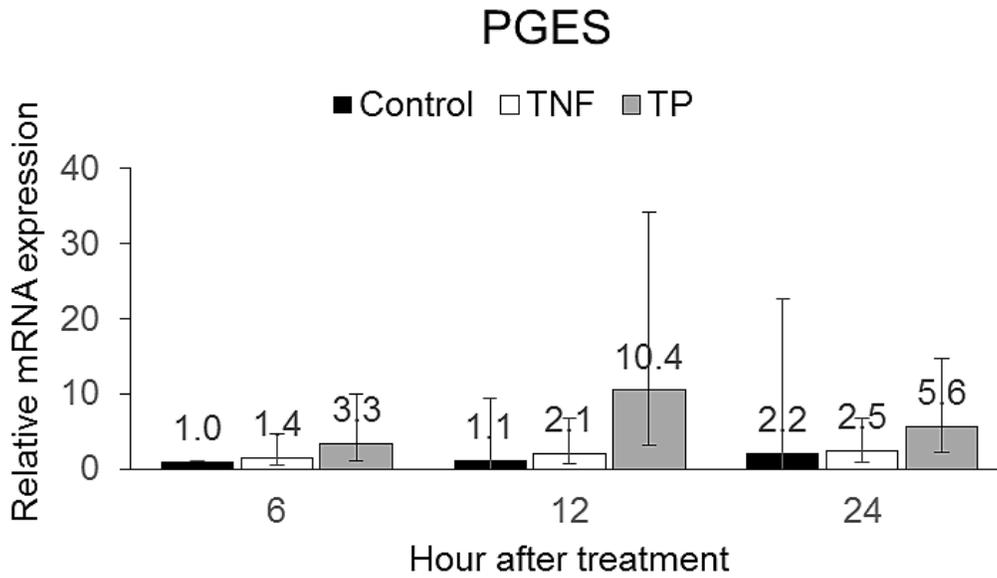


Figure 7-6. Relative endometrial mRNA expression of PGES according to treatments and time. Nonpregnant cows from the control group were used as reference for comparison. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 cfu/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution.

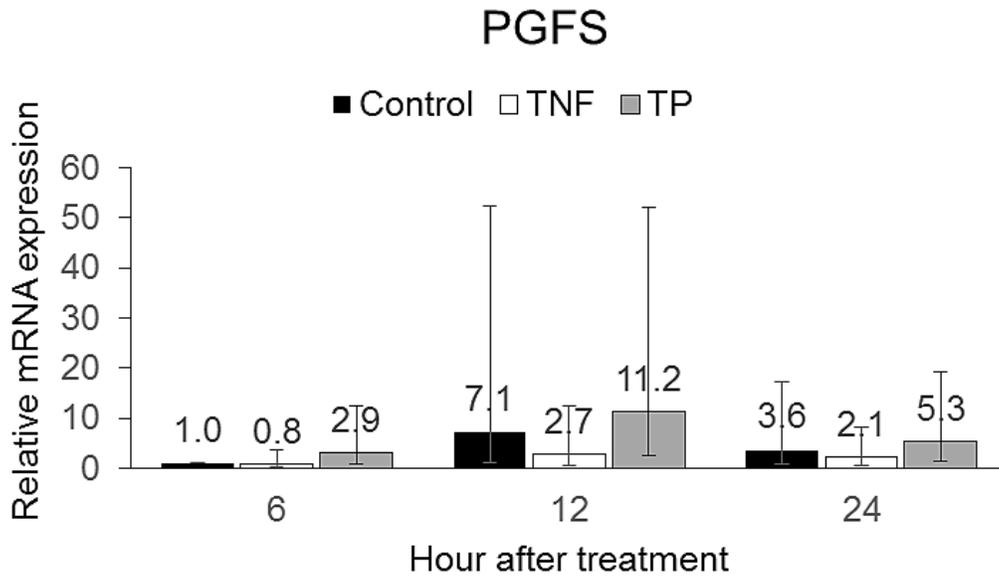


Figure 7-7. Relative endometrial mRNA expression of PGFS according to treatments and time. Nonpregnant cows from the control group were used as reference for comparison. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 cfu/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution.

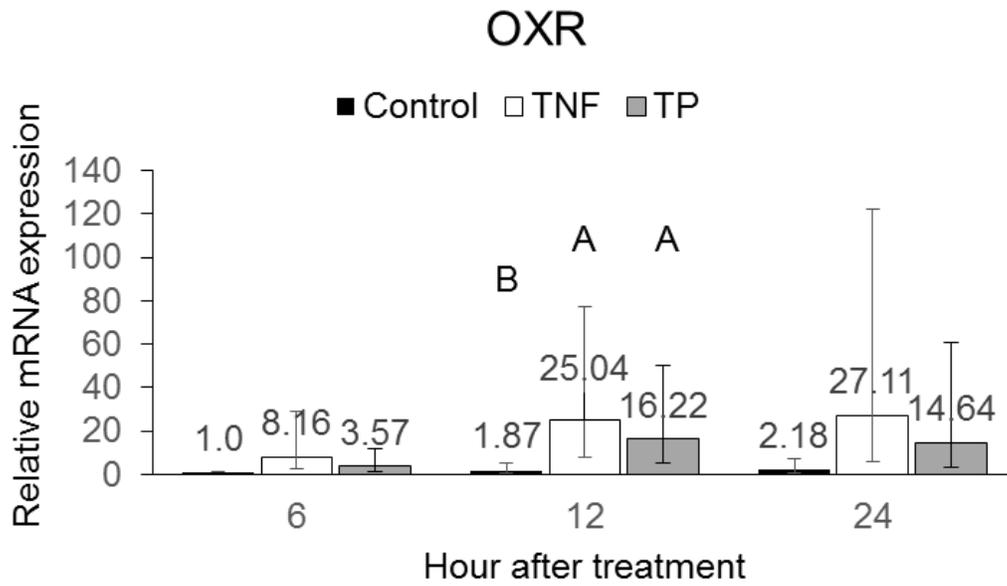


Figure 7-8. Relative endometrial mRNA expression of OXR according to treatments and time. Nonpregnant cows from the control group were used as reference for comparison. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 cfu/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution. Within same hour after treatment different capital letters means tendency to be different (^{A, B}; $P < 0.10$).

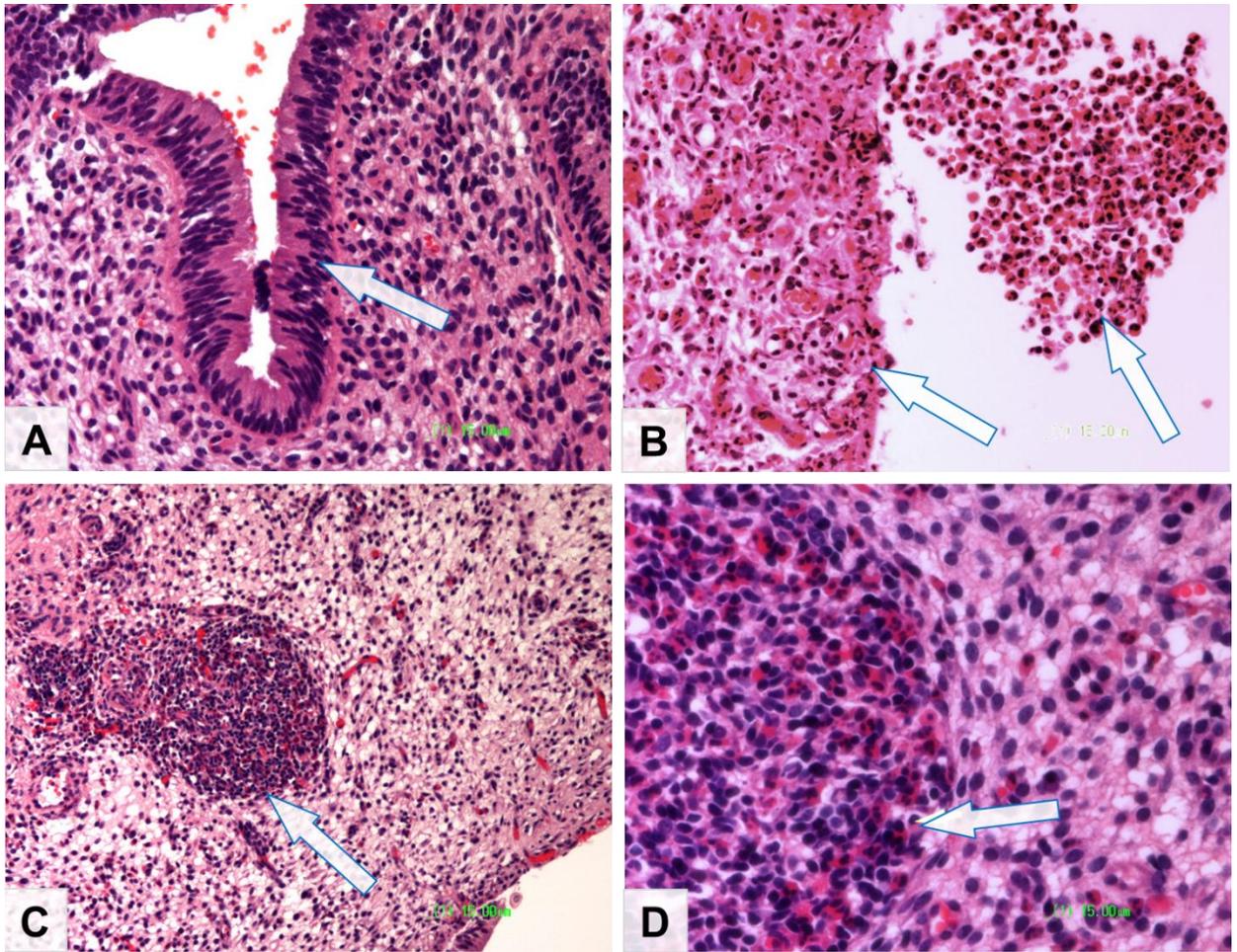


Figure 7-9. Uterine tissues pictures stained with hematoxylin and eosin. A - endometrium of control cow with arrow pointing intact epithelium and absence of inflammation; B - endometrium of *T. pyogenes* cow with arrow pointing damaged epithelium and neutrophils in the periglandular area; C - endometrium of *T. pyogenes* in a smaller magnification with arrow pointing massive accumulation of neutrophils; D - endometrium of *T. pyogenes* with arrow pointing massive accumulation of neutrophils.

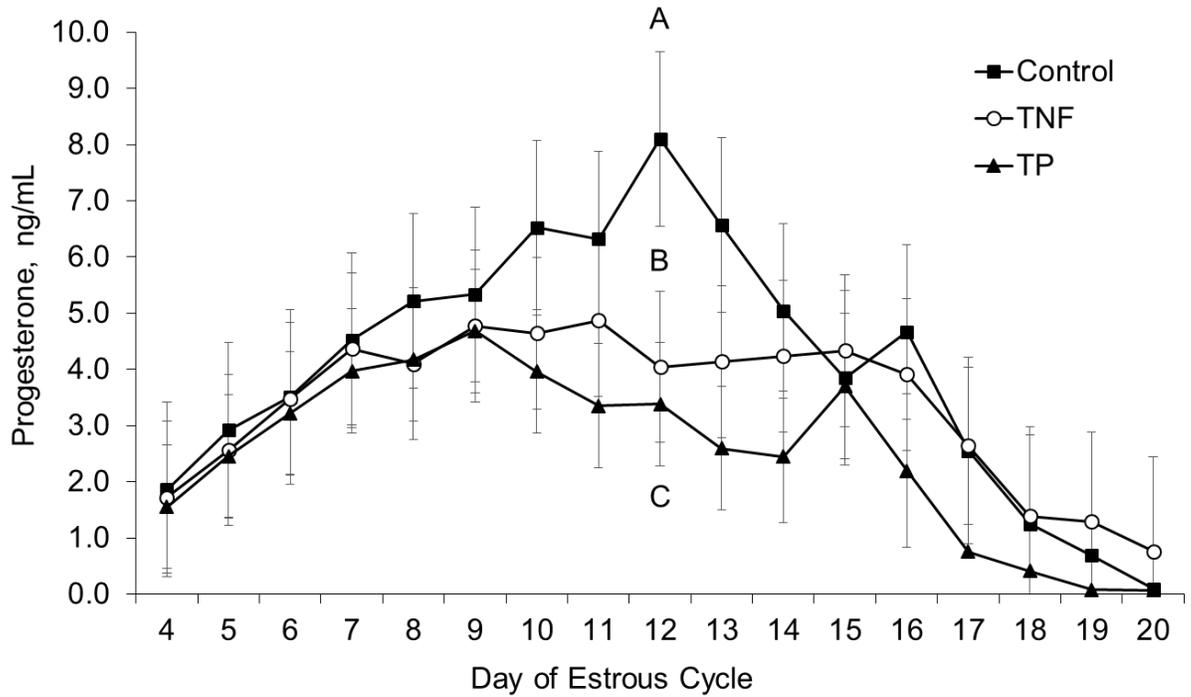


Figure 7-10. Concentrations of progesterone in plasma according to day of the estrous cycle. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 CFU/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution. Within day, concentrations of progesterone tend to be differed (A, B C; $P < 0.10$).

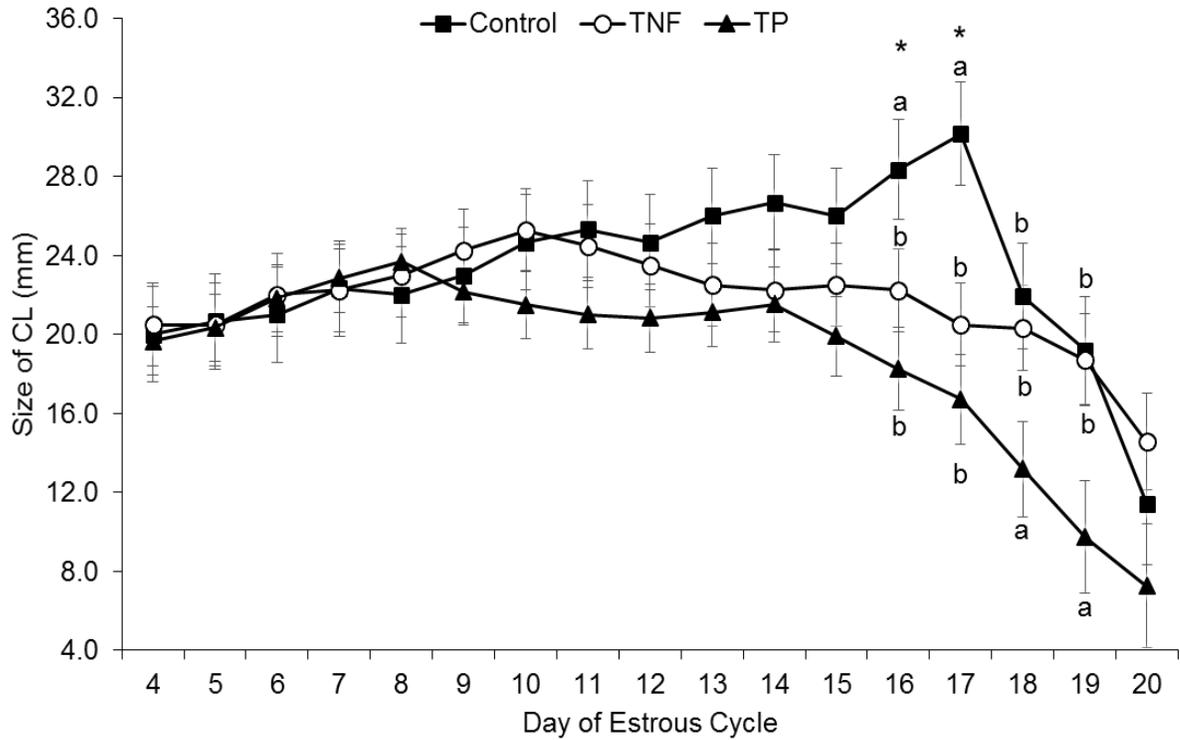


Figure 7-11. Size of the corpus luteum according to day of the estrous cycle. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 CFU/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution. *Means an interaction between treatment and day ($P < 0.001$). Within day, size of the corpus luteum differed (^{a, b}; $P < 0.10$).

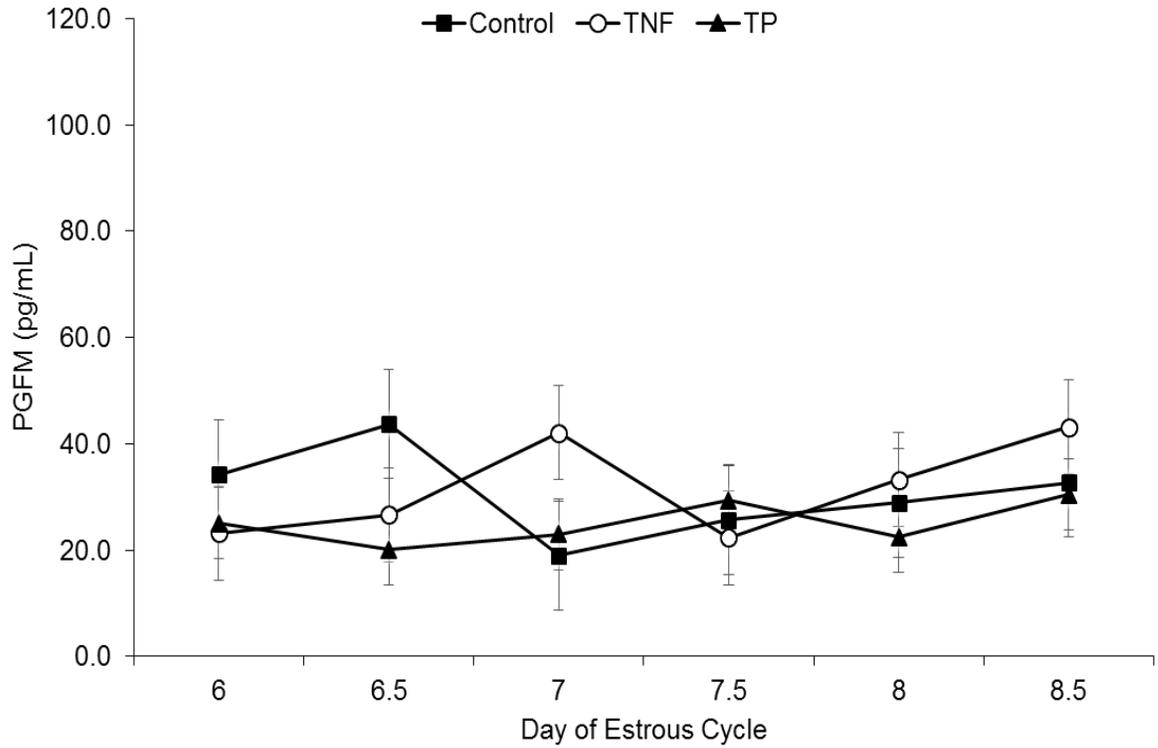


Figure 7-12. Concentrations of prostaglandin F metabolite (PGFM) in plasma according to day of the estrous cycle. TP = intrauterine infusion of 10 mL of saline solution containing 10^9 CFU/mL of *T. pyogenes*; TNF = intrauterine infusion of 10 mL of saline solution containing 1 μ g of tumor necrosis factor α ; and control = intrauterine infusion of 10 mL of saline solution.

CHAPTER 8

EFFICACY OF AMPICILLIN TRIHYDRATE FOR TREATMENT OF METRITIS AND SUBSEQUENT FERTILITY IN DAIRY COWS

Objectives were to evaluate the efficacy of ampicillin trihydrate for treatment of metritis in dairy cows compared with ceftiofur hydrochloride and the subsequent effects on pregnancy per artificial insemination (P/AI) for the 1st postpartum AI. Rectal temperature was measured daily for the first 12 d in milk (DIM), and fever was characterized by rectal temperature ≥ 39.5 °C. Vaginal discharge was scored at 4, 6 and 8 DIM, and on any day a cow had fever. Cows with vaginal discharge score 5 (reddish/brownish foul smell) were diagnosed with metritis and cows with metritis and rectal temperature ≥ 39.5 °C were diagnosed as puerperal metritis. Cows with metritis (n = 528) were blocked by parity and type of metritis (metritis only or puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin (n = 259) or 2.2 mg/kg of ceftiofur (n = 269) once daily for 5 d. Day of diagnosis of metritis was considered study d 1. A cohort of 268 cows without metritis was selected randomly as controls at 12 DIM based on the same parity and day of calving. In cows with metritis, rectal temperature was measured on study d 1 to 7, and 12, and vaginal discharge was scored on study d 5, 7, and 12. Metritis cure was characterized by vaginal discharge < 5 . At 32 DIM, vaginal discharge was scored for diagnosis of purulent vaginal discharge (PVD, vaginal discharge score > 2 , mucopurulent discharge). At day 39 DIM a uterine cytology samples were collected to evaluate subclinical endometritis. At 53 and 67 DIM ovaries were scanned to determine cyclic status. Pregnancy status was diagnosed on d 34 and 62 after 1st AI. Data were analyzed using the PROC GLIMMIX of SAS. Cure rates of metritis for ampicillin were greater than for ceftiofur on d 5 (37.1% vs. 25.2%) and 7 (57.2% vs. 46.3%) after metritis diagnosis, but not different on d 12 (82.0% vs.

85.0%). Cows with puerperal metritis had lower cure rates than cows with metritis only on d 5 (39.7 vs. 23.2%), 7 (62.9 vs. 40.5%), and 12 (88.1 vs. 77.7%). Incidence of fever after treatments, did not differ between treatments (ampicillin = 20.8% vs. ceftiofur = 19.3%), but mean rectal temperature tended to be less for ceftiofur than ampicillin cows (ampicillin = 39.15 °C vs. ceftiofur = 39.10 °C). Cows receiving ampicillin had lower prevalence of PVD than those treated with ceftiofur (57.7 vs. 67.8%), but they were both greater than no metritis cows (21.9%). Subclinical endometritis incidence was the same for ampicillin and ceftiofur (30.0 vs. 25.4%), but they were both greater than no metritis cows (14.5%). The proportion of cyclic cows at 67 DIM did not differ among treatments (ampicillin = 74.8% vs. ceftiofur = 75.0% vs. no metritis = 75.1%). First service P/AI did not differ among treatments at 34 (ampicillin = 28.9% vs. ceftiofur = 29.1% vs. no metritis = 32.0%) and 62 d after AI (ampicillin = 28.0% vs. ceftiofur = 28.3% vs. no metritis = 30.5%). Ampicillin was an efficacious therapy for metritis. Rate of cure was faster for ampicillin than for ceftiofur, but on d 12 both treatments resulted in similar cure rates. Although ampicillin reduced the prevalence of PVD, and no metritis cows had less PVD and subclinical endometritis than those with metritis, P/AI for the 1st insemination did not differ among treatments.

Introductory Remarks

Metritis is a prevalent postpartum disease in lactating dairy cows characterized by abnormally enlarged uterus and a fetid, watery red-brown fluid to viscous off-white purulent uterine discharge that can be accompanied or not of fever within 21 days postpartum, but more frequently diagnosed in the first week postpartum (Sheldon et al., 2006). The incidence rates of dairy cows developing metritis ranges from 10 to 36% (Goshen and Shpigel, 2006; Santos et al., 2010; Chapinal et al., 2011).

The economic losses caused by metritis are striking ranging from to \$328 to \$380 per affected cow, and the losses are caused by reduced milk production, delayed pregnancy, cost with treatment, and increased culling and death (Drillich et al., 2001). Additionally, cows diagnosed with metritis have an increased risk to develop both clinical and subclinical endometritis (Galvão et al., 2009; Martinez et al., 2012). The main bacteria isolated from cases of uterine infection include *Escherichia coli*, *Trueperella* (formerly *Arcanobacterium*) *pyogenes*, and anaerobic bacteria such as *Prevotella* (formerly *Bacteroides*) species and *Fusobacterium necrophorum* (Griffin et al., 1974; Noakes et al., 1989; Sheldon et al., 2002). Recently, the expressions of some specific virulence factors by these bacteria were associated with increase risky for development of uterine diseases (Bicalho et al., 2012). *Escherichia coli* expressing the adhesin type I fimbriae *fimH* identified in the uterus of cows in the first 3 days postpartum was significantly associated with development of metritis and endometritis. *Fusobacterium necrophorum* expressing the leukotoxin/hemolysin *lktA* in the first 3 days or between days 8 and 12 postpartum was associated with endometritis. *Trueperella pyogenes* expressing the type I fimbriae adhesin *fimA* and the pyolysin *plo* between 8 and 10 d or between 34 and 36 d postpartum was associated with endometritis (Bicalho et al., 2012). Therefore, it has been suggested that the presence of *E. coli* expressing virulence factor *fimH* in the uterus of cows in the first few days postpartum paves the way for the other bacterial infection coordinating the initial process of tissue damage and development of uterine diseases. Thus, it is reasonable to suggest that a reduction on the extent of *E. coli* load in the uterus of metritic cows might mitigate the negative

impact of the disease and minimize the risk of subsequent chronic uterine infections such as clinical and subclinical endometritis.

Ampicillin is a beta-lactam antibiotic that acts as an irreversible inhibitor of dd-transpeptidase, an essential enzyme that bacteria use to make their cell walls. Therefore, ampicillin generally inhibits the third and final stage of bacterial cell wall synthesis in binary fission, which ultimately leads to cell lysis. Ampicillin has received FDA approval for use in dairy cattle and it is indicated for therapy of infections caused by *E. coli* (Burrows, 1993; Lehtolainen et al., 2003), but to date, no published study has evaluated efficacy of ampicillin treatment of metritis in dairy cows.

We hypothesized that ampicillin would be an effective therapy for metritis resulting in similar clinical cure and subsequent reproductive performance compared with cows treated with ceftiofur, a common antibiotic labeled and prescribed for treatment of metritis in the United States. The objectives of study were to evaluate the efficacy of ampicillin trihydrate for treatment of metritis in dairy cows compared with ceftiofur hydrochloride and subsequent effect on pregnancy per AI (P/AI) to the first service.

Materials and Methods

The University of Florida Institute of Food and Agricultural Sciences Animal Research Committee approved all procedures in this study.

Cows, Housing, and Diets

The study was conducted on a single dairy farm located in central Florida. The lactating herd was composed by approximately 4,500 cows with a yearly rolling herd average milk yield of approximately 11,000 kg. A total of 528 cows diagnosed with metritis were enrolled in the study from October of 2012 to January of 2013.

Additionally, a cohort of 268 herdmates without metritis was enrolled in the study at 12 DIM to be used as controls. Metritic primiparous (n = 264) and multiparous cows (n = 264), and non-metritic primiparous cows (n = 134) and multiparous cows (n = 134) cows were housed together during the first 2 wk postpartum, and separately thereafter. Cows were housed in free-stall barns with sand-bedded stalls and equipped with sprinklers and fans for forced evaporative cooling. Cows received the same TMR to meet or exceed the nutrient requirements for a lactating Holstein cow producing 45 kg/d of milk with 3.5% fat and 3.2% true protein when DM intake is 25 kg/d (NRC, 2001). Diets consisted of ryegrass silage, corn silage, wet brewer's grains, dried distiller's grains, earlage, ground corn, citrus pulp, solvent-extracted soybean meal, expeller soybean meal, corn gluten feed, molasses, minerals, and vitamins.

Experimental Design, Treatments, and Body Condition Scoring

Rectal temperature was measured using an electronic thermometer (GLA Agricultural Products, San Luis Obispo, CA), immediately after the morning milking. Cows with a rectal temperature ≥ 39.5 °C were considered febrile. Vaginal discharge retrieved using the Metrichick (Metrichick, Simcro, New Zealand) was scored at 4, 6 and 8 DIM, and on any day a cow had fever. A vaginal discharge scoring system based on Sheldon et al. (2006) was used in the current study and as follows: 1 = clear or translucent mucus; 2 = mucus containing flecks of white or off-white pus; 3 = discharge containing $\leq 50\%$ white or off-white mucopurulent material; 4 = discharge containing $> 50\%$ purulent material; and 5 = watery, reddish/brownish color of foul smell. Cows with vaginal discharge score 5 were diagnosed as having metritis. Cows with a vaginal discharge score 5 and rectal temperature ≥ 39.5 °C were diagnosed as puerperal metritis. Cows with metritis (N = 528) were blocked by parity (primiparous vs.

multiparous) and type of metritis (only metritis = 312 or puerperal metritis = 216) and, within each block, assigned randomly to receive 11 mg ampicillin/kg of BW i.m. (n = 259) as ampicillin trihydrate (Polyflex, Boehringer Ingelheim Vetmedica, St. Joseph, MO) or 2.2 mg of ceftiofur/kg of BW i.m. (n = 269) as ceftiofur hydrochloride (Excenel RTU sterile suspension, Zoetis, Madison, NJ) once daily for 5 d. The day of diagnosis of metritis was considered study d 1. A cohort of 268 cows without metritis was selected randomly at 12 DIM based on the same day of calving and same parity to match herdmates diagnosed with metritis. The body condition of all cows was assessed at enrollment in the study using a scoring system 1 (emaciated) to 5 (obese) according to Ferguson et al. (1994) as depicted in the Elanco BCS chart (Elanco, 2009). Cows that had dystocia based on any type of assistance during delivery, a stillbirth calf, twin calves, or occurrence of retained fetal membranes were classified as having calving-related disorders.

Rectal Temperatures, Vaginal Discharge Evaluation, and Cure Definitions

Cows diagnosed with metritis had rectal temperature recorded from study d 1 to 7, and again on study d 12 after morning milking (Figure 8-1). Additionally, all cows that had vaginal discharge scored using the same 1 to 5 scoring system mentioned above on study d 5, 7 and 12. Cure of metritis was defined based on vaginal discharge and rectal temperature. Initially, cure was determined based solely on a vaginal discharge < 5, or absence of watery, reddish/brownish color of foul smell discharge on d 5, 7 and 12 of study. In addition, cure of was also determined based on vaginal discharge < 5 and rectal temperature < 39.5 °C. Finally, cure on d 12 of the study was also determined based on vaginal discharge score < 5, rectal temperature < 39.5 °C, and no additional

health problem or concurrent antimicrobial other than the specified respective treatment before d 12 in the study.

Evaluation of Purulent Vaginal Discharge, Subclinical Endometritis, and Estrous Cyclicity

Samples of vaginal discharge and uterine endometrial cytology were collected from all cows diagnosed with metritis and from the cohort of cows without metritis. Vaginal discharge was collected at 32 ± 3 DIM using the metricheck device to determine occurrence of purulent vaginal discharge (PVD), formerly known and classified as clinical endometritis (Sheldon et al., 2006; Dubuc et al., 2010). Cows with vaginal discharge score > 2 were classified as having PVD. Uterine cytology samples were collected on $d 39 \pm 3$ postpartum using the cytobrush technique (Kasimanickam et al., 2005) using a stainless steel gun protected by a one-way plastic tube protector (Continental plastics, Delaval, WI). After collecting the endometrial cytology, the cytobrush was rolled onto a slide and air dried immediately. The slides were transported to the laboratory and stained using diff-quick stain kit (IMEB, San Marcos, CA). Three technicians blinded to the treatments read the slides. Two-hundred cells were counted in each slide using a microscope at 400 x magnifications to determine the proportion of PMNL relative to the total leukocytes and endometrial cells counted. Cows with a proportion of PMNL $\geq 5\%$ were classified as having subclinical endometritis (Gilbert et al., 2005).

Estrous cyclicity was evaluated at 50 ± 3 and 64 ± 3 DIM by ultrasonographic examination of the ovaries using a portable ultrasound scanner equipped with a 7.5 MHz transrectal probe (Easi-Scan, BCF Technology, Rochester, MN). Cows with at least a CL > 15 mm recorded on one of the two examination days were considered to

be estrous cyclic, whereas those without a visible CL > 15 mm in both examinations were considered anovular.

Reproductive Management

Cows enrolled in this study were subjected to a reproductive program as described in Figure 7-2. Cows receiving the first postpartum AI were presynchronized with 2 i.m. injections of 25 mg of PGF_{2α} (5 mL of Lutalyse, 5 mg/mL of dinoprost as tromethamine salt; Zoetis, Madison, NJ) administered 14 d apart, at 50 ± 3 and 64 ± 3 DIM. At the second injection of PGF_{2α}, cows' tailheads were painted daily with chalk and those identified in estrus by removal of tail chalk were artificially inseminated on the same morning. Cows not observed in estrus within 12 d of the second PGF_{2α} of the presynchronization protocol were enrolled in the 5-d timed AI program at 76 ± 3 DIM (Figure 8-2). Briefly, the 5-d timed AI program consisted of an i.m. injection of 86 µg of GnRH (2 mL of Cystorelin sterile solution, gonadorelin diacetate tetrahydrate equivalent to 43 µg of gonadorelin/mL; Merial Ltd., Duluth, GA) followed by 2 i.m. injections of PGF_{2α} on d 5 and 6 of the protocol. A second i.m. injection of GnRH was administered concurrently with AI at 72 h after the first PGF_{2α}. Pregnancy was diagnosed by transrectal ultrasonography on d 34 ± 3 after AI. The presence of an amniotic vesicle containing an embryo with a heartbeat was used as the criteria to determine pregnancy. Pregnant cows on d 34 ± 3 were reexamined for pregnancy by transrectal palpation 4 wk later, on d 62 of gestation. Pregnancy per AI was calculated by dividing the number of cows diagnosed pregnant at d 34 or 62 after AI by the number of cows receiving AI. Pregnancy loss was calculated as the number of cows that lost a pregnancy between d 34 and 62 after AI divided by the number of cows diagnosed pregnant on d 34 after AI. Cows that were detected in estrus before study d 32 were reinseminated and

considered nonpregnant. Inseminations were performed by six technicians with semen from 13 Holstein sires.

Statistical Analysis

Sample size calculation was performed using the POWER procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). The sample size was calculated to provide sufficient experimental units to detect statistical significance ($\alpha = 0.05$; $\beta = 0.20$) when cure of metritis by d 7 and d 14 differ by 10 percentage units (51 vs. 41%) and by 7 percentage units (84 vs. 77%), respectively. It was assumed that 41% and 77% of the cows treated with ceftiofur would experience clinical cure on d 7 and 14, respectively, after initiation of treatments (Chenault et al., 2004). Additionally, sample size calculation aimed to allow identification of an increase of 8 percentage units (39 vs. 30%) in P/AI after the first insemination when considering that 30% of the cows treated with ceftiofur would become pregnant following the first postpartum AI. Under these assumptions, between 210 and 240 experimental units per treatment were deemed necessary. Because of potential attrition, a minimum of 250 cows were planned for enrollment.

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS/STAT, SAS Institute Inc., Cary, NC) fitting a binary distribution. Treatment was forced in the final models, but covariates and the interaction between treatment and covariates were sequentially removed from the model if $P > 0.10$. The models for cure of included the fixed effects of treatment (ampicillin vs. ceftiofur), type of metritis (only metritis vs. puerperal metritis), parity (primiparous vs. multiparous), calving related disorders (yes or no), and interactions between treatment and parity, treatment and type of metritis type, and treatment and calving related disorders, and the random effect of block. Two models were built for analysis of purulent

vaginal discharge, subclinical endometritis, and estrous cyclic status. The first model included only cows randomly assigned to treatments and excluded cows without metritis, and the statistical models were identical to those described for cure of metritis. The second model evaluated all cows, including cows without metritis, and type of metritis was removed from the explanatory variables. The models for pregnancy per AI and pregnancy loss were similar to those used to analyze purulent vaginal discharge, but also included type of insemination (after detected estrus or timed AI), technician, and sire.

The continuous data with repeated measures over time were analyzed using the GLIMMIX procedure of SAS (SAS/STAT version 9.3; SAS Institute Inc., Cary, NC) with models fitting a Gaussian distribution. Data were tested for normality of residuals, and non-normally distributed data were transformed before analysis. Rectal temperature was analyzed with the fixed of treatments (ampicillin vs. ceftiofur), type of metritis (metritis only vs. puerperal metritis), parity, the interactions between treatment and type of metritis, treatment and parity, parity and type of metritis, and treatment and type of metritis and parity. Block and cow nested within treatment were the random terms in the models. The covariance structure that resulted in the smallest Akaike's information criterion was selected for the model.

Differences with $P \leq 0.05$ were considered significant and those with $0.05 < P \leq 0.10$ were considered tendencies.

Results

The prevalence of metritis in the farm throughout the period of the study was 36.1 % (528/1,463). Of the 528 cows enrolled in the study, 40.9% were considered to

have puerperal metritis on the day of diagnosis (216/528), resulting in an overall incidence of puerperal metritis of 14.8% (216/1,463).

Rectal Temperatures and Incidence of Fever

On the day of study enrollment, the rectal temperature was greater ($P < 0.05$) for ampicillin than ceftiofur. Nevertheless, the mean rectal temperature of cows after treatments were initiated did not differ between treatments (Table 8-1). An interaction ($P < 0.001$) between treatment and day in the study was observed for rectal temperature because cows receiving ceftiofur had lower temperatures between d 2 and 3 of treatment, whereas cows receiving ampicillin had lower temperatures on d 6 and 7 of treatment (Figure 8-3). As anticipated, type of metritis influenced ($P < 0.001$) rectal temperature, and it was greater for cows with puerperal metritis than those with metritis only. Incidence of fever did not differ between treatments and averaged 20.2% throughout the 12 d post-treatment evaluation (Table 8-1). Cows with puerperal metritis had greater ($P < 0.001$) incidence of fever than those with metritis only. Incidence of fever from study d 2 to 12 decreased ($P < 0.001$) over time, from 23.8% on study d 2 to 17.0 on study d 12. No interaction between treatment and type of metritis, or parity, or day in the study were observed for incidence of fever.

Metritis Cure Based on Vaginal Discharge Score < 5

Of the 528 cows randomly assigned to treatments, 526 were evaluated for cure on d 5, 525 were evaluated on d 7, and 517 on d 12. Before study d 12, 11 cows were not evaluated for vaginal discharge score, 5 ampicillin and 6 ceftiofur. The reasons were because they were culled (1 ampicillin, 2 ceftiofur) or died (4 ampicillin, 4 ceftiofur).

Clinical cures based on the criterion of vaginal discharge score < 5 were greater for ampicillin than ceftiofur on d 5 ($P < 0.01$) and 7 ($P = 0.02$) of the study; however, no

difference ($P = 0.40$) in cure rates on d 12 were detected between treatments (Figure 8-4A). Cows with puerperal metritis had reduced cure on d 5 ($P < 0.001$), 7 ($P < 0.001$), and 12 ($P < 0.01$) compared with cows with metritis only (Figure 8-5A). Nevertheless, no interaction between treatment and type of metritis was observed for cure based on vaginal discharge < 5 on d 5, 7 and 12 after initiation of treatments. Multiparous cows had increased ($P < 0.01$) cure than primiparous on study d 5, but not on d 7 and 12 (Figure 8-6A). Cows with calving-related problems had reduced ($P = 0.02$) cure on d 5 and tended ($P = 0.07$) to have reduced cure on d 7 compared with cows with normal calving (Figure 7-7A). However, on d 12 of the study the cure of metritis was the same for cows with and without calving related disorders. There were no interactions between treatment and type of metritis, parity, or calving-related disorders for cure based on vaginal discharge score < 5 on d 5, 7 and 12 of the study.

Metritis Cure Based on Vaginal Discharge Score < 5 and Rectal Temperature < 39.5 °C

When cure rates were analyzed according to the criteria of vaginal discharge score < 5 and concurrent rectal temperature < 39.5 °C, cows treated with ampicillin had increased ($P < 0.01$) cure of metritis on and 7 compared with cows treated with ceftiofur, but the proportion of cows with metritis cured on d 5 ($P = 0.18$) and d 12 ($P = 0.76$) did not differ between treatments (Figure 8-4B). Cows with puerperal metritis had reduced ($P < 0.01$) clinical cure throughout the 12 d observational period compared with cows with metritis only (Figure 8-5B). Multiparous cows tended ($P = 0.07$) to have increased clinical cure on d 5 and 12 after the initiation of treatments than primiparous cows (Figure 8-6B). Cow diagnosed with calving-related problems had reduced ($P = 0.001$)

clinical cure on study d 5 than those with normal calving, but this difference was no longer present on study d 7 and 12 (Figure 7-7B).

Metritis Cure Based on Vaginal Discharge Score < 5, Rectal Temperature < 39.5 °C, and no Additional Antimicrobial Therapy

When cows that received any additional antimicrobial therapy during the 12 d observational period were considered a failure of the original treatment, then clinical cure declined for both treatments. On d 12, the proportion of cows considered clinically cured was similar ($P = 0.63$) between ampicillin and ceftiofur (58.2 vs. 60.4%). Cows with puerperal metritis had reduced ($P = 0.02$) clinical cure than those with metritis only (54.0 vs. 64.3%). On the other hand, primiparous cows had greater ($P = 0.03$) proportion of cows cured than multiparous cows (63.9 vs. 54.5%). Cows with calving-related problems had similar clinical cure on d 12 compared with cows with normal calving, and it averaged 59.2%.

Purulent Vaginal Discharge and Subclinical Endometritis

A total of 760 cows were evaluated for PVD on d 32 postpartum, 248 cows in ampicillin, 252 ceftiofur, and 260 no metritis. Cows receiving ampicillin had reduced ($P = 0.03$) prevalence of PVD on d 32 postpartum compared with those treated with ceftiofur, but they were both greater ($P < 0.01$) than cows not diagnosed with metritis (Figure 8-8A). The benefits of ampicillin in reducing PVD were observed in cows with only metritis (ampicillin = 49.1 vs. ceftiofur = 68.4%), but not in those with puerperal metritis (ampicillin = 68.5 vs. ceftiofur = 66.9%). The prevalence of subclinical endometritis on d 39 postpartum did not differ ($P = 0.38$) for cows treated between cows treated with ampicillin and ceftiofur, but they were both greater ($P < 0.01$) than no metritis cows (Figure 8-8B) and not interactions with other variables were detected.

Estrous Cyclicity, Pregnancy per AI, and Pregnancy Loss

Of the initial 796 cows enrolled in the study (259 Ampicillin, 269 Ceftiofur, and 268 no metritis), 29 cows in Ampicillin (14 sold, 7 dead, 4 pyometras, and 4 with adhesions of the reproductive tract), 42 Ceftiofur (21 sold, 13 dead, 3 pyometras, and 5 adhesions of the reproductive tract), and 21 no metritis (16 sold, 4 dead, 1 pyometra) did not receive the first AI or contributed with analysis of P/AI and pregnancy loss. Therefore, 749 cows contributed with data for resumption of estrous cyclicity and 704 cows contributed with data for P/AI.

The percentage of estrous cyclic by 64 ± 3 DIM did not differ among treatments and averaged 75% (Table 8-2). A greater ($P < 0.01$) proportion of multiparous cows were cyclic at 64 DIM than primiparous cows (79.1 vs. 70.9%). For cows randomly assigned to treatments, type of metritis did not influence ($P = 0.91$) resumption of estrous cyclicity and 75.5 and 75.0% of cows with metritis only and cows with puerperal metritis resumed ovulation by 64 DIM.

Pregnancy per AI to first service on d 34 and 62 after first insemination did not differ among treatments (Table 8-2). For cows randomly assigned to treatments, type of metritis did not influence ($P < 0.95$) P/AI on d 34 or 62 after insemination. On d 64, P/AI were 26.7 and 26.4% for cows with metritis only and cows with puerperal metritis, respectively. Similar to P/AI, pregnancy loss between 34 and 62 d of gestation did not differ among treatments.

Discussion

As anticipated, ampicillin was an efficacious alternative treatment for metritis and showed clinical efficacy similar to or better than ceftiofur. Metritic cows treated with

ampicillin trihydrate had faster cure rate than those treated with ceftiofur hydrochloride. Although the study design does not allow for detection of spontaneous cure, it is known that a large proportion of cows diagnosed with puerperal metritis not receiving antimicrobial therapy have remission of the symptoms within 14 d of diagnosis (Chenault et al., 2004; McLaughlin et al., 2012). Treatment with ampicillin lead to greater proportion of cows clinically cured on d 7 after initiation of treatments when the criteria for cure was either vaginal discharge < 5 or a combination of vaginal discharge < 5 concurrent with rectal temperature < 39.5 °C. This benefit was observed in both, cows with metritis only and cows with puerperal metritis, which indicates that ampicillin cured metritis and puerperal metritis at a faster rate than ceftiofur.

Early in the course of treatment, cows receiving ceftiofur had lower rectal temperature than those treated with ampicillin, but this difference reversed after study d 5 after which cows treated with ampicillin had lower rectal temperature than those treated with ceftiofur. The lower body temperature for cows treated with ampicillin after the course of therapy is another indication that clinical efficacy in curing metritis was at least similar to that of ceftiofur. As anticipated, cows presenting puerperal metritis, a more acute debilitating form of the disease, had reduced cure rates at 5, 7 and 12 d after diagnosis, and antimicrobial therapy with either ampicillin or ceftiofur were equally efficacious at resolving puerperal metritis within the first 12 d after diagnosis.

Ampicillin and ceftiofur are both beta-lactam antibiotics that bind irreversibly to bacterial enzyme dd-transpeptidase blocking the formation crosslinks between this enzyme and peptidoglycan, which compromises the formation of rigid cell wall synthesis in binary fission, which ultimately leading to cell lysis (Katzung, 2007). Ampicillin is

classified as amino-penicillin because of the presence of amino group as part of its core. This amino group is critical for ampicillin to penetrate the outer membrane of gram-negative bacteria, which gives the drug its broad spectrum by killing both gram-positive and gram-negative bacteria (Katzung, 2007). Ceftiofur has also effectiveness against gram-positive and gram-negative bacteria and is resistant to beta-lactamase, therefore, preventing the action of these enzymes on the degradation of the beta lactam ring, which inactivates many antibiotics of this class (Collatz et al., 2006). Ampicillin and ceftiofur are both effective against gram-negative bacteria such as *E. coli* (Lehtolainen et al., 2003; Sheldon et al., 2004), which is thought to initiate the infection and promote the initial tissue damage that facilitates establishment of other bacterial groups in the uterus. Efficacy against *E. coli*, particularly those expressing the virulence factor fimH (Bicalho et al., 2012), might limit the extent of pathogenic bacteria colonization of the uterus and reduce the intensity of disease.

It is interesting to note that 12.6% of the cows with metritis only developed fever after the antimicrobial therapy had been established. These cows were not diagnosed with fever on the first day of treatment, but developed rectal temperature ≥ 39.5 °C despite treatment with ampicillin or ceftiofur. In general, treating cows affected by puerperal metritis with antimicrobials reduces body temperature (Chenault et al., 2004; McLaughlin et al., 2012). Interestingly, the decline in body temperature is also observed in metritic cows that remain untreated (Chenault et al., 2004; McLaughlin et al., 2012), indicating normal resolution of the disease by the cow's immune system. Nevertheless, the proportion of cows that initiated antimicrobial therapy that develops fever has not

been reported and it seems acceptable that a small portion of the treated cows might become worse or not respond to the antibiotics.

Administration of ceftiofur at the prescribed dose of 2.2 mg/kg results in concentrations of ceftiofur derivatives above those capable of inhibiting the growth of utero-pathogenic bacteria (Drillich et al., 2006c; Sheldon et al., 2004). When ampicillin is administered to cattle, concentrations increase immediately and reach approximately 0.9 µg/mL of plasma, followed by a decline to values of approximately 0.3 and 0.4 µg/mL for the next 24 h (Gehring et al., 2005). Therefore, the similar clinical efficacy of ampicillin and ceftiofur are likely related to their ability to maintain concentrations of antibiotics sufficient to inhibit the growth of the major pathogens that cause uterine disease.

Many studies evaluating the clinical efficacy of antibiotics for therapy of metritis usually only evaluated the remission of the disease with reduction in fever and improvements in uterine discharge score (Chenault et al., 2004; McLaughlin et al., 2012). However, because of extensive spontaneous cure even in cows with puerperal metritis (Chenault et al., 2004; McLaughlin et al., 2012), it is critical that the evaluation of therapy goes beyond clinical cure. In fact, proper treatment of metritis can influence milk yield and reproductive performance (Goshen and Shpigel, 1996). Despite a faster cure and a small reduction in the prevalence of PVD, cows treated with ampicillin had similar prevalence of estrous cyclicity and P/AI at first postpartum insemination compared with cows treated with ceftiofur. It has been reported consistently in the literature that PVD has detrimental impacts on fertility (Gilbert et al., 2005; Dubuc et al., 2011), and one would anticipate that reductions in the prevalence of PVD and

subclinical endometritis would benefit fertility at first AI. However, it is possible that the changes in prevalence of PVD and cytological endometritis caused by either metritis or treatment of metritis were insufficient to impact fertility. All cows in the study were subjected to a presynchronized timed AI protocol, but it is unlikely that hormonal treatments, particularly PGF_{2α}, benefited uterine health that would influence P/AI (Dubuc et al., 2011). Therefore, the improved cure rates and rectal temperature on d 7 after metritis diagnosis and reduced prevalence of PVD identified in cows treated with ampicillin in comparison with ceftiofur did not translate in improved reproductive performance in the current study.

Although cows without metritis had a remarkable reduction in the incidence of PVD and subclinical endometritis in comparison with metritic cows treated with ampicillin and ceftiofur, no difference in the prevalence of estrous cyclic cows and P/ AI for the first postpartum insemination were identified between cows without metritis and those diagnosed with metritis. These results were surprising considering the well-known negative associations among between uterine diseases and subsequent fertility (Dubuc et al., 2011). At this point, it is unclear why cows with metritis had similar P/AI and risk of pregnancy loss compared with those not diagnosed with metritis. It is possible that early diagnosis and prompt therapy might have minimized the negative impact of metritis on fertility. Goshen and Shpigel (2006) allocated cows diagnosed with metritis to receive 4 treatments with 5 g of chlortetracycline as intrauterine boluses over the course of two weeks or to remain as untreated controls. The P/AI at first postpartum insemination were 38.3, 42.5, and 18% in cows without metritis, cows with metritis treated with chlortetracycline, and cows with metritis that remained as untreated

controls, respectively. Therefore, it is possible that proper antimicrobial therapy immediately after the diagnosis of metritis might reestablish fertility similar to that of unaffected cows.

Conclusion

Ampicillin was an efficacious alternative therapy for metritis resulting in faster cure rates than ceftiofur; however, by d 12 after the diagnosis of metritis, no differences between treatments were observed. Ampicillin reduced the prevalence of cows with PVD on d 32 postpartum compared with cows receiving ceftiofur, but treatment did not affect the prevalence of cytological endometritis in cows previously diagnosed with metritis. Similarly, treatment did not affect the resumption of estrous cyclicity by 64 d postpartum, P/AI at first AI, and the risk of pregnancy loss. Although, cows without metritis had reduced prevalence of PVD and subclinical endometritis compared with metritic cows, estrous cyclicity and P/AI did not differ between those without metritis and metritic cows treated with ampicillin or ceftiofur. Type of metritis is an indicator of the severity of disease and cows with puerperal metritis have poorer cure rates than those with metritis alone. Nevertheless, type of metritis did not influence subsequent estrous cyclicity, P/AI or risk of pregnancy loss.

Table 8-1. Effect of antibiotic treatment on mean rectal temperature (RT) and incidence of fever in cows with only metritis or cows with puerperal metritis (metritis and RT \geq 39.5 °C)

Parameter	Treatment ¹				TRT	<i>P</i> ² Type	TRT* Type
	AMP		CEFT				
	Metritis ³	Puerperal Metritis	Metritis	Puerperal Metritis			
Cows, n	152	107	160	109	-	-	-
Mean RT, ⁴ °C	39.04	39.31	39.02	39.22	0.09	0.01	0.20
Fever incidence, ⁵ %	12.5	33.2	12.9	28.2	0.47	0.01	0.34

¹ Cows with metritis were blocked by type of metritis and assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d.

² TRT = effect of treatment; TM = effect of type of metritis; TRT x TM = interaction between TRT and TM.

³ Metritis = cows with metritis only based on vaginal discharge score 5 (watery, reddish/brownish color of foul smell) and rectal temperature < 39.5 °C; P. metritis = puerperal metritis based on vaginal discharge score 5 and rectal temperature \geq 39.5 °C.

⁴ RT = mean rectal temperature from d 2 to 12 after enrollment.

⁵ Fever = incidence of fever (RT > 39.4°C) from d 2 to 12 after enrollment.

Table 8-2. Effect of treatment on estrous cyclicity, pregnancy per AI and pregnancy loss following the 1st postpartum insemination.

	Treatments ¹			<i>P</i>
	Ampicillin	Ceftiofur	No Metritis	
	Adjusted means (n/n)			
Estrous cyclic ²	74.8 (184/245)	75.9 (185/246)	75.1 (198/258)	0.96
Pregnancy per AI				
Day 34	28.9 (61/230)	29.1 (70/227)	32.0 (84/215)	0.87
Day 62	27.9 (58/230)	28.3 (65/227)	30.5 (76/215)	0.91
Pregnancy loss	5.8 (3/61)	6.1 (5/70)	11.3 (8/76)	0.52

¹ Cows with metritis were blocked by type of metritis and assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. No metritis cows were randomly selected at 12 d postpartum based on the day of calving and parity.

² Estrous cyclic by 64 d postpartum based on the presence of a CL in at least one of the two ovaries examined on d 50 ± 3 and 64 ± 3 DIM

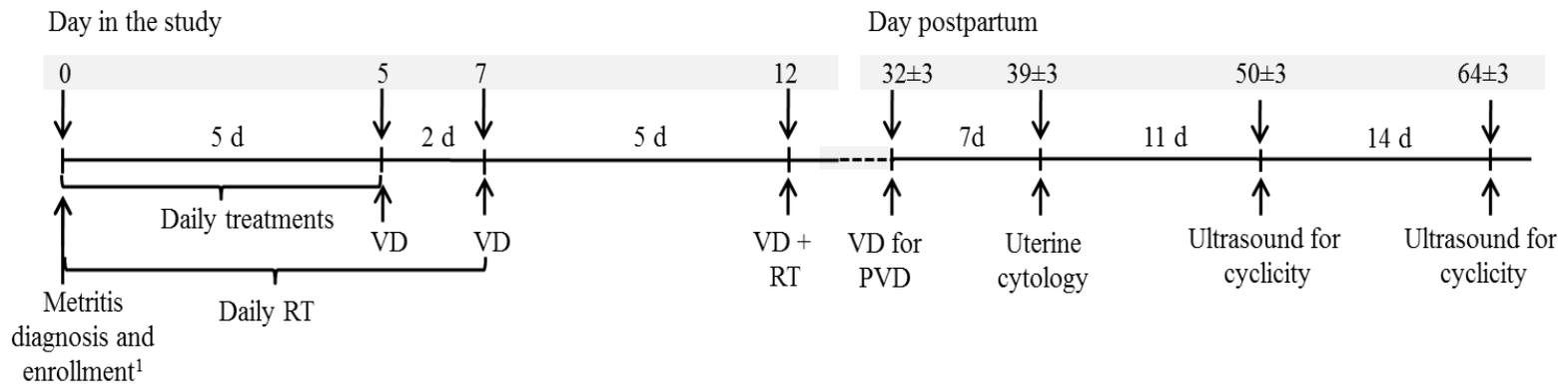


Figure 8-1. Diagram of treatments for metritis, monitoring of cure, and evaluation of uterine health. Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. Puerperal metritis was determined by VD score of 5 and rectal temperature (RT) ≥ 39.5 °C. PVD = purulent vaginal discharge.

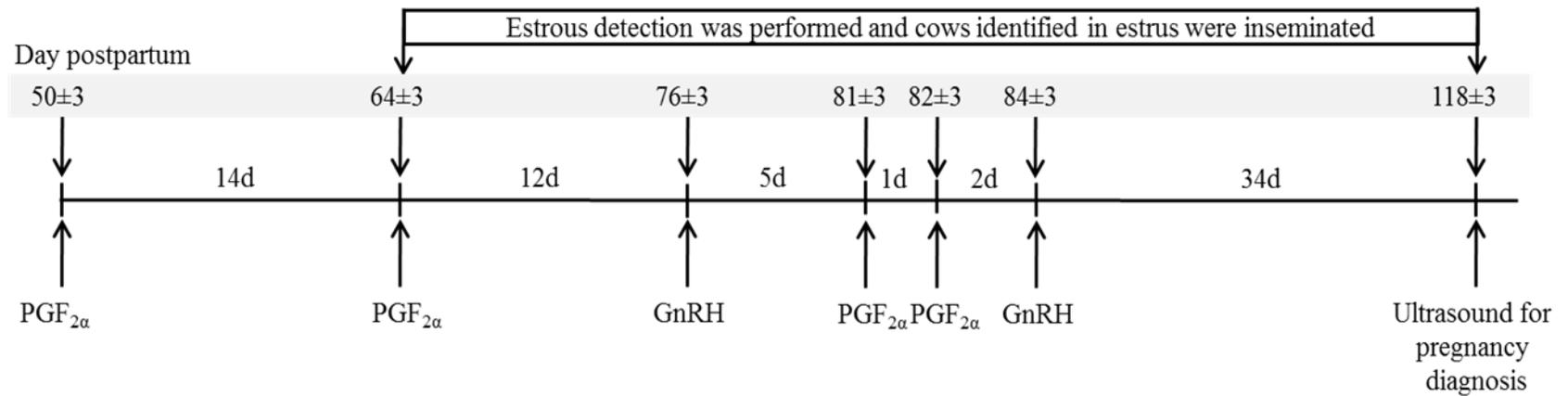


Figure 8-2. Diagram of reproductive program used for first insemination and pregnancy diagnosis. Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. Puerperal metritis was determined by VD score of 5 and rectal temperature (RT) ≥ 39.5 °C. Cows treated for metritis and a cohort of herdmates without metritis were subjected to a presynchronized 5-d timed AI program. Cows observed in estrus any time after the second 64 ± 3 d postpartum were inseminated.

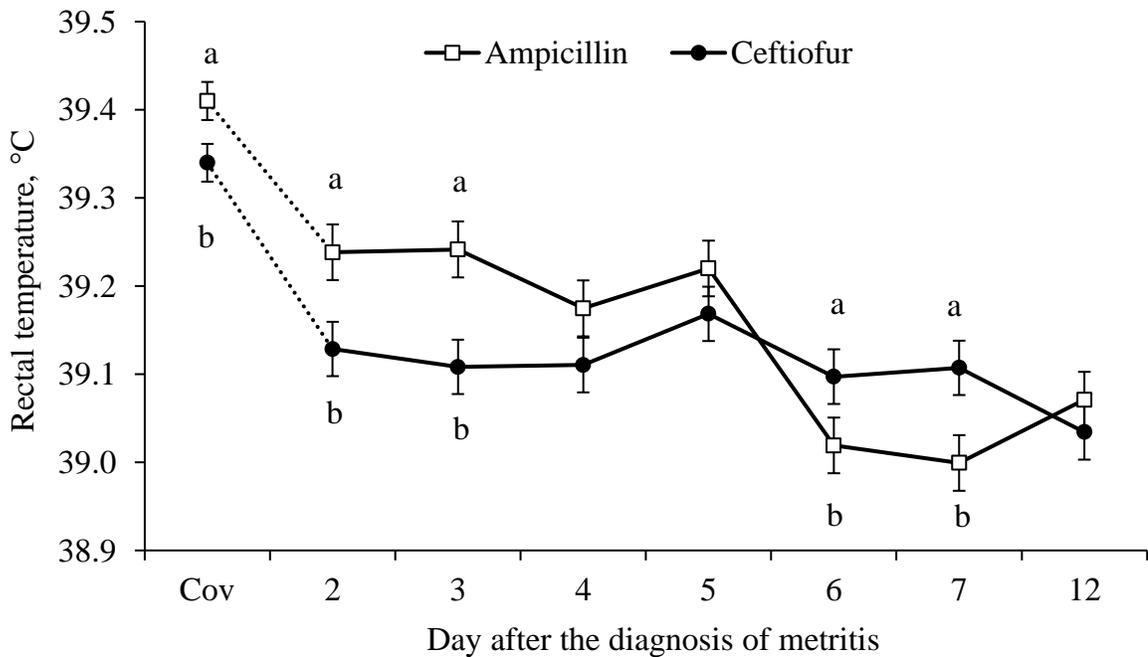


Figure 8-3. Rectal temperatures on d 2, 3, 4, 5, 6, 7 and 12 after the diagnosis of metritis according to treatment. Cov = covariate value measured on the day of study enrollment. Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. Effect of treatment ($P = 0.09$), type of metritis ($P < 0.001$), day ($P < 0.001$), interaction between treatment and type of metritis ($P = 0.20$), and treatment and day ($P < 0.001$). Within a day, different letters denote statistical difference ($^{a,b} P \leq 0.05$) between treatments.

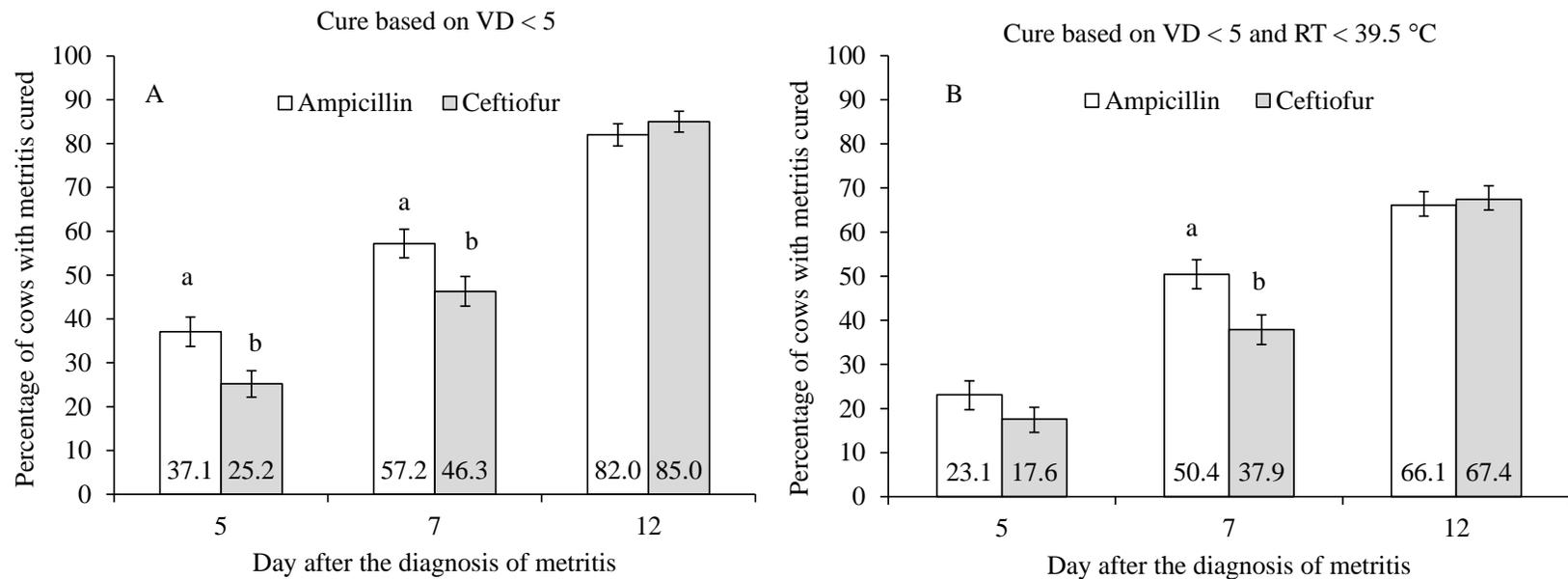


Figure 8-4. Adjusted proportions (\pm SEM) of cows with metritis cured on d 5, 7 and 12 after initiation of treatments with ampicillin or ceftiofur. Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. On panel A, cure of metritis was based on VD < 5. On panel B, cure of metritis was based on VD < 5 and rectal temperature (RT) < 39.5 °C. Within a day, different letters denote statistical difference ($P \leq 0.05$) between treatments.

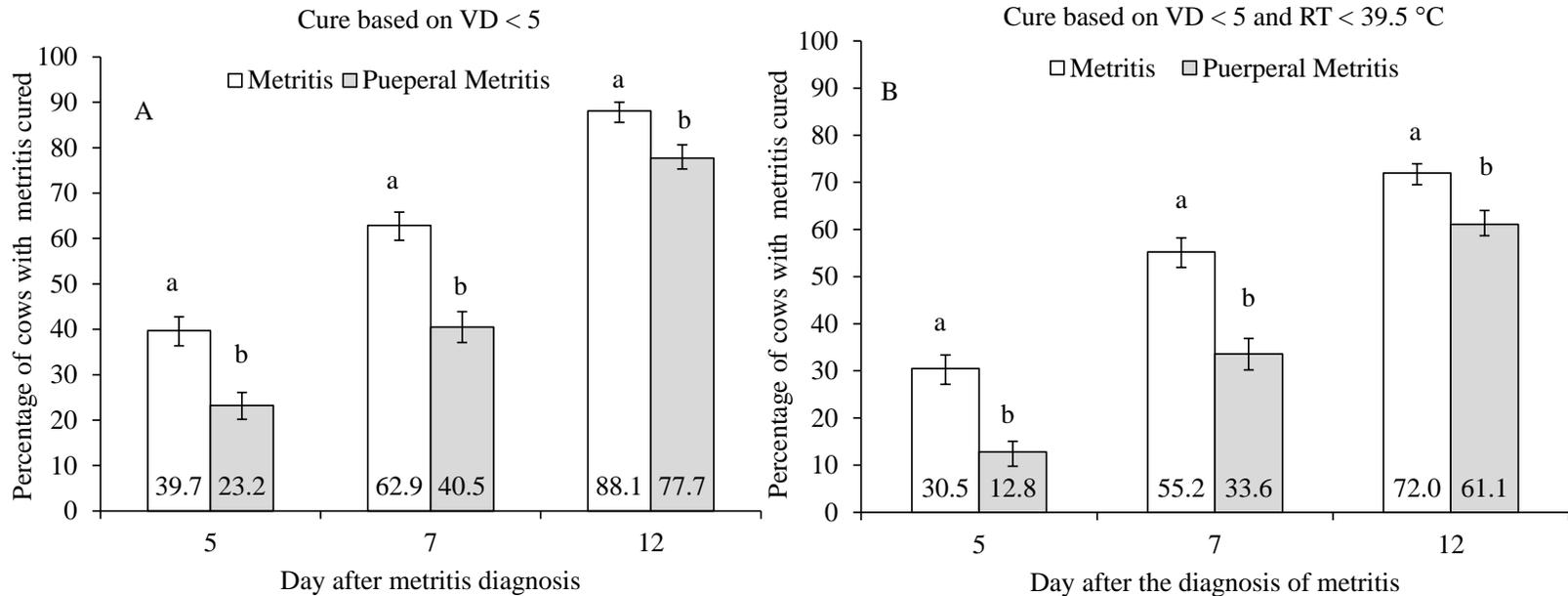


Figure 8-5 Adjusted proportions (\pm SEM) of cows with metritis cured on d 5, 7 and 12 after initiation of treatments according to initial diagnosis of metritis only or puerperal metritis. Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. On panel A, cure of metritis was based on VD < 5. On panel B, cure of metritis was based on VD < 5 and rectal temperature (RT) < 39.5 °C. Within a day, different letters denote statistical difference ($P \leq 0.05$) between treatments.

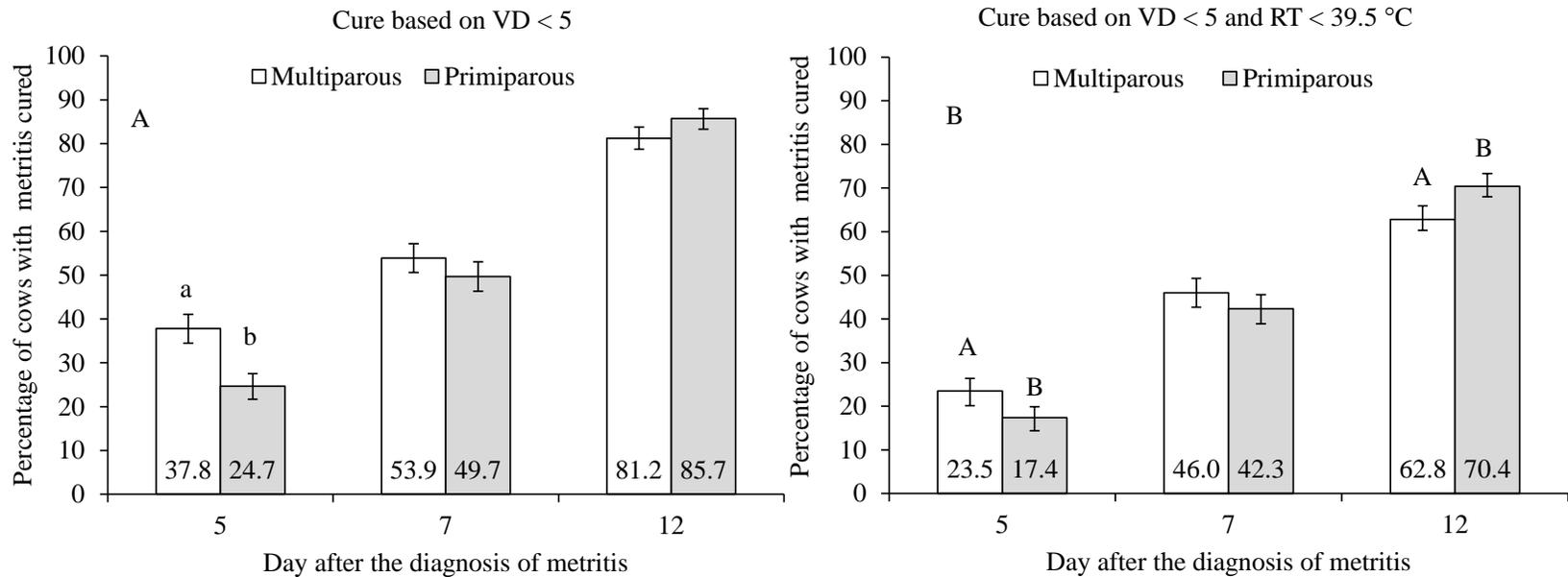


Figure 8-6. Adjusted proportions (\pm SEM) of cows with metritis cured on d 5, 7 and 12 after initiation of treatments according to parity. Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. On panel A, cure of metritis was based on VD < 5. On panel B, cure of metritis was based on VD < 5 and rectal temperature (RT) < 39.5 °C. Within a day, different letters denote statistical difference (^{a,b} $P \leq 0.05$) between treatments. Within a day, different letters denote tendency for statistical difference (^{A,B} $P \leq 0.10$).

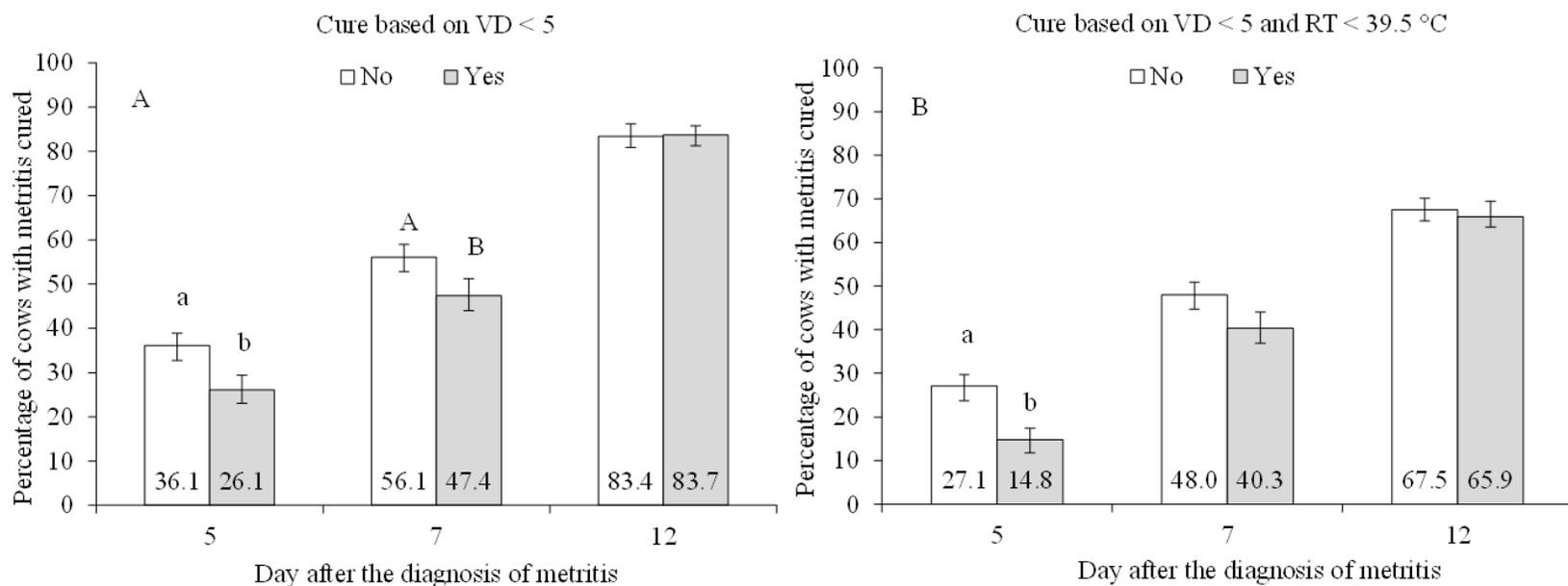


Figure 8-7. Adjusted proportions (\pm SEM) of cows with metritis cured on d 5, 7 and 12 after initiation of treatments according to diagnosis of calving-related disorders (dystocia, stillbirth, twins, and retained placenta). Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. On panel A, cure of metritis was based on VD < 5. On panel B, cure of metritis was based on VD < 5 and rectal temperature (RT) < 39.5 °C. Within a day, different letters denote statistical difference (^{a,b} $P \leq 0.05$) between treatments. Within a day, different letters denote tendency for statistical difference (^{A,B} $P \leq 0.10$).

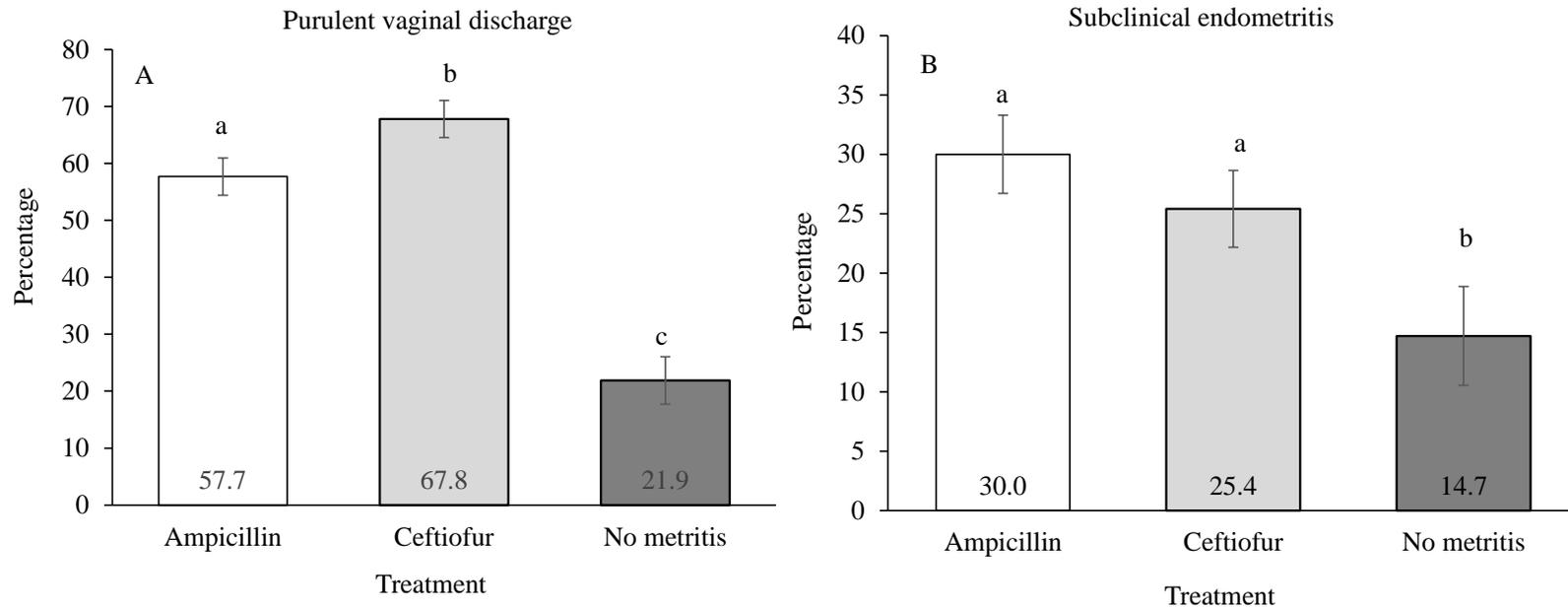


Figure 8-8. Adjusted proportions (\pm SEM) of cows with purulent vaginal discharge on d 32 ± 3 postpartum (panel A) or with cytological subclinical endometritis on d 39 ± 3 postpartum (panel B). Cows diagnosed with metritis were blocked by parity and type of metritis (only metritis vs. puerperal metritis) and, within each block, assigned randomly to receive 11 mg/kg of ampicillin or 2.2 mg/kg of ceftiofur once daily for 5 d. Metritis was defined as vaginal discharge (VD) score of 5 based on discharge of watery brown/red foul smell. For panel A, effect of treatment ($P < 0.001$). For panel B, effect of treatment ($P < 0.001$). Within a day, different letters denote statistical difference ($^{a,b,c} P \leq 0.05$).

CHAPTER 9 CONCLUSIONS AND FUTURE DIRECTIONS

The studies presented in this dissertation contributed to advance the fields of reproductive physiology of dairy heifers, reproductive management of dairy cows, and uterine health of dairy cows having direct implication to dairy sciences and the dairy industry.

The results presented in Chapter 3 revealed that In spite of the long re-insemination interval for second and third AI, cows receiving 3TAI became pregnant at a faster rate than cows receiving a single timed AI before introduction to natural service. The improved reproductive performance of 3TAI cows resulted in 15% greater hazard of pregnancy, 17% greater risk of pregnancy, and 9 fewer days nonpregnant than 1TAI cows. The faster pregnancy rate was likely the result of a combination of increased breeding of nonpregnant cows associated with improved probability of pregnancy to a breeding, which improved reproduction during the first 84 d in the study. Therefore, in herds in which detection of estrus is not carried out, a combination of AI and natural service is used, bulls are managed to optimize their fertility, and the resynchronized timed AI is implemented using the Ovsynch protocol with a CIDR insert, it is advantageous to allow cows multiple inseminations before bull exposure for natural service to optimize pregnancy rate. Results from this study indicate that in herds in which detection of estrus is not carried out, and a combination of AI and natural service is used, cows should receive at least three timed AI before bull exposure for natural service.

The results of Chapter 4 showed that administration of GnRH on the first day of the 5-d timed AI protocol did not influence P/AI or pregnancy loss of dairy heifers when

a single treatment with PGF_{2α} on day 5 of the protocol is used. Although ovulation on study day 0 and presence of new CL 5 d later was low, treatment with GnRH increased ovulation rate in heifers compared with no GnRH treatment. As consequence of the increased ovulation and formation new CL, the proportion of heifers with low progesterone at AI when receiving a single PGF_{2α} treatment 5 d later was less for those receiving GnRH than controls. Timing of induction of ovulation with GnRH relative to AI influenced P/AI of heifers not displaying estrus on the day of the timed AI. In general, P/AI was better when heifers received the ovulatory stimulus concurrent with AI at 72 h after PGF_{2α} than 16 h before timed AI. Therefore, when heifers are subjected to the 5-d timed AI program with a single treatment of PGF_{2α}, it is suggested that the initial GnRH is not necessary and the period of proestrus should be 72 h with administration of GnRH to induce ovulation concurrent with timed AI.

The results of Chapter 5 complement those of study 1 in Chapter 4 showing increased follicle turnover at initiation of 5-d timed AI program by using GnRH combined with two doses of PGF_{2α} administered on d 5 and 6 to optimize luteolysis successfully improved P/AI in dairy heifers. Results of the current study demonstrate similar concepts of previous work with lactating dairy cows reinforcing the need for estrous and ovulation synchronization protocols to incorporate physiological principles to optimize fertility in dairy heifers. Follicle turnover by inducing ovulation with GnRH, although low in dairy heifers, was beneficial to fertility. However, the benefit of GnRH to optimize fertility requires two doses of PGF_{2α} administered 24 h apart to increase regression of a newly formed CL. The P/AI of approximately 60% obtained in the current study supports the use of the 5-d timed AI protocol as an alternative breeding program for reproductive

management of heifers when detection of estrus is not used. Finally, it was demonstrated that high concentrations of progesterone when GnRH was administered suppressed the LH release and impaired ovulation. Further research is needed to determine if additional increase in ovulation to the initial GnRH of the 5-d timed AI protocol can further improve fertility in dairy heifers.

The results from Chapter 6 demonstrated that treatment with one or two injections of PGF_{2α} in early lactation before cows were subjected to a presynchronized timed AI protocol was unable to improve uterine health and measures of fertility in lactating dairy cows. Subclinical endometritis impaired P/AI and maintenance of pregnancy in lactating dairy cows, particularly when associated with PVD, and the negative effect of subclinical endometritis was observed when the inflammatory process persisted until 46 DIM. Interestingly, when both PVD and subclinical endometritis were associated or when subclinical endometritis persisted by 46 DIM, pregnancy loss increased. Future research should focus on understanding the mechanism leading to persistent inflammatory diseases in dairy cows and to develop new strategies that can mitigate the negative impact of subclinical endometritis on fertility.

Chapter 7 revealed that intrauterine infusion with *T. pyogenes* disrupts luteal function and leads to early demise of CL at least in part of the cows. Although, *T. pyogenes* clearly induced endometrial inflammation, we were unable to identify a consistent response in endometrial mRNA expression of genes linked to inflammation and the luteolytic cascade that could support a direct anticipated interplay between the bacterium-induced inflammatory response and factors triggering the early demise of the

CL. Future research should focus on investigating the immune response and activation of the endometrial luteolytic cascade in cows that undergo early demise of the CL.

The results from Chapter 8 lead to the conclusion that ampicillin was an efficacious alternative therapy for metritis resulting in faster cure rates than ceftiofur; however, by day 12 after the diagnosis of metritis, no differences between treatments were observed. Ampicillin reduced the prevalence of cows with PVD on day 32 postpartum compared with cows receiving ceftiofur, but treatment did not affect the prevalence of cytological endometritis in cows previously diagnosed with metritis. Similarly, treatment did not affect the resumption of estrous cyclicity by 64 days postpartum, P/AI at first AI, and the risk of pregnancy loss. Although, cows without metritis had reduced prevalence of PVD and subclinical endometritis compared with metritic cows, estrous cyclicity and P/AI did not differ between those without metritis and metritic cows treated with ampicillin or ceftiofur. Type of metritis is an indicator of the severity of disease and cows with puerperal metritis have poorer cure rates than those with metritis alone. Nevertheless, type of metritis did not influence subsequent estrous cyclicity, P/AI, or risk of pregnancy loss. Future research should investigate the cost benefit of using ampicillin in comparison to ceftiofur and characterize the microbiome of cows before, during and after treatment with ampicillin to determine how this antimicrobial therapy affects the microbial uterine flora and leads to cure of metritis.

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BIOGRAPHICAL SKETCH

Fábio Soares de Lima was born on January 30, to Gaspar de Lima and Dolores Soares de Lima in Muzambinho, Minas Gerais, Brazil. He is the third of four children that grew up on a dairy farm surrounded by cows from an early age. After graduation from high school he was accepted and enrolled in the College of Veterinary Medicine at São Paulo State University, where he had an opportunity to work under the supervision of Dr. José Luiz Moraes Vasconcelos. In 2004, during the clinical year of his veterinary studies, he spent 5 months at the University of Wisconsin, in Madison working on research projects that involved physiology of reproduction, health and reproductive management of dairy cows under the supervision of Dr. Milo Wiltbank. After his graduation from São Paulo State University, he worked for 18 months at the University of California, Davis in the Veterinary Medicine Teaching and Research Center in Tulare, under the supervision of Dr. José Eduardo P. Santos, conducting research in the areas of dairy cow nutrition, reproduction, and health. In July 2006, he was accepted as clinical resident in the Food Animal Reproduction and Medicine Program in the College of Veterinary Medicine at the University of Florida. In 2007, he started a Master of Science program in Clinical Sciences under the supervision of Dr. Carlos A. Risco that was concluded in 2009. After completion of his MSc he remained at University of Florida and received his PhD degree from the Department of Animal Sciences under advisement of Dr. José Eduardo P. Santos. After completion of his PhD degree he will move to Cornell University to work as postdoctoral associate with Dr. Rodrigo Bicalho.