EFFECT OF SUBCLINICAL ENDOMETRITIS ON OVARIAN AND UTERINE RESPONSE TO A TIMED AI PROTOCOL IN DAIRY COWS

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled:

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Presented by Roger Molina-Coto

A candidate for the degree of Master of Science

We hereby certify that in our opinion it is worthy of acceptance

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Dedication

There is no way to pay off the contribution from all people that helped to get done this experience in USA, thanks for making easier, worthy and enjoyable these two years.

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Abbreviations

AI	Artificial insemination
BCS	Body condition score
BHB	Beta hydroxybutyrate
BoHV-4	Bovine herpesvirus 4
BUN	Blood urea nitrogen
CB1	Cytobrush exam 1
CB2	Cytobrush exam 2
CIDR	Controlled internal drug release
CL	Corpus luteum
CXCL	Chemokine C-X-C motif 8
CXCR	C-X-C chemokine receptors
DIM	Days in milk
ELISA	Enzyme-linked immunosorbent assay
Foll	Ovarian follicle evaluation
HMGCR	3 - hydroxy -3- metil-glutaryl - CoA reductase
HSD	Hydroxyl-steroid dehydrogenase
IGF1	Insulin like growth factor 1
IGFBP	Insulin like growth factor binding protein
IL	Interleukin
INFt	Interferon tau
ISG	Interferon stimulated genes
LAB	Lactic acid bacteria

Larg2F	The second largest follicle
LargF	The largest follicle
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
MUC	Mucine
Mx	Myxovirus
NEFA	Non esterified fatty acids
NK-cells	Natural killer cells
NSAID	Non-steroidal anti-inflammatory drugs
Ov	Ovulation
P/AI	Pregnancies per artificial insemination
P4	Progesterone
PAM	Pathogen associated molecules
PG	Prostaglandin
PGES	Prostaglandin E synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFM	Prostaglandin F metabolite
PGHS2	Prostaglandin H synthase type 2
PMN	Polymorphonuclear neutrophils
Preg	Pregnant
PTA	Predicting transmitted ability
PTAFR	Platelet-activating factor receptor
PTGS	Prostaglandin endoperoxide synthase

PVD	Purulent vaginal discharge
RTP	Receptor transporter protein
SCE	Subclinical endometritis
STAR	Steroidogenic acute regulatory protein
TLR	Toll like receptor
TNF	Tumor necrosis factor
UH	Uterine health
US	Ultrasound scanning

ACADEMIC ABSTRACT

EFFECT OF SUBCLINICAL ENDOMETRITIS ON OVARIAN AND UTERINE RESPONSE TO A TIMED AI PROTOCOL IN DAIRY COWS

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During parturition the uterus of the dairy cow is more exposed to the environment. Bacteria colonize the uterus causing infection. The immunological response of the cows is able to clear infection, however, around 20% of cows remain with infection and a prolonged inflammatory process after calving. Uterine disease with clinical or subclinical signs [subclinical endometritis (SCE)] can develop. Uterine disease impairs fertility by decreasing pregnancy rates, increasing days open and embryonic losses.

There are theories about how uterine infection affects reproductive physiology, however, some of the conclusions are only based on in vitro model studies. The short or long term effects of uterine disease on the ovary and uterus are not clear.

This study evaluated the effects of SCE diagnosed by cytobrush on ovarian and uterine response to a timed AI protocol in dairy cows. Ovarian structures were evaluated by ultrasound at specific time points of the timed AI protocol. Blood samples were taken to evaluate plasma progesterone and IGF1 concentrations. Uterine performance was evaluated by using ISG15 expression in response to INFt secretion from the embryo. Pregnancy rate was evaluated by using different methods of pregnancy detection.

Overall, no major alterations were found in cows diagnosed with SCE on ovarian or uterine response to a timed AI protocol. Pregnancy rate also was similar in uterine

healthy cows and cows with SCE at the first exam but 100 % of embryonic losses were found from day 25 to 45 after timed AI based on the second cytobrush exam.

1 Chapter.

Literature Review

1.1 Definition of uterine disease

Metritis is a uterine infection that manifests as clinical disease. It typically presents fetid vaginal discharge between 3 to 9 days after calving. The cow may also present fever, which makes it a systemic illness, that reduces cow performance in a short time (Esposito et al., 2014; LeBlanc, 2014). The definition of the infection changes according to the presence of clinical symptoms and time postpartum. Clinical endometritis has been defined as uterine inflammation without systemic illness after 20 days in milk (DIM). A mucopurulent vaginal discharge may also be present (Esposito et al., 2014). Cows with metritis and cows experiencing problems at calving have an increased incidence of endometritis and subclinical endometritis (SCE) (Bittar et al., 2014). Although metritis tends to be associated with purulent vaginal discharge (PVD) and mixed bacterial infection, SCE is not usually associated with PVD or bacterial infection (LeBlanc, 2014). Subclinical endometritis does not present any clinical symptoms. Leucocyte infiltration, mainly polymorphonuclear neutrophils (PMN), and edema are the main signs of this disease. Diagnosis of SCE is commonly done by techniques such as cytobrush or uterine flushing (Esposito et al., 2014).

1.2 Incidence of uterine disease

The reported incidence of disease in dairy cows from calving to artificial insemination is 42%. This percentage includes diseases with a local infection such as uterine infection, but also systemic diseases such as digestive and respiratory diseases. The presence of disease in dairy cattle decreases fertilization and conceptus development (Ribeiro et al., 2015). Incidence of PVD and SCE at 35 (DIM) was 17.1 and 36.2%

respectively (Denis-Robichaud and Dubuc, 2015a). de Boer et al. (2015) reported 10, 15 and 7% incidence of PVD at 0, 21 and 42 DIM respectively. Prunner et al. (2014b) reported an incidence of SCE of 21% from 20 to 30 DIM. In a crossbred dairy herd, between 32 to 70 DIM, 26% of cows were diagnosed with SCE by cytobrush (cutoff = 5% PMN) (Carneiro et al., 2014). At 65 DIM cows were assessed for SCE by cytobrush, and a threshold of 5% of PMN was used. The average of PMN for SCE cows was 16.02% and for control cows was 3.7% (Brodzki et al., 2014a).

Subclinical endometritis in repeat-breeder cows has not been evaluated as fully as in first service cows. Pothmann et al. (2015) studied cows with 3 or more unsuccessful services in a row and assessed SCE by cytobrush technique (cutoff: 5% PMN). The incidence of SCE was 12.7%. More than 90% of the diagnosed SCE was found in multiparous cows. These findings indicate that uterine disease affects significantly dairy herds.

1.3 Development of uterine disease

Bacteria are present in the uterus right after calving. Jeon et al. (2015) evaluated the population of bacteria in the uterus by using metagenomics. The first sampling was within the first 20 minutes after calving. The uterine environment was rich in bacteria. Metritic and healthy cows share similar bacteria after calving, but quickly differ for cows that develop metritis at that time. *Bacteroides* and *Fusobacterium* were the most common genus identified in metritic cows in the first six days after calving. *Candidatus blochmannia, Scherichia Sneathia* and *Pedobacter* were associated with uterine health (Jeon et al., 2015).

Some cows are able to clear infection by 2 or 3 weeks postpartum, but some do not. Neutrophils are the primary immune cells attempting to clear the bacterial infection.

Concomitant with the bacteria, dairy cows have metabolic disturbances such as elevated non-esterified fatty acids (NEFA) and low glucose concentrations (LeBlanc, 2014). Abnormal metabolic profiles are associated with delayed clearance of bacteria. The metabolic status affects neutrophil function and generates inflammatory factors (LeBlanc, 2014). Recruitment and activation of PMN is the main immune response to bacterial infection in the uterus. The function of PMN is vital to clear the infection and avoid the transition from physiological to pathological infection (Kimura et al., 2014). The immune system of cows is affected by all the challenges of the transition period, including impaired neutrophil function, low lymphocyte and antibody responsiveness and diminished cytokine releases (Esposito et al., 2014). A successful immune response in a healthy cow is able to resolve the inflammatory state postpartum to a regular or homeostatic state within a period of 1 to 3 weeks. Cows that develop endometritis or SCE are not able to do this and maintain the inflammatory response for a longer period (Foley et al., 2015).

Bacterial colonization or growth within the uterine tissue releases endotoxins called pathogen associated molecules (PAM). Gram negative bacteria, for instance, release a lipopolysaccharide (LPS). Endometrial cells have receptors that are able to detect PAM. Once a receptor is activated, it triggers cytokines and chemokines to recruit and activate immune defense cells (Healy et al., 2014).

The pathway through which uterine disease causes reproductive failure is still uncertain. Endotoxins from bacteria, however, may play a role. An ex vivo model was developed to evaluate the effect of LPS, on theca cells of different stages of ovarian follicular development (Magata et al., 2014). The theca cells of pre-selected follicles

were more susceptible to LPS when stimulated with progesterone than cells from post-selected follicles (in this case, pre or post selected follicles refer to follicular wave stages). No difference was found when theca cells were stimulated with estradiol. Healey et al. (2016) showed that intermediates of the mevalonate pathway are associated with inflammatory response when endometrial cells are stimulated by LPS. Lipopolysaccharide stimulation on endometrial cells produced less inflammatory signals [Interleukin -1 and C - X - C protein chemokine ligand (IL1 and CXCL8 respectively)] when an inhibitor of squalene synthase was used. The concentration of inflammatory signals was similar, however, when an inhibitor of 3 - hydroxy - 3 - metyl-glutaryl - CoA reductase (HMGCR) was used. Cholesterol concentrations did not affect inflammatory signals.

1.4 Diagnosis of uterine disease

Different methods for diagnosis of reproductive tract disease in cows have been used. Vaginoscopy (Hartmann et al., 2016), palpation (Hartmann et al., 2016), ultrasonography (Polat et al., 2015; Toni et al., 2015), uterine flush (Bicalho et al., 2016), cytobrush (Madoz et al., 2014; Denis-Robichaud and Dubuc, 2015b; Ledgard et al., 2015; Pascottini et al., 2015; Polat et al., 2015; Hartmann et al., 2016), cytotape (Pascottini et al., 2015), metricheck device (Denis-Robichaud and Dubuc, 2015b; Ledgard et al., 2015; Bicalho et al., 2016), gloved hand (Toni et al., 2015) and biopsies (Hartmann et al., 2016) (Madoz et al., 2014) are some of the methods used to assess metritis, clinical and subclinical endometritis and cervicitis.

Plasma estradiol concentrations have shown to affect the sensitivity of vaginal discharge (Metricheck and gloved hand) and ultrasound evaluations (presence of fluid into the uterus and diameter of the cervix) for the diagnosis of uterine disease (Silper et

al., 2016). Polymorphonuclear neutrophils, however, are considered good indicators for SCE because they are not significantly affected by the estrous cycle stage or circulating progesterone concentrations (Madoz et al., 2013).

Recently, the percentage of PMN from uterine cytology showed a high correlation with gene expression related to inflammatory response. Genes with a high correlation were C – X – C motif chemokine receptor 2 (CXCR2), Interleukin -1A (IL1A), Interleukin – 1B (IL1B) and Interleukin – 8 (IL8) where the correlation was above 0.6 (Peter et al., 2015).

1.5 Molecular biology approaches to diagnose uterine disease

The presence of PMN in luminal epithelial cells is an indicator for SCE. Other biomolecules are currently used to diagnose uterine diseases as well. Gene expression of cytokines such as IL1A, IL6, IL17A, tumor necrosis factor – α (TNFα), and enzymes such as prostaglandin H synthase type – 2 (PGHS2) and prostaglandin E synthase (PGES) are highly correlated with the percentage of PMN in the uterus between 29 to 36 DIM. The cytokines are involved in PMN recruitment and inflammatory responses (Johnson et al., 2015). Specifically, IL1A has been recognized as the signal that triggers inflammation in response to a combination of endometrial cell damage and bacteria (Healy et al., 2014).

Different types of uterine samples have been used to evaluate these biomolecules, and they have also been evaluated in peripheral blood. Cows with SCE at 45 to 55 DIM have more mononuclear and PMN cells in peripheral whole blood than cows without SCE. Among mononuclear cells, B-cells, natural killer cells (NK-cells) and CD172α positive monocytes were elevated. At the same time, mRNA expression was greater for CXCL8, TNF and IL12 which are potential indicators for SCE in peripheral blood (Duvel

et al., 2014). Some proteins have been correlated with the percentages of PMN as well. Ledgard et al. (2015) found a strong correlation between percentages of PMN and cathelicidin, PGLYRP1, SERPINB1 and S100A9. These proteins are products of immune cells in response to a current infection and also during a prolonged inflammatory response.

In addition to gene expression analyses, ELISA tests have been used to determine concentrations of cytokines and acute phase proteins at 60 DIM from SCE and healthy cows. Serum TNFα, IL6, IL10, haptoglobin and serum amyloid A (SAA) had greater concentrations in SCE than healthy cows. In uterine washes, only IL1, IL10 and haptoglobin were different. The evidence suggests that these molecules are candidates for biomarkers of uterine disease (Brodzki et al., 2015b). Evaluating the same molecules at an earlier DIM (5, 22 and 40 DIM), the authors found that these molecules were not appropriate indicators of uterine disease at 5 DIM but they are at 22 and 40 DIM from serum or uterine washes (Brodzki et al., 2015a). Kim et al. (2014) using commercial ELISA tests, evaluated cytokine concentrations (TNFα, IL6 and IL10) from uterine washes at 28 DIM. These cytokines were greater in cows diagnosed with clinical endometritis than in cows with SCE or healthy cows. Conversely, concentrations were not different when they were measured systemically (in serum). Some proteins and cytokine concentrations, however, failed to differentiate between uterine diseases. Kasimanickam et al. (2014) showed that cows with metritis or SCE had greater mRNA expression for mucine – 1 (MUC1), toll like receptor – 4 (TLR4), IL1β, IL8, TNFα, insulin like growth factor – 1 (IGF1) and IGF binding protein – 2 (IGFBP2) than healthy cows. These genes were similar between cows diagnosed with SCE and healthy cows

except for IL1β and TNFα. Similarly, Johnson et al. (2015) did not find differences between clinical metritis and subclinical endometritis in the expression of cytokines and enzymes related to the synthesis of prostaglandins, but both differed from healthy cows.

The mRNA expression of cytokines (IL1A, IL1B, IL6 and TNFα) have shown expected changes with respect to the resolution of uterine disease over the time. Cytokine concentrations were less in cows categorized as mild, moderate and severe metritis at 42 DIM than 4 days after calving. These results are consistent with the expected infection resolution of uterine disease (Heppelmann et al., 2015).

In order to evaluate short and long term effects of uterine diseases, the transcriptome of specific genes have been analyzed in healthy, SCE and clinical endometritis cows. In one study, the first diagnosis of SCE was done approximately at 27 DIM by using cytobrush and vaginoscopy. The same evaluation was done weekly until 48 DIM. Long term effects of uterine disease were evaluated based on the first and last examination of the female reproductive tract. A greater mRNA expression of chemokines and enzymes such as prostacyclin synthase and prostaglandin D2 synthase was found in cows with endometritis. When data were analyzed for each individual week (current uterine health status), cows with endometritis presented greater RNA expression of prostaglandin endo peroxidase synthase - 1 (PTGS1), prostaglandin endo peroxidase synthase – 2 (PTGS2), prostaglandin endo peroxidase synthase – 3 (PTGS3), prostaglandin D synthase (PTGDS), CXCL1/2, CXCL3, CXCL5, CXCR2, IL1A, IL1B, IL6, IL8, TNF, matrix metalloproteinase -1 (MMP1) and platelet activating factor receptor (PTAFR) at 48 DIM but not during earlier sampling days. This demonstrates a strong late immune response in cows with endometritis (Peter et al., 2015).

Acute phase proteins, such as haptoglobin, did not differ in cows with or without clinical endometritis during the first 8 weeks postpartum. Their concentrations were significantly different by week, suggesting no relationship between systemic inflammation and clinical endometritis (Yasui et al., 2014). Early postpartum (10 DIM), serum haptoglobin concentrations distinguished between metritic versus healthy cows when cows were multiparous cows, but not if they were primiparous cows (Burfeind et al., 2014).

There is not a lot of information about uterine disease in repeat breeder cows. Kasimanickam et al. (2014) showed that repeat breeder cows with subclinical endometritis had greater expression of MUC1, TLR4, IL1β, IL6, IL8, TNFα, IGF1 and IGFBP2 than normal cows (non-repeat breeder cows). Only MUC1, IGF1, IGFBP2 had greater expression in repeat breeder cows without SCE when compared with normal cows.

1.6 Uterine disease and reproductive outcomes

It is known that uterine disease causes impaired reproductive performance. Subclinical endometritis evaluated by cytobrush or uterine lavage at 35 or 49 DIM, showed that its incidence increases the number of days open and reduces pregnancies per artificial insemination (P/AI) (Vieira-Neto et al., 2014). During the same period (35 and 49 DIM) Galvao et al. (2009) reported 30 and 40 more days open which is consistent with the 30-day delay reported by Madoz et al. (2013). Also conception rate at the first service was reduced.

The effect of uterine disease on reproductive performance has not been the same for all categories of cows. Multiparous cows are more susceptible to metritis. Metritis results in delayed time to first insemination and longer days open in multiparous cows but

it did not affect primiparous cows (Toni et al., 2015). In the case of SCE diagnosed by cytobrush at 35 DIM with a cutoff of 5% PMN, the hazard to pregnancy was lower. Also, days open were 42 days longer in cows with SCE than in healthy cows (Bicalho et al., 2016). Not only are cows less likely to get pregnant when associated with uterine disease, there are also greater pregnancy losses. Clinical endometritis and retained placenta also increased the probability of pregnancy losses. Cows with retained placenta had 3.36 times greater odds and cows with clinical metritis had 2.16 times greater odds of losing pregnancy than cows without those diseases (Machado et al., 2015).

Evaluation of PVD by gloved hand at day 26 showed that cows with PVD had delayed resumption of cyclicity when compared with healthy cows. Pregnancies per artificial insemination were greater in healthy cows than cows with PVD. Purulent vaginal discharge was associated with more pregnancy losses (Maquivar et al., 2015). In beef cows, based on the percentage of PMN from uterine lavage between days 28 to 68 post-partum, SCE caused a 40 day delay to conception compared with healthy cows (Ricci et al., 2015).

The percentage of PMN along luminal epithelial cells is an indicator of SCE, but it is important to consider the DIM when the diagnostic test is done. When cows presented ≥25% of PMN at 0 DIM, for instance, they had a better chance for pregnancy than cows with less than 25% (de Boer et al., 2015). Cows with larger percentages of PMN at 21 DIM, however, had less chance for pregnancy. This shows that an early strong immune response increases fertility but a protracted inflammatory response decreases fertility (de Boer et al., 2015). This was confirmed by Peter et al. (2015) who showed that cows with endometritis around 48 DIM (late response) presented greater

concentrations of biomolecules related to the inflammatory response. Heppelmann et al. (2016) also found greater expression of IL1A, IL1B and TNFα in cows with endometritis than in healthy cows from d 0 to 65 after calving. This provides evidence for protracted inflammatory response in cows with endometritis.

The location where the sample is taken from the cow can also affect the outcome. Brodzki et al. (2014a) sampled the uterus and collected peripheral blood. They found that local uterine immune mechanisms were different than systemic immune mechanisms for healthy and SCE cows at 65 DIM. Even when systemic and local immune reactions were different, phagocytic activity of immune cells was lesser in cows diagnosed with SCE independent of the sample type (blood or uterus).

Intrauterine, cervical or vaginal sampling can also give different outcomes. Clinical endometritis assessed by gloved hand is not an accurate measurement because infection and inflammation from cervix or vagina can be interpreted as uterine infection, which is not always true (Madoz et al., 2014). Cervicitis and endometritis can be independent diseases that both cause poor reproductive performance. Endometritis is more severe than cervicitis solely, but if both occur together, reproductive performance is poorer (Hartmann et al., 2016).

The transcriptome in SCE cows depended on sample location as well. A group of healthy cows and cows with SCE were slaughtered at 29 DIM for transcriptome evaluation in liver and adipose tissue. Subclinical endometritis altered gene expression in liver and adipose tissue. The result indicated not only the known local effects, but also that systemic effects can decrease fertility. Subclinical endometritis was associated with changes in liver transcription for genes related to coagulate cascade, steroid hormone

synthesis, apoptosis, inflammation and oxidative stress. In adipose tissue, inflammation, oxidative phosphorylation, dynamics of long chain fatty acids and others were also altered in cows with SCE (Akbar et al., 2014).

Metagenomics analysis to evaluate the population of bacteria also differ by the type of sample evaluated. Cows analyzed at weeks 1, 4 and 7 after calving presented different bacteria population during each week, but also were different if the sample was from a uterine flush or biopsy (Knudsen et al., 2016).

Salilew-Wondim et al. (2016) evaluated the transcriptome in healthy cows and cows with clinical and subclinical endometritis. Both diseases caused dysregulation of genes, clinical endometritis in 177 genes and SCE in 28. Twenty-six out of the twenty-eight dysregulated genes in cows with SCE were also significantly dysregulated in cows with clinical endometritis. Genes involved in immune process, cell adhesion, regulation of neurogenesis, regulation of apoptotic signaling pathway, G-protein couple receptor signaling pathway and chemotaxis were affected by clinical or SCE.

1.7 Risk factors associated with uterine disease

Dubuc et al. (2010) identified risk factors for uterine disease. The authors determined that risk factors are not the same for metritis, PVD or SCE. High NEFA concentration pre-partum, dystocia, retained placenta and greater haptoglobin are the major risk factors for metritis. Twinning, dystocia, metritis and increased haptoglobin are associated with PVD, whereas low body condition score (BCS) at calving, hyperketonemia and increased haptoglobin during the first week post-partum were the identified risk factors for SCE.

1.7.1 Nutritional and metabolic factors

The effect of trace minerals on retained placenta, metritis and clinical endometritis were evaluated. Calcium, Mg, Mo and Zn were lesser in cows with retained placenta. Calcium, Mo, P, Se and Zn were lesser in cows with metritis and Ca, Cu, Mo and Zn were lesser in cows with SCE compared with healthy cows (Bicalho et al., 2014a). Salehi et al. (2016) found greater expression of genes associated with inflammation in cows with SCE (IL1B, IL8, IL10) and TNFα but did not find any differences when healthy and SCE cows were supplemented with oilseeds which are recognized to suppress inflammation. Other supplements such as monensin and high starch diets improved the activity of immune cells (PMN, phagocytes and monocytes), but they did not improve uterine health in cows with SCE diagnosed by uterine lavage at 8 and 47 DIM. Further investigation must be done to determine the effects of increased immune cell activity on conception risk (Yasui et al., 2015).

Metabolic pressure after calving, negative energy balance, and metabolic diseases such as ketosis increase the incidence of uterine disease (Williams, 2013). Metabolites such as NEFA and beta hydroxyl butyrate (BHB) were not different from 3 weeks before to 3 weeks after calving in cows that developed endometritis from 40 to 60 DIM. Energy balance pre-partum (based on intake of Mcal/d) was similar, but negative energy balance was more severe in the first 3 weeks postpartum in cows with endometritis than in healthy cows. Energy balance was positive at week 6 for both groups but significantly greater in healthy cows (Yasui et al., 2014). Severity of negative energy balance influences uterine involution postpartum and the ability to clear infection. Repare and regeneration of uterine tissue during involution is mediated by metabolic actions and hormones at the cellular level (Wathes et al., 2009). Wathes et al. (2007) reported that

severe negative energy balance is associated with more uterine inflammation and altered tissue remodeling and immune response.

Low body condition score has been associated with greater incidence of SCE (Carneiro et al., 2014). In addition to low body condition score (BCS), glucose concentration and blood urea nitrogen (BUN) are associated with incidence of SCE as well. Cows diagnosed with SCE between weeks 5 to 7 post-partum had low BCS, low glucose concentration and greater BUN (Senosy et al., 2012). Other studies failed to link BCS with SCE, for example, Akbar et al. (2014) did not find an association between SCE and BCS. Interestingly, milk yield and blood metabolites such as NEFA, protein, albumin, globulin, magnesium, glutamate dehydrogenase and aspartate aminotransferase did not differ for healthy cows and cows with SCE diagnosed between 22 and 25 DIM (threshold of 18% of PMN) (Akbar et al., 2014). Duvel et al. (2014) also reported that concentration of Ca, BHB, NEFA and progesterone in serum did not differ between the healthy and SCE cows.

1.7.2 Infectious factors

The presence of bacteria affects conception. The process through which bacteria exerts its effects is still uncertain. It is clear that the presence of bacteria is related to clinical and/or subclinical uterine inflammation (Ghanem et al., 2015). The presence of specific bacterial populations in the uterus is also a factor involved in uterine disease. Cows with *Trueperella pyogenes* had a greater risk of presenting PVD, purulent uterine lavage, SCE and poor reproductive performance (Prunner et al., 2014a; Ghanem et al., 2015; Wagener et al., 2015; Bicalho et al., 2016). It is known that *T. pyogenes* secretes an exotoxin called pyolysin. This toxin causes hemolysis and cytological lysis in a cholesterol-dependent manner. Stromal cells have greater contents of cholesterol, so they

are more susceptible to pyolysin than luminal epithelial cells or immune cells. *T. pyogenes* will also cause more severe effects when luminal epithelial cells are damaged (Amos et al., 2014).

Wagener et al. (2014) showed that in addition to *Escherichia coli, T. pyogenes* and *Bacillus spp*., *Streptococcus uberis* is associated with uterine health in postpartum dairy cows. Cytobrush samples were taken at 3, 9, 15 and 21 DIM. They demonstrated that the presence of *S. uberis* at day 3 increased the risk of *T. pyogenes* infection at day 9 after calving.

The effect of bacteria can vary according to the physiological stage of the cow. Prunner et al. (2014b) found that the presence of *T. pyogenes* during the first four weeks after calving was associated with clinical metritis in primiparous cows but not in multiparous cows.

Prunner et al. (2014b), found that *E. coli, T. pyogenes, Streptococcus spp*. *Staphylococcus spp*. *Corynebacterium spp*. and *Bacillus spp*. were the most frequent bacteria isolated from the uterus between 20 to 30 DIM. Their work is in agreement with Heppelmann et al. (2015), who found that *E. coli, Bacillus spp*. and *T. pyogenes* were the most common. Isolation of *E. coli* at 0 DIM did not have an effect at 21 DIM on the isolation of *T. pyogenes* but *T. pyogenes* was associated with PVD (de Boer et al., 2015; Ledgard et al., 2015).

Twenty-seven percent of cows with SCE between 45 to 55 DIM showed presence of *E. coli,* but none tested positive for *T. pyogenes* (Duvel et al., 2014). Brodzki et al. (2014b) found that SCE cows were similar in bacteria population at 5 DIM, but significant differences were shown at 22 and 40 DIM. *E. coli, T. pyogenes, F.*

necrophorum, *P. melaninogenicus*, *Staphylococcus spp*. and *Streptococcus spp*. were isolated at 5 DIM, but only *T. pyogenes* was isolated in healthy cows at 22 and 40 DIM. Subclinical endometritis cows presented the same type of bacteria at 22 and 40 DIM compared with 5 DIM except for the absence of *Streptococcus spp*. at 40 DIM.

Bacteriology has been used as a tool to diagnose SCE but negative bacterial samples are sometimes positive for SCE when assessed by biopsy or cytobrush (Madoz et al., 2014). This finding opens the discussion about the nature of inflammation in SCE cows, whether sterile inflammation is present without current infection or bacteriology has some limitations. Prunner et al. (2014b) reported that from endometrial samples taken from 20 to 30 DIM for bacterial growth, only 60% had growth indicating that either a sterile environment existed or the culture technique had failed.

Bovine herpesvirus 4 (BoHV4) is the only virus directly associated with endometritis after calving. It attacks macrophages and produces chronic infections. It spreads easily in endometrial cells causing damage on endometrial epithelial and stromal cells (Sheldon et al., 2009b).

1.7.3 Management factors

Management factors and complications at parturition can increase the incidence of uterine disease. Retained placenta, calving difficulty and calving assistance have been identified as risk factors for clinical and subclinical endometritis and increases in days open (Healy et al., 2014; Prunner et al., 2014b; Wagener et al., 2014). The vaginal laceration score (VLS) which is related with calving difficulty, was evaluated by Vieira-Neto et al. (2016). Cows with severe vulvo-vaginal laceration were also cows more susceptible to develop uterine disease.

Cows suffering from dystocia tend to have a greater population of bacteria 3 weeks after calving and a greater concentration of inflammatory signals such as IL8, IL1B and IL1A (Healy et al., 2014).

Type of housing also affected the incidence of SCE in beef cows. Cows allocated and inseminated in tie stalls had a greater incidence of SCE than cows inseminated in free stalls. In the same study, parity was not a factor associated with SCE (Ricci et al., 2015). Stocking density at 100 and 80% of headlocks was evaluated in dairy cattle but no effects were found for retained placenta, metritis or PVD (Silva et al., 2014).

1.7.4 Genetic factors

A study evaluated sire predicting transmitted ability (PTA) for milk production and its association with health including the association with metritis, but no effect was found (Bicalho et al., 2014b). Whereas, Moore et al. (2014) showed that cows selected for high fertility had a lesser PVD and cytological endometritis than cows with low fertility traits.

Changes in DNA methylation have not been associated with SCE, so changes in gene expression are not believed to occur through an epigenetic mechanism. Genes that regulate immune response are more highly expressed in SCE cows than in healthy cows. Bacteriological findings do not correlate with the expression of inflammatory mediators in cows that had cleared bacteria previously. This suggests a lack of control of the immune system once infection has been cleared (Walker et al., 2015).

1.7.5 Immune response

Progesterone and estradiol may affect immune response. Specifically, *E. coli* and LPS response was tested on endometrial tissue from beef and dairy cattle. Endometrial tissue was tested in an ex vivo model, whereas endometrial epithelial samples, stroma

cells and peripheral blood leucocyte cells were evaluated by an in vitro model. In regard to progesterone and estradiol, cattle breed, *E. coli* or LPS stimulation had no effect on inflammatory mediators such as IL1B, IL6 and IL8. Data indicate that immune responses ex vivo and in vitro differ from in vivo evaluations where in all cases bacteria and endotoxins increase inflammation (Saut et al., 2014).

Impaired immune response is also a major factor for the development of uterine infection and inflammation in dairy cows. A group of cows were assessed by cytobrush at 5, 22 and 40 DIM. Subclinical endometritis was diagnosed at 22 DIM by using a cutoff of 18 % PMN. Cows with SCE showed lesser phagocytic activity of granulocytes and monocytes than control cows. Some biological markers such as CD4 +, CD14 + and CD25 + leukocyte cells were in lesser concentrations in cows with SCE in peripheral blood. CD21 + and CD8 + lymphocyte cells were significantly decreased in SCE cows from uterine flushes (Ghanem et al., 2015).

1.8 Effects of uterine disease on ovarian structures and their physiology

Metritis delays ovarian activity after calving (Vercouteren et al., 2015). The same effect was seen for SCE (> 8 % PMN at 25 DIM). Cows with SCE had delayed resumption of ovarian activity after calving (Salehi et al., 2016).

Some studies have examined the effects of uterine disease on ovarian structures and associated hormones. It is still unclear how uterine disease mediators affect ovaries, pituitary or hypothalamic function. A combination of all possible candidates may be necessary (Sheldon et al., 2009a).

The delay in the resumption of cyclicity in cows with uterine disease is related to changes in ovarian activity. The number of ovarian follicles in healthy cows was shown to be greater than in cows with PVD. Additionally, healthy cows had lower incidence of

cystic follicles (Maquivar et al., 2015). Tsousis et al. (2009) also had found a greater incidence of ovarian cysts in cows with clinical endometritis diagnosed from 14 to 42 DIM.

Anovulation is associated with SCE in dairy cows. Cows diagnosed with SCE at 42 DIM were less likely to ovulate between 63 to 70 DIM than healthy cows (Burke et al., 2010). Onset of ovarian function was also altered by SCE. Dubuc et al. (2012) showed that cows with SCE had a delayed resumption of ovarian activity after calving.

Early cyclicity post-partum is related with health and fertility, however, studies report different outcomes with respect to uterine disease and cyclicity. Galvao et al. (2010) reported that cows that started to cycle by 21 DIM had less incidence of SCE by 49 DIM and greater pregnancy per artificial insemination than anovulatory cows by 49 DIM. Carneiro et al. (2014) did not find any relationship between SCE, cyclicity, parity or DIM. Cows resuming ovarian cyclicity sooner after calving showed greater cytokine mRNA expression of inflammatory signals at 40 to 60 DIM than cows that started to cycle later (Heppelmann et al., 2015).

The effect of metritis on luteal size, progesterone concentration and enzymes involved in steroidogenesis during the first four estrous cycles after calving were tested. The concentration of progesterone was similar over time. The size of the corpus luteum was smaller in the metritic cows but only for the first estrous cycle. The amount of luteal RNA for steroid acute regulatory protein (StAR), Cytochrome P450 and 3B - hydroxyl steroid dehydrogenase (3β – HSD) was similar for healthy and metritic cows (Struve et al., 2013). Sheldon et al. (2009a) reported that uterine disease causes a decrease in

follicular size and estradiol concentrations. Progesterone concentrations were affected as well.

Long term effects of SCE on ovarian function have been identified. Green et al. (2011) showed that cows diagnosed with SCE at 21 DIM (cutoff: 18% PMN) had a self-cure rate of 81% by 42 DIM, but the initial SCE affected pre-ovulatory follicles at 63 DIM. Estradiol, dehydroepiandrosterone and androstenedione concentrations were significantly less in 8 to 10 mm follicles. Cortisol tended to be greater and testosterone lower in this same category of follicles.

Senosy et al. (2011) used a different approach to evaluate reproductive disorders. They evaluated the effect of ovarian structures on the incidence of endometritis. The presence of corpus luteum from week 3 to 7 after calving or follicular size was not associated with the incidence of endometritis. Delayed uterine involution was associated with the presence of endometritis in weeks 6 and 7 after calving.

Uterine pathogen load is also associated with ovarian structures and reproductive performance early postpartum. On 13 and 15 DIM, cows with greater uterine pathogen load had lesser concentration of estradiol and smaller follicles. On 24 and 26 DIM, the diameter of the corpus luteum was less and progesterone concentrations were reduced in cows with greater uterine pathogen load (Williams, 2013). The effect of *Arcanobacterium pyogenes* on endometrial and ovarian function was evaluated. Explants of epithelial and stromal endometrium were exposed to heat killed *A. pyogenes*. Killed bacteria did not affect the production of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) or PGE_2. Whereas, when explants were exposed to bacteria free filtrate from *A. pyogenes*, $PGF_{2\alpha}$ and PGE_2 concentrations increased. The effect of intrauterine infusion of bacteria free filtrate from

A. pyogenes on the estrous cycle was also evaluated. Infusion was done during the first nine days of the estrous cycle. No differences were found in the emergence of the follicular wave, dominant follicle and corpus luteum size. Peripheral plasma concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol, progesterone, metabolites of prostaglandins and acute phase proteins were unchanged (Miller et al., 2007). Kaneko and Kawakami (2008) confirmed that uterine infusion of *A. pyogenes* in cyclic cows did not change follicular dynamics or follicular size, however, uterine bacteria inoculation was associated with rapid regression of corpus luteum and an increase in prostaglandin F metabolite (PGFM).

The endotoxin LPS has been associated with extended luteal phases of estrous cycles. Lipopolysaccharide induces inflammatory mediators that shift the production of $PGF_{2\alpha}$ to PGE (Sheldon et al., 2009a). Herath et al. (2009) also showed *in vivo* and *in vitro* evidence for production of PGE compared with $PGF_{2\alpha}$ in cows with *E. coli* infection. Luttgenau et al. (2016), however, found the opposite effect in heifers. Intrauterine infusion of LPS decreased progesterone concentrations and reduced the lifespan of the corpus luteum in the cycle when LPS was infused, but also in the subsequent cycle.

1.9 Effects of uterine disease on estrous synchronization response

Synchronization of estrous cycles can give different reproductive outcomes with respect to uterine disease. The effect of clinical and subclinical endometritis at the initiation of a Presynch - Ovsynch protocol for timed artificial insemination (TAI) was tested. Uterine disease was diagnosed by ultrasonography and visual evaluation of vaginal discharge. Pregnancy rate at first service did not differ for cows with clinical and subclinical endometritis compared with healthy cows (Kasimanickam et al., 2006).

Uterine disease, however, affected the response to estrous synchronization when a controlled internal drug release (CIDR) was used. Cows without uterine disease were 1.9 times more likely to respond to a synchronization protocol using a CIDR and 2 times more likely to be pregnant after artificial insemination than cows with uterine disease (McNally et al., 2014).

1.10 Effects of uterine disease on the uterus

Uterine disease induces a protracted inflammatory response with recruitment of immune cells chemokines and cytokines that are combating uterine disease but also are causing uterine tissue disruption. This prolonged inflammatory stage can affect transport of spermatozoa, failures in embryonic attached and poor placental development (Sheldon et al., 2009b).

Angiosclerosis is a degenerative vascular lesion with deposition of collagen and/or elastic fibers in the vessel wall. It is also an effect of endometritis that has not been studied in full extent in dairy cows. Heppelmann et al. (2016) found that cows with endometritis presented greater incidence of angiosclerosis on the endometrium than uterine healthy cows, which possibly affects uterine functions.

1.11 Treatment of uterine disease

Systemic and local treatments have been used to treat uterine diseases. Cephapirin, which is a first generation cephalosporin antibiotic, is an approved treatment for purulent vaginal discharge in some countries. Denis-Robichaud and Dubuc (2015b) used uterine infusion to treat PVD and endometritis at 35 DIM. Cephapirin infusion did not reduce the prevalence of uterine disease or ovarian activity. Pregnancy at first service in cows with PVD, endometritis or both was always greater in cows receiving the Cephapirin infusion. These results were in agreement with the work of Runciman et al.

(2008) where intrauterine infusion with Cephapirin did not affect cure of endometritis. When cows had a major risk of severe uterine disease, within the first 21 DIM, however, the use of Cephapirin improved conception at first service. Other uterine infusions such as Formosulphatiazole have been compared with Cephapirin to treat endometritis diagnosed at 28 DIM. Formosulphatiazole and Cephapirin were treatments and propylene glycol uterine infusion was used as control. Both antibiotic treatments improved uterine health of endometritic cows. Bacteriology culture and clinical scores for endometritis were significantly greater for the propylene glycol group. Reproductive outcomes were not reported in this study (Mari et al., 2012).

Ceftiofur, a third generation Cephalosporin antibiotic, has been used to treat uterine disease. It was shown to decrease the incidence of endometritis. Ceftiofur was effective only in cows that did not suffer retained placenta and with 2 or more parities. A positive effect was not found in other categories of cows (Dubuc et al., 2011). The large variation among studies for dose, route of administration, and duration of treatment do not enable a clear position with respect to effectiveness of Ceftiofur to treat metritis. Furthermore, the definition of the uterine disease, definition of cure, reproductive outcome and experimental designs lack consistency in studies using Ceftiofur (Reppert, 2015).

Other antibiotic therapies for metritis have included the use of intramuscular amoxicillin and intramuscular amoxicillin plus oxytetracycline uterine infusion. The use of amoxicillin plus oxytetracycline uterine infusion produced better reproductive performance than only intramuscular amoxicillin in cows with metritis diagnosed during the first 21 DIM. The combined treatment increased the percentage of pregnant cows at

first service and decreased the percentage of open cows at 150 DIM (Armengol and Fraile, 2015).

The use of antimicrobials and other drugs to treat uterine diseases such as retained placenta, clinical endometritis and puerperal metritis in cattle and other species, has been reviewed by Pyorala et al. (2014).

Subclinical endometritis does not have a treatment. Dini et al. (2015) proposed uterine lavage as an alternative to reduce the presence of PMN in the uterus but this treatment has to be tested for reproductive outcomes in larger studies. Up to 90 % of self-cure has been reported for SCE in dairy grazing cows (Priest et al., 2013).

Subclinical endometritis includes a uterine inflammatory response. Non-steroidal anti-inflammatory drugs (NSAID) have been evaluated, therefore, to treat SCE. Carprofen, which is a NSAID, was tested in cows with SCE diagnosed at 14 DIM (cutoff: 14 % PMN). The SCE cows received 3 injections of Carprofen between 21 to 31 DIM. The treatment did not affect the percentage of PMN, anovulatory interval or milk production at 42 DIM. Pregnancies per artificial insemination were similar between groups at 42 DIM as well, however, cows with more than 14 % of PMN at 14 DIM (severe SCE) had better conception when treated with Carprofen than cows in the same category without the Carprofen treatment (Priest et al., 2013).

Hormonal treatments for uterine disease have been also tested. The use of $PGF_{2\alpha}$ has been the most studied but its outcomes are not clear enough. Drillich et al. (2005) suggested $PGF_{2\alpha}$ as the gold standard for treatment of chronic endometritis. Galvao et al. (2009) and Dubuc et al. (2011), however, showed that treatment with $PGF_{2\alpha}$ did not improve the condition of clinical or subclinical endometritic cows. Galvao et al. (2009)

reported no effect of $PGF_{2\alpha}$ on endometritis but they indicated that $PGF_{2\alpha}$ improved the overall conception rate in endometritic cows.

Diet supplementation with selenium was tested with respect to its effect on SCE. Supplementation with different sources of selenium in diet before and after calving did not reduce the incidence of SCE at 30 DIM but cows with SCE had lower fertilization rate than healthy cows (Cerri et al., 2009).

Some lactic acid bacteria (LAB) have shown to decrease the infection and inflammation in cultured endometrial cells, but more research using in vivo models is needed to see if uterine infusion of LAB can improve uterine health status (Genis et al., 2016).

Homeopathic remedies have been used as alternatives to prevent instead of cure endometritis. Different homeopathic treatments were tested from the first day of calving until 27 DIM. Intramuscular administration of these remedies did not decrease the incidence of endometritis at 21 and 27 DIM (Arlt et al., 2009).

1.12 Interferon stimulated genes for early pregnancy detection

Interferon tau (INFt) is a family 1 interferon that is secreted by the embryonic trophoblast between 10 to 25 days after conception with greater concentration between 14 to 16 days (Roberts et al., 1996; Roberts, 2007). The INFt is secreted into the uterine lumen for maternal recognition of pregnancy in cattle. It decreases expression of estrogen and oxytocin receptors and also increases expression of interferon stimulated genes (ISG) with the final outcome of blocking luteolysis (Stewart et al., 2001). In order to test which ISG were differentially expressed when cows get pregnant, a set of candidate genes were tested [Myxovirus-resistance protein - 1 (MX1), Myxovirus resistance protein - 2 (MX2), β2 microglobulin, interferon stimulated gene protein - 15 (ISG15), INF regulatory factor-

1 and INF regulatory factor - 2]. From this set of genes, only MX1, MX2 and ISG15 were considered appropriate indicators for pregnancy when tested on days 18 and 20 after insemination (Gifford et al., 2007). Green et al. (2010) evaluated expression of MX2, ISG15 and 2'- 5'- olygoadenylate synthetase (OAS1) at day 14, 16 and 18 after insemination from peripheral blood leucocytes. The authors reported that on day 18 MX2 and ISG15 were suitable indicators of pregnancy in primiparous cows. The same test was less sensitive for multiparous cows. Upregulation of ISG15 and MX1 was also identified in liver samples of Holstein cows at day 18 after breeding (Meyerholz et al., 2016). In beef cattle, Pugliesi et al. (2014) suggested the evaluation of expression of OAS1, MX1, MX2 and ISG15 in peripheral blood samples at day 20 after insemination as a tool for early pregnancy diagnosis.

Interferon stimulated genes and progesterone have a complex functional relationship. Progesterone allows up and down regulation of some genes such as different types of interferons. Specifically, for INFt, progesterone facilitates secretion of this signal for maternal recognition of pregnancy (Bazer et al., 2008). Even when progesterone influences secretion of INFt, progesterone supplementation by intravaginal devices (CIDR) from day 4 to 18 after insemination, did not improve expression of ISG15 or receptor transporter protein - 4 (RTP4) at day 16 after breeding. Treatment with CIDR tended to decrease expression of those genes at day 19 (Monteiro et al., 2014).

1.13 Insulin like growth factor - 1 and fertility in dairy cows

Insulin like growth factor – 1 (IGF1) belongs to the somatotrophic axis. It is a link between nutrition and reproductive function. Concentrations of IGF1 change with energy balance. Severe negative energy balance after calving is associated with low concentrations of IGF1 (Wathes et al., 2011). Underfed animals had lower concentrations

of IGF1 than animals that were full-fed (Valour et al., 2013). There are IGF binding proteins (IGFBP) that regulate IGF1 concentration. These binding proteins are also driven by metabolic status (Clempson et al., 2012). For example, oviductal mRNA expression of IGFBP2 and IGFBP6 is reduced in cows under severe negative energy balance. These specific binding proteins have great affinity for IGF2 which may perturb embryo development in the oviduct (Fenwick et al., 2008). Insulin like growth factor-1 receptors have been identified in ovarian structures, uterine tissue and embryos during early stages of development (Kawashima et al., 2007). Severe negative energy balance may affect the recovery of the uterus after parturition through IGF1. Low concentrations of IGF1 affect tissue regeneration making the uterus more susceptible to uterine diseases and infertility (Wathes et al., 2011).

Concentrations of peripheral IGF1 are greater in primiparous than in multiparous cows (Taylor et al., 2004). Concentration of IGF1 vary depending on parity. Fertility is improved when IGF1 concentrations are greater (Grimard et al., 2013). Insulin like growth factor - 1 concentrations in peripheral blood are highly correlated with gene expression in oviduct of post-partum cows between 4 to 15 days of the estrous cycle (Valour et al., 2013). Reduced systemic concentrations of IGF1 are associated with failure in ovarian function and failure in early embryo development (Wathes et al., 2007).

Blood IGF1 has also been associated with greater probability of getting pregnant, resumption of estrous cyclicity, and normal fertility in dairy cattle (Kawashima et al., 2007; Velazquez et al., 2008). Taylor et al. (2004) reported that the likelihood to become pregnant was greater (11 times more likely) when concentration of IGF1 at one week after calving was more than 25 ng/ml. It was also greater (5 times more likely) when

IGF1 was more than 50 ng/ml at the moment of first service. Patton et al. (2007) proposed that IGF1 was a reproductive indicator when measured during the first two weeks after calving because they found that greater concentrations of IGF1 were associated with greater probability of pregnancy at first service and fewer days open. Gene expression analyses in cows with high and low fertility showed that liver samples from high fertility cows had 34% greater expression of IGF1 than low fertility cows (Cummins et al., 2012).

Nicolini et al. (2013) showed that polymorphisms of the bovine gene IGF1/SNABI can produce different reproductive outcomes such as a faster resumption of cyclicity post-partum. Also polymorphisms of IGFBP2 influenced milk production and fertility (Clempson et al., 2012).

2 Chapter. EFFECT OF SUBCLINICAL ENDOMETRITIS ON OVARIAN AND UTERINE RESPONSE TO A TIMED AI PROTOCOL IN DAIRY COWS

2.1 Introduction

Dairy cows face significant challenges around parturition. These include calving, the initiation of lactation, the transition from dry to lactating cow management, diminished dry matter intake, high nutrient demand, body reserve mobilization and uterine involution. All these changes produce a cow that is more vulnerable to get sick. Metabolic diseases such as sub-acute ruminal acidosis, fatty liver, clinical and subclinical hypocalcemia and ketosis are part of the risks after calving (Mulligan and Doherty, 2008). Cows also face diseases such as metritis and endometritis that collectively affect biological and productive parameters on dairy farms (Esposito et al., 2014). High producing dairy cows are additionally challenged perhaps by milk production itself or through the sum of metabolic challenges. High producing cows are more susceptible to uterine disease. Williams (2013) reported, for example, that cows producing more than 35 kg of milk per day have greater incidence of uterine disease.

Infection takes place in the uterus of the cow right after calving. The cow is usually able to clear the infection within a few days after calving, but if this does not happen, then metritis can develop. Other forms of uterine disease and inflammation such as subclinical endometritis may also take place (Bittar et al., 2014).

Uterine disease affects fertility in dairy cows. The mechanisms of how uterine disease affects fertility have been hypothesized (LeBlanc, 2008; de Boer et al., 2014; LeBlanc, 2014; Eckel and Ametaj, 2016).There are both short and long term effects of uterine disease on ovarian structures and ovarian and uterine responses.

Subclinical endometritis is a uterine disease that does not present clinical symptoms and cannot be easily diagnosed on farms. Nonetheless it produces deleterious effects on reproduction in dairy cows. We hypothesized that subclinical endometritis (SCE) affects ovarian function so that the response to a timed artificial insemination protocol is compromised. It also affects uterine function to prevent the establishment and maintenance of pregnancy.

2.2 Objectives

The general objective of this study was to categorize cows for SCE and evaluate the ovarian response to a TAI protocol and also evaluate embryonic survivability at different stages of pregnancy after TAI in cows with different SCE status. The specific objectives were: **1.** Assess polymorphonuclear neutrophils (PMN) by cytobrush to define the subclinical endometritis status. **2.** Determine the pregnancy rate at different stages of pregnancy after TAI for cows with different SCE status. **3.** Determine if SCE affects the response of the ovary to the TAI protocol. **4.** Measure ISG15 expression as an indicator of early embryonic development in cows with different SCE status.

2.3 Materials and methods

This study followed the research animal care and welfare regulations established by the Institutional Animal Care and Use Committee (ACUC) of the University of Missouri.

2.3.1 Study design

Holstein lactating cows from Foremost Dairy Farm in Columbia, Missouri were used for the experiment. Cows included in the experiment were managed in free stalls, milked twice a day and fed a total mixed ration. Data was collected during two periods, the first period from November 2014 to April 2015 (N=56 cows) and the second period

from October 2015 to May 2016 (N=51 cows). Weekly groups of cows between 30 to 39 DIM were included for the study. Size of weekly groups was from 1 to 11 candidate cows. Cows were inseminated from 68 to 77 DIM in a timed artificial insemination (TAI) protocol. The protocol was the Presynch Ovsynch56 that consisted of an injection of $PGF_{2\alpha}$ (PG1) (5 mL Lutalyse; 25mg dinoprost tromethamine, IM; Zoetis Inc., Florham Park, NJ), 14 days later a second injection of $PGF_{2\alpha}$ (PG2). Fourteen days after PG2, an injection of GnRH (2 mL Factrel; 100 µg gonadorelin, IM; Zoetis Inc.), 7 days later a third injection of $PGF_{2\alpha}$ (PG3) with a second GnRH injection 56 hours after the PG3; finally, TAI 16 hours after the second GnRH injection. Forty-three percent of cows were first parity cows; the remaining of cows were second or greater parity. Figure 2-1 shows a timeline of the experiment.

2.3.2 Ovarian structure evaluations by ultrasound

Ovaries were imaged by using ultrasound (Aloka SSD500, Tokyo, Japan) using a 7.5 MHz probe (UST-556I). Ovarian follicles were counted, measured and classified in three categories with respect to the follicular diameter (class 1: 2 to 5 mm; class 2: 6 to 9 mm; class 1: ≥10 mm). Corpora lutea (CL) were also counted and measured in two dimensions (length and width). If the CL had a lacuna (fluid filled center), then the diameter of the lacuna was measured. Average of width and length of the CL was defined as the CL diameter. Study days for ovarian ultrasound evaluation are shown in Figure 2-1.

Ovulation was categorized as early if the preovulatory follicle was present at d 35 and absent at d 37; on-time if present at d37 and absent at d 39; late if present at d 37 and still present at d 39 but not at d 45; and no ovulation if the preovulatory follicle was present at d 37, 39 and 45 of the experiment.

2.3.3 Uterine Cytology

Two endometrial cytologies were performed per cow (Figure 2-1). Endometrial cytology was assessed by using a cytobrush (Cytobrush Plus, Cell collector, CooperSurgical, Inc, Berlin, Germany). The cytobrush was cut to 3 to 5 cm length, threaded on to a steel rod (approximately 60 cm long) and placed into an artificial insemination (AI) gun with a steel rod in place of the plunger. The assembly was covered by an AI sheath (Continental Sheaths, USA) and a plastic sanitary sleeve (Continental Coveralls, WI, USA). The perineal region of the cow was cleaned with a paper towel. The cytology instrument was inserted through the vagina to the uterine body by manipulating the tract transrectally. The plastic sleeve was broken at the cervical interface. The cytobrush was exposed to the uterine body by pushing it forward with the steel rod. The exposed cytobrush was rolled three times against the uterine body wall. Then, the cytobrush was retracted into the AI gun and removed from the cow covered by the AI gun and insemination sheath.

The cytobrush was rolled on a clean microscope slide and fixed with CytoPrep fixative (Cat No. 12-570-10, Fisher Scientific Co., Pittsburgh, PA). The sample was air dried and taken to the laboratory where it was stained with Protocol Hema 3 Stain Set (Fisher Scientific Company, VA, USA) following manufacture instructions. In brief, the slide was placed five times for one second each in Hema 3 fixative solution, followed by the same procedure in Hema 3 Solution 1 and Hema 3 Solution 2, respectively. The slide was rinsed in deionized water and air dried.

During the first period of the study, one cytobrush sample was taken for the microscope slide. For the second period, one additional sample following the same procedure, was taken. The additional cytobrush sample was stored in RNA Stabilization

Reagent (RNAlater, QIAGEN, Hilden, Germany) into a 2 ml collection tube. The sample was placed on ice for shipping to the laboratory and frozen at -20 ˚C within less than 2 hours for further analysis out of the scope of this thesis.

The stained slide was scored at 250X using a Labourlux S microscope (Leitz, Portugal). Each slide was read by a single examiner. One hundred total cells were counted from 5 different random places on each slide (20 cells per place). The counted total number of PMN divided by the counted total cells multiplied by 100 was defined as the percentage of PMN of the sample.

2.3.4 Blood collection, plasma samples and hormone assays

Blood samples were collected from the caudal tail blood vessels (Figure 2-1). Samples were collected in 10 ml vacutainer tubes [Monoject EDTA (K3) 0.10 mL 15% EDTA solution] and immediately placed on ice before being brought to the laboratory.

Blood samples were centrifuged (Beckman GS-6R centrifuge) at 3000 rpm (1500 x g) at 4 ˚C for 15 minutes. The plasma was collected and placed in polypropylene tubes and frozen at - 20 ˚C for further analysis.

Concentrations of progesterone were measured by validated RIA assay (Pohler et al., 2016) using the liquid-liquid phase double antibody precipitation assay reagents supplied by MpBio (cat # 07 - 170105). Briefly, plasma (100 µL) was co-incubated at 37 ˚C with antisera and 125I-progesterone for 1 hour at 37 ˚C. Thereafter, a goat-anti-rabbit antisera was added, incubated for 15 minutes at RT and the antibody-antigen complex precipitated by centrifugation at 3000 g for 30 minutes at 4 ˚C. The supernatant was decanted and the assay vials containing the precipitated pellets were counted on a Perkin Elmer 1420 gamma counter for 2 min per vial. Slopes of the standard curve (range 0.001 to 5 ng/tube) and serial dilutions of a bovine serum pool (range 20 µL to 200 µL) were

parallel (slopes = - 1.48 and - 1.29, respectively, P > 0.6) and y intercept values were - 1.43 and 1.65 (respectively; natural log\logit calculations). Minimum detectable concentrations were 0.001 ng/tube (i.e. 0.01 ng/100 μL). Within assay coefficients there was less than 5 % variation.

Plasma concentrations of IGF1 were determined using a competitive, liquid-liquid phase, double-antibody IGF1 radioimmunoassay procedure as described previously by Lalman et al. (2000). Assay procedures and validation information were as follows. Plasma samples were thawed, mixed thoroughly, and 10 μL of plasma sample pipetted into individual wells of a 96 deep-well plate. Immediately thereafter, 400 μl of 1M glycine (pH 3.2) was added to acidify each sample followed by the addition of 500 μL of PABET+P (consisting of 0.1% gelatin, 0.01 M EDTA, 0.9 % NaCl, 0.01 M PO4, 0.01 % sodium azide, 0.05 % Tween-20, 0.02% Protamine SO4, pH = 7.5). The acidified-diluted aliquots were then individually sealed within each well and incubated in a constant temperature oven at 37 ˚C for 48 hours. Thereafter, each acidified-diluted sample was neutralized by addition of 90 μL of 0.5 NaOH before being submitted to the IGF1 assay. IGF1 assay procedures were adapted from those described by Holland et al. (1998). Recombinant human IGF1 was used for iodination and standards (UBI – 01 - 141, Amgen Corp., Thousand Oaks, CA, USA). Antiserum (UB3 - 189) was provided by the National Hormone and Pituitary Program and used at a final assay tube dilution of 1:10,000. Sample (40 μL of the acidified-diluted sample; in triplicate determinations), antisera, and PABET + P were combined (total volume balanced to 300 μL with PABET + P) and incubated at 4 ˚C for 24 hours. Iodine 125labeled IGF1 (125I - IGF1; 25,000 cpm) was then added, and incubation continued at 4 ˚C for an additional 16 hours. The

antigen-antibody complex was then precipitated following a 15 minute, 22 °C incubation with 100 μL of a precipitated sheep-anti-rabbit second antiserum, by centrifugation at 3,000 × g for 30 minutes, and the supernatant discarded by aspiration. Assay tubes containing the precipitated antigen - 1 antibody complex were counted for 1 minute on a Wallac 1470 gamma counter (Perkin Elmer, Turku, Finland). IGF1 standards and pooled aliquots of bovine serum extract were linear (log/logit transformation; R2 > 0.97) and parallel over an IGF - I mass of 1.5 to 15 ng/tube and an acid extracted serum volume of 2.5 to 100 μL per tube. Total specific binding was 39 %, the minimum detectable concentration was 1.5 ng/tube, percentage recovery of mass was > 97 % across the range of 2.5 to 100 μL of sample and the inter- and intra-assay CV were < 5 %. Extraction recoveries were also assessed for concentrations of IGF - I in 2.5-, 5-, 7.5-, 10-, 25-, 40-, and 100- μl volumes of the acidified-diluted bovine serum sample and found to equal or exceed 99% over the 2.5 to 100 μL range tested. Parallelism was assessed and verified between standard concentrations of IGF - I and the acidified-diluted serum sample containing IGF - I in volumes ranging from 2.5 to 100 μL.

2.3.5 RNA extraction and cDNA synthesis

Peripheral leukocyte RNA was extracted from 1.5 mL of whole fresh blood. The blood sample was collected using identical procedures for plasma collection. The RNA extraction and purification was done by using the QIAamp® RNA Blood Mini Kit (QIAGEN, Hilden, Germany) by following the manufacture instructions. In brief, the blood sample was placed on ice and taken from the farm to the laboratory. In the laboratory, isolation of leukocytes was performed and samples were stored at - 80 ˚C until purification of RNA. Isolated leukocytes were thawed at room temperature and processed in QIAamp mini spin columns. On-column DNase digestion was performed to

remove genomic DNA. Once RNA was extracted and purified it was eluted two times in 30 µl of RNase-free water (final volume approx. 60 µL). Concentration of RNA was measured by using a spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE, USA) using 1 µL of the eluted RNA solution. The 260/280 ratio was used to evaluate purity of the sample. Additionally, the integrity of RNA was confirmed by using agarose gels (1.5 % agarose). The buffer was 0.5 X solution of Tris-borate-EDTA (TBE) plus ethidium bromide (0.005 % v/v).

The high capacity cDNA reverse transcription kit (Applied Biosystems™ Vilnius, Lituania) was used for cDNA synthesis in a 20 µL reaction. Five hundred nanograms of RNA were used. The Mastercycler Gradient block (Eppendorf, Hamburg, Germany) was set for cDNA (37 ˚C for 2 hour). Then, the cDNA samples were stored at - 20 ˚C until RTPCR analysis.

2.3.6 Real Time Polymerase Chain Reaction

The 7500 Real Time PCR System (Applied Biosystems, Singapore) was used for quantitative PCR. Polypropylene 96 well plates were used (GeneMate, BioExpress T-3060-1) sealed with thermal sealing tape for PCR (MidSci, MO, USA, TS-RT2RR-100). The SYBR® Green PCR Master Mix (10 µL), the forward and reverse primers for the genes specified (Table 2-1; [2.5 µL each one (10X)]), cDNA sample (1 µL) and molecular biology water were used in a 20 µL reaction for qPCR. The 7500 System was set for 50 ˚C for 2 minutes followed by 95 ˚C for 10 minutes and 45 cycles of 95 ˚C for 15 seconds and 60 ˚C for 1 minute. A melting curve was performed at the end of each run. A 1 : 4 serial dilutions were used as a standard curve (high, medium, low and null concentration) which was run in all plates in triplicate. Samples were run in duplicate. Expression of each gene was calculated based on CT values from the 7500 System

Software. By using the formula 2 ^ (average CT values of each sample minus the average CT values of the medium dilution of the standard curve). The ISG15 expression was normalized by using bovine cyclophilin (Bov Cyclo) gene expression. The ratio of ISG15/Bov Cyclo was reported for statistical analysis. Intra and inter plates coefficient of variation was always less than 3 % for the standard curve.

2.3.7 Farm records

Reproductive, productive and health data were retrieved from PCDart Dairy Records Management Software (DRMS) and from the Herd Management Software that were used at Foremost Dairy Farm. Reproductive data that were collected through first service were used for the study. Parity was included in the analysis in two categories, first parity cows and cows with parity two or greater.

2.3.8 Pregnancy detection

Pregnancy was assessed by different methods (Figure 2-1). Peripheral blood samples from study days 57, 59 and 61 (d 18, 20 and 22 after breeding) were used to extract RNA from leukocytes to evaluate gene expression for ISG15.

At day 64 of the experiment (d 25 after breeding) a plasma sample was used for pregnancy diagnosis by using a commercially Bovine Pregnancy Test Kit (IDEXX Laboratories, Inc. Maine, USA) that detected pregnancy associated glycoproteins (PAG). The test was done following manufacturer instructions for bovine plasma. Samples were run in duplicate. The plates were read at 450 nm in a spectrophotometer (Synergy HT, Biotek Instruments, VT, USA).

Pregnancy detection at d 71 of the experiment (d 32 after artificial insemination) was performed by the College of Veterinary Medicine of the University of Missouri by using ultrasound. Pregnancy confirmation at d 83 (d 45 after artificial insemination) was

based on a beating heart in the embryo. The embryonic evaluation was done by ultrasound (Aloka Prosound SSD500, Tokyo, Japan) using a 5 MHz probe (UST - 588).

2.3.9 Analysis of data

All data was analyzed by using the 9.4 version of SAS software (SAS Institute Inc, Cary, NC, USA). Distribution of cows for the first and second cytobrush exam were analyzed using Proc Freq of SAS.

A total of 107 cows were retained for statistical analysis. Follicle and CL structures, IGF1 and progesterone, and responses to the synchronization timed AI protocol were analyzed by regression models using the GLM procedure of SAS when single days of the experiment were analyzed. Also, for the same response variables, when multiple days were analyzed for the same variable, the MIXED procedure was used. Class variables for the model were parity and SCE1 and day when applicable. Interactions were tested when using either GLM or MIXED and removed from the model if not significant.

The ISG15 ratio data were analyzed and receiver operator characteristic (ROC) curves were produced. Data were analyzed for each independent day (d 18, 20 and 22 after timed AI). The Logistic procedure of SAS was used with positive pregnancy diagnosis based on PAG outcome as the event. The sensitivity was defined as the proportion of cows identified pregnant by ISG15 ratio from the cows identified pregnant by PAG. The specificity was defined as the proportion of cows identified not pregnant by ISG15 ratio from the cows identified not pregnant by PAG. PROC FREQ was used to evaluate the agreement between the pregnancy diagnosis made by each individual day by ISG15 versus the pregnancy outcome from PAG. The effects of parity and PAG were also tested for the ISG15 ratio. The Duncan's test to separate means was used when

information was analyzed with the GLM procedure and LSMEANS with Tukey's adjustment was used to separate means when the MIXED procedure was used. Chi square test was used to evaluate differences Statistical significance was declared at $P \leq 0.05$, tendencies between $P \geq 0.05$ to 0.09. Pregnancies per AI based on different days after timed AI and from different methods of pregnancy detection were calculated by using Microsoft Excel.

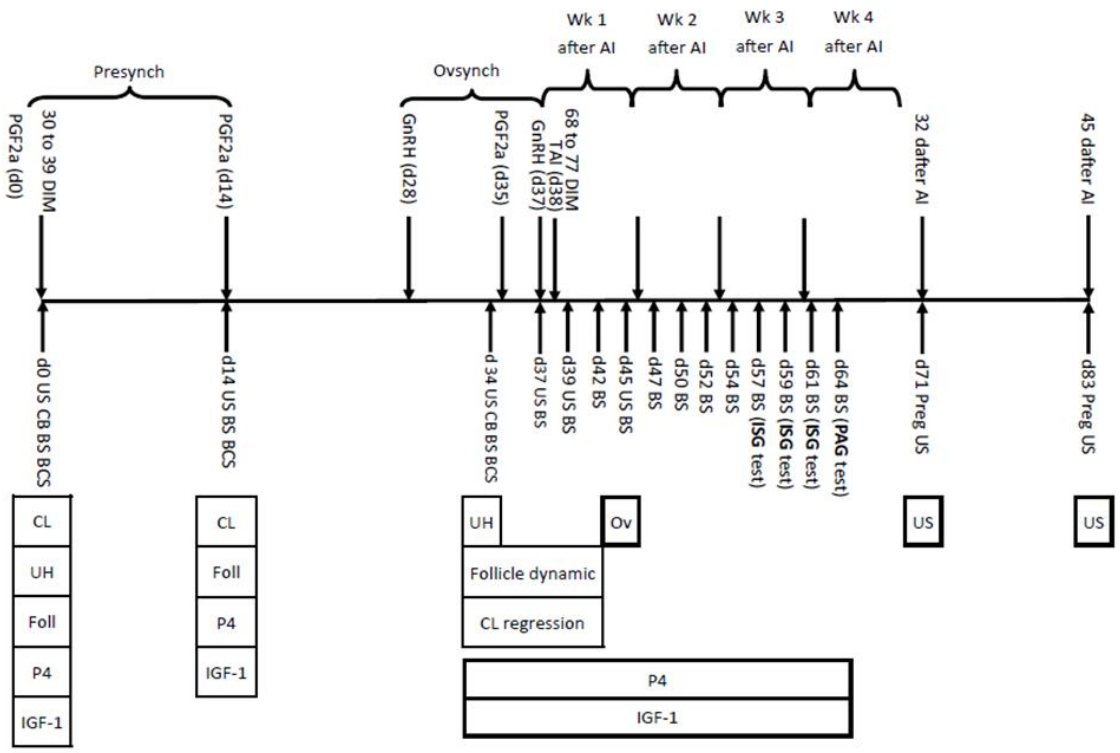

Figure 2-1.Study design for evaluation of SCE on uterine and ovarian response in dairy cows.
CL: corpus luteum; UH: uterine health; Foll: follicles; P4: progesterone; IGF1: Insulin like growth factor-1; PGF_{2a}: prostaglandin F_{2a}; CB: cytobrush; US: transrectal ultrasound; BS: blood sample; GnRH: gonatropin releasing hormone; Ov: ovulation; TAI: timed artificial insemination; ISG: interferon stimulated genes; PAG: pregnancy associated glycoproteins; Preg: pregnancy detection

Table 2-1. Nucleotide sequence of the primers for ISG15 and Bov Cyclo genes

Gene	GenBank	Primer	Primer sequence	Primer location
ISG15	NM_174366	Forward	5’- CAG CCA ACC AGT GTC TGC AGA GA -3’	14 to 36
		Reverse	5’- CCA GGA TGG AGA TGC AGT TCT GC -3’	284 to 306
Bov Cyclo	NM_178320	Forward	5’- CAC CGT GTT CTT CGA CAT CG -3’	23 to 42
		Reverse	5’- ACA GCT CAA AAG AGA CGC GG -3’	65 to 84

3 Chapter. Results

3.1 Validation of early pregnancy detection by using ISG15 expression to test uterine response to SCE

A total of 147 samples were extracted for RNA from 49 cows during the first year. One hundred and fifty-three samples were extracted for year 2. A total of 12 samples did not yield enough RNA for PCR procedures (less than 50 ng/mL). These 12 samples were from year 1 and 2. From year 1, 2 samples for d 18 and 3 samples for d 20 were not used in PCR. From year 2, 2 samples for d 18, 3 samples for d 20 and 2 samples for d 22 were not used in PCR also for low yields of RNA. The range of RNA yields obtained was from 0. 816 to 28.7 μg.

The average coefficient of variation for triplicates of the standard curve used in year 1 was 0.61 % for ISG15 and 0.36 % for Bov Cyclo whereas, in year 2, it was 0.44 % and 0.34 % for ISG15 and Bov Cyclo respectively.

Data for ISG15 ratio was used to develop ROC curves to estimate pregnancy at days 18, 20 and 22 after breeding. These curves are presented in Figure 3-1. The cut points with corresponding sensitivity, specificity and kappa statistic as a measure of agreement between pregnancy detection by ISG15 and PAG are shown in Table 3-1. The cut points are the numbers of ISG15 ratio above the which cows are considered pregnant. The sensitivity and specificity between days for each individual year were testes by Chi Square. The sensitivity and specificity were similar for all cases ($P > Chi\ sq. > 0.1$).

Parity was a significant effect on ISG15 expression; for both years, primiparous cows had greater ISG15 expression than multiparous cows (for year 1, d 18 $P = 0.1$, d20 $P = 0.03$ d 20 $P = 0.05$; for year 2, d 18 $P = 0.01$, d 20 $P = 0.03$, d 22 $P = 0.09$). In Figure 3-2 the effect of parity for each year of study and day are shown.

The expression of ISG15 differed by pregnancy status, in Figure 3-3 the ISG15 ratio is shown for pregnant and open cows for each year of the study and day.

Combining the results from all different pregnancy detection methods used in this study [ISG15 ratio at d 18, 20 and 22 and the pregnancy outcomes by PAG (d 25) and ultrasounds (d 32 and 45)] the overall percentage of pregnancies per AI is shown in Figure 3-4. The percentages of pregnancy were similar among days (Pr > Chi sq. = 0.3).

3.2 Effects of subclinical endometritis on ovarian and uterine response

3.2.1 Cytology results to determine SCE status [cytobrush exam at d 0 of the experiment (CB1)]

A total of 107 cows were examined by cytobrush to assess percentage of PMN as indicator of SCE status. The exam was performed between 30 to 39 DIM. Figure 3-5 shows the distribution of cows with different percentages of PMN.

Based on data shown in Figure 3-5, and by using a cut point of ≥ 6 % of PMN (Dubuc et al., 2010) the uterine health status was defined. Seventy-six cows (71.0 %) fell into the category of uterine healthy cows and 31 cows (28.9%) were diagnosed with SCE. The average PMN % of cows without SCE was 0.80 ± 1.17 (mean ± standard deviation) while the average PMN of cows diagnosed with SCE was 39.34 ± 28.33 (mean ± standard deviation).

3.2.1.1 Effects of SCE on ovarian structures within a timed AI protocol

The largest follicle (LargF), the second largest follicle (Larg2F) and the number of follicles categorized by follicle diameter (Fclass1, Fclass2 and Fclass3) were measured and tested for SCE status.

3.2.1.1.1 Ovarian follicle evaluation at d 0 of the experiment

Cows were evaluated by using ultrasound at d 0 of the experiment (day of the first PG). There was not effect (P > 0.1) of SCE on LargF, Larg2F or number of follicles in

the three diameter classes. The diameter of the LargF was greater in cows with parity ≥ 2 (P = 0.03) compared with parity 1 (Figure 3-6).

3.2.1.1.2 Ovarian follicle evaluation at d 14 of the experiment

The same ovarian evaluation done at d 0 was done at d 14 of the experiment (d of PG2). There was no effect of SCE on LargF, Larg2F or number of follicles in specific diameter classes. The diameter of the LargF differed by parity (P = 0.04). Parity ≥ 2 cows had larger follicle than parity 1 cows.

3.2.1.1.3 Ovarian follicle evaluation at d 34, 37 and 39 of the experiment

The effect of day during this stage of the timed AI protocol was highly significant on the LargF and Larg2F (P < 0.05) (Figure 3-7). At d 37 the second GnRH of the timed AI protocol was administered which induced ovulation and change in sizes of this follicles. The three classes of follicles were also different by day (P < 0.05) (Figure 3-8). The number of small follicles increased at day 39 as results of initiation of a new follicle wave after ovulation.

Cows with SCE differed for the size of the largest follicle during this stage of the timed AI protocol (P = 0.04) (Figure 3-9). Healthy cows had a greater diameter follicle. The Larg2F, and classes of follicles were not affected by SCE (P > 0.05).

3.2.1.2 Effects of SCE on corpora lutea based on cytobrush test

The diameter of the corpus luteum (CL), the diameter of the lacuna of the CL (if present) and net CL tissue (CL tissue = CL diameter-lacuna diameter) were measured and tested for the effect of SCE status.

3.2.1.2.1 Ovarian corpora lutea evaluation at d 0 of the experiment

The diameter of the CL, lacuna or CL tissue did not differ by SCE status (P > 0.1). There were no effects of parity on the CL on d 0 of the experiment (P > 0.1).

3.2.1.2.2 Ovarian corpora lutea evaluation at d 14 of the experiment

There were no effects of SCE or parity on the CL, lacuna or CL tissue (P > 0,1).

3.2.1.2.3 Ovarian corpora lutea evaluation at d 34, 37 and 39 of the experiment

The PG3 was administered on d 35 of the experiment. The effect of day was significant (P < 0.001) for the CL, lacuna and CL tissue. The CL decreased in diameter after PG3 (Figure 3-10). The diameter of CL tissue tended to be affected by SCE (P = 0.09) being greater in cows with SCE. The CL or lacuna did not differ (Figure 3-11). Parity did not affect any CL measurement (P > 0.1).

3.2.1.2.4 Ovarian corpora lutea evaluation at d 45 of the experiment

Corpora lutea were measured at d 45 of the experiment (6 days after timed AI) but there was no effect of SCE or parity (P > 0.1).

3.2.1.3 Cyclicity response to the Pre- Synch Ovsynch56 timed AI protocol

3.2.1.3.1 Cyclicity of cows at administration of $PGF_{2\alpha}$ (PG)

The percentage of cycling cows at d 0 (PG1), d 14 (PG2) and d 34 (d before PG3) did not differ by SCE status or parity (P > 0.1) (Figures 3-12 and 3-13 respectively). The percentage of cycling cows increased as the timed AI protocol progressed (Pr > Chi sq. > 0.001). The overall means ± standard deviation for percentage of ovulation at PG1, PG2 and PG3 were 57.5 ± 4.9, 76.7 ± 4.2 and 90.0 ± 3.0 respectively.

3.2.1.3.2 Pre-ovulatory follicle size and ovulation at timed AI

The diameter of the pre-ovulatory follicle was not affected by SCE status or parity (P > 0.1). The pre-ovulatory follicle size for parity 1 was 15.07 ± 0.41 and 15.15 ± 0.42 for parity 2 or greater. Cows with SCE had a pre-ovulatory follicle of 14.82 ± 0.49 mm, whereas uterine healthy cows had a 15.40 ± 0.33 mm pre-ovulatory follicle (lsmeans ± standard error).

Ovulation at timed AI was not affected by SCE or parity (P > 0.1). In Table 3-2, the number and percentages of cows with early, on-time, late or no ovulation with respect to the programmed timed AI are shown. These percentages were also similar for SCE status (Pr > Chi Sq. = 0.2).

3.2.1.4 Progesterone profiles at specific points of the timed AI protocol

Days 0, 14, 18, 34 and 39 were used to evaluate the response of cows to the Pre-Synch Ovsynch56 timed AI protocol based on progesterone concentrations. A cut point of ≥ 1 ng/mL of progesterone in plasma was used to determine the presence of a functional CL. Effects of SCE and parity were tested. No effect of SCE or parity was found (P > 0.1) for the presence of CL a d 0, 14 and 34 or for the absence of functional CL at d 18 and 39 (two and three days after PG2 and PG3 respectively).

3.2.2 Hormone response to the Presynch Ovsynch56 protocol of cows with or without SCE

3.2.2.1 Insulin like growth factor-1 (IGF1) concentrations

3.2.2.1.1 Insulin like growth factor-1 concentrations at d 0 of the experiment

At d 0 of the experiment, cows diagnosed with SCE tended to have greater plasma IGF1 concentrations (P = 0.07). Insulin like growth factor-1 concentrations were greater for parity 1 than parity ≥ 2 (P = 0.01). Pregnancy was subsequently determine by using pregnancy associated glycoproteins (PAG) as indicator of pregnacy status. Cows that got pregnant did not differ IGF1 concentration with non pregnant cows (P = 0.5) (Figure 3-14).

3.2.2.1.2 Insulin like growth factor-1 concentrations at d 14 of the experiment

Plasma IGF1 concentrations on d 14 were similar for cows with or without SCE (P = 0.1). Cows with parity 1 had greater concentrations of IGF1 than cows with parity ≥ 2 (P < 0.001). Non pregnant cows, based on a subsequent evaluation by using PAG test, tend to have (P = 0.07) greater IGF1 concentrations than pregnant cows (Figure 3-15).

3.2.2.1.3 Insulin like growth factor-1 concentrations at d 34, 37 and 39 of the experiment

The day of the experiment had a significant effect on IGF1 concentrations ($P < 0.001$) with the greatest concentration the day before timed AI (Figure 3-16). Which is related with greater estradiol concentrations from the preovulatory follicle.

During the same stage of the timed AI synchronization protocol, IGF1 concentrations tended to differ by SCE ($P = 0.08$). Cows with SCE tended to have greater IGF1 concentrations than healthy cows. First parity cows had greater concentrations of IGF1 ($P < 0.001$). Pregnant or not pregnant cows after timed AI had similar IGF1 concentrations ($P = 0.7$) (Figure 3-17).

3.2.2.1.4 Insulin like growth factor – 1 concentrations during the first cycle after timed AI

The IGF1 profile for the first cycle after timed AI (d 39 to 61 of the experiment) is shown (Figure 3-18). Days 45 and 59 had greater concentrations of IGF1 compared with d 39 ($P = 0.009$). These increased concentrations are correlated with the first and second follicular waves of the cycle. Concentrations of IGF1 were between 92 to 102 ng/mL during this period.

In figure 3-19, IGF1 concentrations for the first cycle after timed AI are shown. There was an effect of SCE status on IGF1 concentrations ($P = 0.02$). Cows diagnosed with SCE had greater concentrations of IGF1. Parity 1 cows also had greater concentrations of IGF1 than cows parity ≥ 2 during this period ($P < 0.001$). The pregnancy status after timed AI did not affect IGF1 concentrations ($P = 0.11$) The IGF1 concentration in not pregnant cows was 101 ± 3.3 and 93 ± 3.7 for pregnant cows (Lsmeans ± Standard error).

3.2.2.2 Progesterone (P4) concentrations

3.2.2.2.1 Progesterone concentrations at d 0 of the experiment

There was no effect of SCE or parity on P4 at d 0 ($P > 0.1$). Cows that eventually became pregnant did not differ in P4 when compared with not pregnant cows at d 0 of the experiment ($P > 0.1$).

3.2.2.2.2 Progesterone concentrations at d 14 of the experiment

No effects of SCE, parity or pregnancy status after timed AI were found of P4 concentrations at d 14 of the experiment ($P > 0.1$).

3.2.2.2.3 Progesterone concentrations at d 34, 37 and 39 of the experiment

There was an effect of day on progesterone concentrations ($P < 0.001$). The greatest concentration of P4 during this period was at d 34 before PG3, but a significant decrease was observed for days 37 and 39 (Figure 3-20). The effects of SCE, parity and PAG status were tested on progesterone concentrations but no effects were found ($P > 0.1$). Based on the cut point of ≥ 1 ng/mL of P4 the percentage of cows having a CL at d 34 was established. The percentage of cows cycling was 83.2 ± 3.7 (mean ± standard deviation). Then, by using the same P4 cut point, the percentage of cows that did not have low P4 concentrations before breeding (d 37 of the experiment) was 5.7 ± 2.3 (mean ± standard deviation).

3.2.2.2.4 Progesterone concentrations during the first cycle after timed AI (d 39 to 61 of the experiment)

There was a day * PAG interaction ($P < 0.001$). Pregnant cows had greater concentrations of progesterone after day 12 after timed AI compared to not pregnant cows (Figure 3-21).

Cows with or without SCE did not differ progesterone concentrations after timed AI ($P = 0.8$). First parity cows had greater concentration of progesterone than cows with parity ≥ 2 ($P = 0.001$) (Figure 3-22). Pregnant cows differed in progesterone

concentration by parity. First parity cows had greater concentration of progesterone than multiparous cows ($P < 0.001$) (Figure 3-23). Not pregnant cows did not differ in progesterone concentrations by parity ($P > 0.1$).

3.2.2.3 Effects of SCE on uterine expression of ISG15

The samples for evaluation of ISG15 were processed separately for year 1 and year 2. Year was a significant effect on ISG15 ratio ($P < 0.05$). Year 1 showed greater ISG15 ratio than year 2 for d 57, 59 and 61 of the experiment (d 18, 20 and 22 after timed AI respectively).

The effect of SCE on ISG15 ratio was evaluated for pregnant and not pregnant cows at d 57, 59 and 61 of the experiment (d 18, 20 and 22 after TAI respectively). The ratio of ISG15 did not differ for SCE ($P > 0.1$) in pregnant or not pregnant cows (Figure 3-24).

The ISG15 ratio was tested for parity effect in pregnant and not pregnant cows. Parity affected ISG15 expression in not pregnant cows ($P = 0.01$ for d 18; $P = 0.03$ for d 20) but it did not affect in pregnant cows ($P > 0.05$) (Figure 3-25). In not pregnant cows, first parity cows had significantly greater ISG15 expression than cows with parity ≥ 2. The same numerical behavior was observed for pregnant cows but without statistical differences.

3.2.2.4 Effects of SCE on pregnancy

The effect of SCE on pregnancy was evaluated at different days and by different methods of pregnancy detection. Days 57, 59 and 61 of the experiment correspond to days 18, 20 and 22 after timed AI. For these days, pregnancy was determined by ISG15 RNA expression from peripheral blood leukocytes as explained in section 3.1. At day 25 after breeding (d 64 of the experiment) the pregnancy diagnosis was done by PAG, and

finally at day 32 and 45 after breeding (d 70 and 83 of the experiment) the pregnancy detection was done by using ultrasound scanning.

In Figure 3-26, the percentages of pregnancy for each day from all the observations present at each day are shown. No differences for SCE were found on the percentages of pregnancy in any of the days ($P > 0.1$). The actual numbers of pregnant and not pregnant cows were tested for cows with or without SCE at d 18, 20, 22, 25, 32 and 45 after timed AI (Table 3-3). There were not statistical differences ($P = 0.7$ for cows without SCE and $P = 0.5$ for cows with SCE), however, numerically, percentages of pregnancy were very similar between d 18 to d 25 and dropped at d 32 but more severely at d 45 where uterine healthy cows showed a greater percentage of pregnancy.

3.3 Cytology results to determine SCE status [cytobrush exam at d 34 of the experiment (CB2)]

In a total of 107 cows, a second cytobrush exam was done to assess percentage of PMN as indicator of SCE status. The exam was performed between 60 to 73 DIM. Figure 3-28 shows the distribution of cows with different percentages of PMN.

Based on data shown in Figure 3-27, and by using a cut point of ≥ 4 % of PMN (Dubuc et al., 2010) the uterine health status was defined. Ninety-six cows (89.7 %) fell into the category of uterine healthy cows and 11 cows (10.3 %) were diagnosed with SCE. The average PMN % of cows without SCE was 0.35 ± 0.68 (mean ± standard deviation) while the average PMN of cows diagnosed with SCE was 14.18 ± 17.47 (mean ± standard deviation).

Information about percentage of PMN at the first and second cytobrush, pregnancy by different methods of pregnancy detection and parity from the 11 cows diagnosed with SCE during the same week when cows were inseminated is shown in

Table 3-4. From these cows, 4 of them never showed a signal for pregnancy, 2 were negative by ISG15 ratio positive by PAG test and negative by ultrasound. Two of them were positive for some days by ISG15, but negative by PAG test or ultrasound. Two more were diagnosed pregnant by ISG ratio and PAG test but negative by one or the two ultrasounds. From 1 cow, only PAG test and ultrasound information was available which was pregnant for the PAG test, but not pregnant by ultrasound evaluations. Overall, from these 11 cows diagnosed with SCE in the second cytobrush exam, any of them were able to maintain pregnancy by d 45 after timed AI.

Table 3-1. Cut points and agreement indicators for the pregnancy test using ISG15 ratio for year 1 and 2 at three different days after breeding.

Year	Day	Cut point	Sensitivity	Specificity	Kappa value
1	18	0.27	0.91	0.80	0.63
	20	0.96	0.86	0.96	0.75
	22	0.50	0.96	0.88	0.79
2	18	0.07	0.78	0.88	0.61
	20	0.11	0.91	0.84	0.69
	22	0.11	0.92	0.92	0.80

The sensitivity did not differ by day per year 1 or 2 (P = 0.4 and 0.3 respectively). The specificity did not differ by day per year 1 or 2 (P = 0.6 and 0.7 respectively).

Table 3-2. Number and percentages of cows ovulating early, on-time, late and non-ovulatory cows

SCE status	Early Ovulation		Timed Ovulation		Late Ovulation		No Ovulation		Total	
	Number of cows	%	Number of cows	%	Number of cows	%	Number of cows	%	Number of cows	%
Positive	1	3.2	27	87	1	3	2	6.5	31	29
Negative	4	5.3	61	80	5	7	6	7.9	76	71
Total	5	4.7	88	82	6	6	8	7.5	107	100

The categories of ovulation defined in this table did not differ (Pr > Chi sq.= 0.2)

Table 3-3. Number of pregnant and not pregnant cows by SCE status diagnosed at d 0 of the experiment.

Day	Negative SCE			Positive SCE			Total
	P	NP	Total	P	NP	Total	
d 18	32	37	69	13	14	27	96
d 20	33	35	68	11	15	26	94
d 22	34	36	70	14	14	28	98
d 25	35	38	73	13	16	29	102
d 32	29	44	73	12	16	28	101
d 45	28	45	73	7	20	27	100

P = 0.7 for Negative SCE; P = 0.5 for Positive SCE; P > 0.1 for every day

Table 3-4. Information about parity, percentages of PMN, and pregnancy detection outcome by different methods for pregnancy detection for the 11 cows diagnosed with SCE based on the second exam of cytobrush.

Cow	Parity	% of PMN at CB1	% of PMN at CB2	PD at d 18 by ISG15	PD at d 20 by ISG15	PD at d 22 by ISG15	PD at d 25 by PAG	PD at d 32 by US	PD at d 45 by US
2652	3	3	4	NP	NP	NP	NP	NP	NP
2843	2	0	4	NP	NP	NP	NP	NP	NP
2863	2	0	5	NP	NP	NP	NP	NP	NP
2684	3	28	10	NP	NP	NP	NP	NP	NP
2729	1	90	16	NP	NP	NP	P	NP	NP
2591	4	0	40	NP	NP	NP	P	NP	NP
3011	1	2	5	P	NP	NP	NP	NP	NP
2954	1	2	4	P	NP	P	NP	NP	NP
2135	7	35	7	P	P	P	P	NP	NP
2474	5	35	5	P	P	P	P	P	NP
2940	2	18	56	.	.	.	P	NP	NP

CB1 = First cytobrush exam (30 - 39 DIM); CB2 = Second cytobrush exam (60 to 73 DIM); PD = Pregnancy detection; US = ultrasound scanning; P = pregnant; NP = not pregnant.

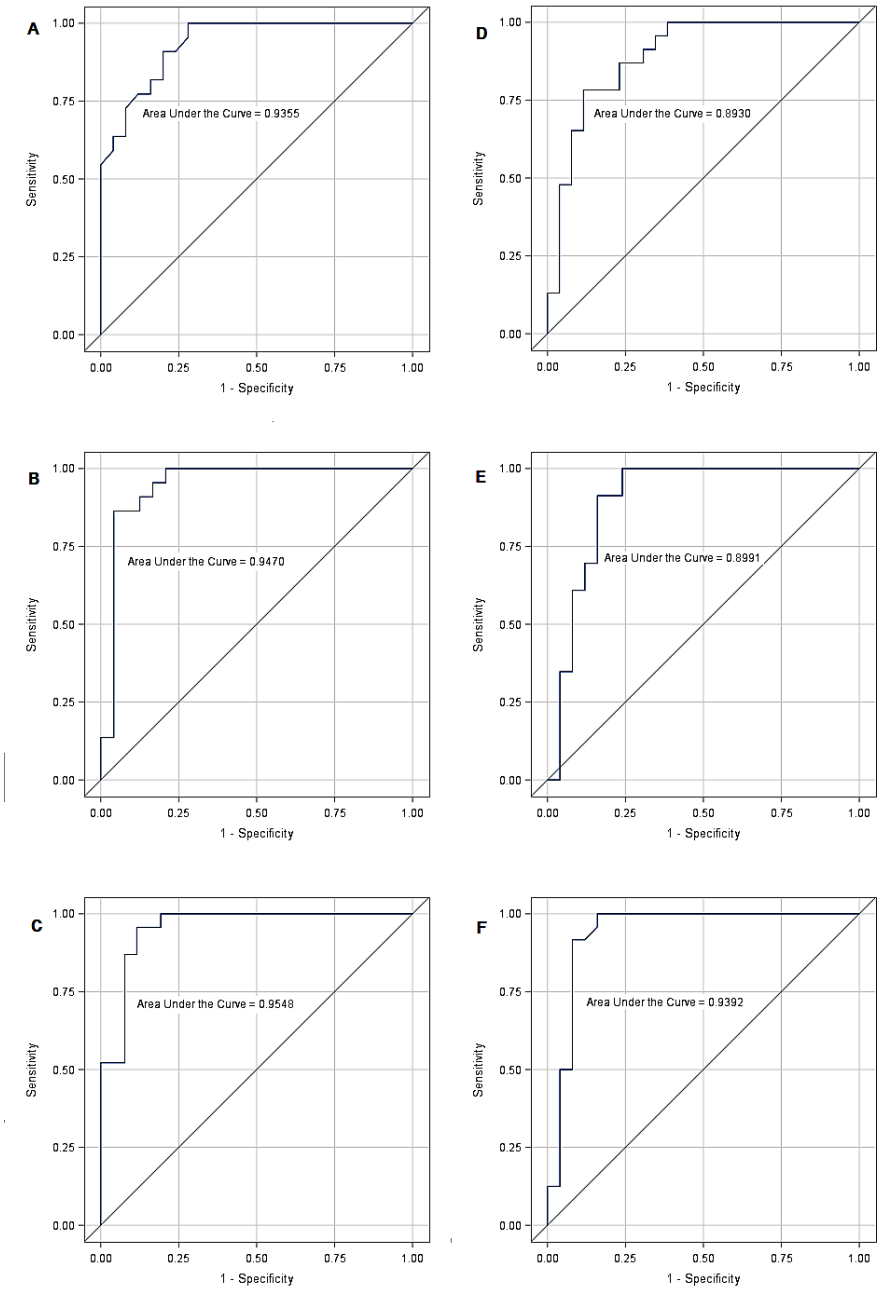

Figure 3-1. Receiver operating characteristic (ROC) curves for an early bovine pregnancy test in lactating dairy cows undegoing a Presynch Ovsynch program for timed AI based on ISG15 expression at different days after timed AI. A) Area under the curve for pregnancy test at d 18 for year 1. B) Area under the curve for pregnancy test at d 20 for year 1. C) Area under the curve for pregnancy test at d 22 for year 1. D) Area under the curve for pregnancy test at d 18 for year 2. E) Area under the curve for pregnancy test at d 20 for year 2. E) Area under the curve for pregnancy test at d 22 for year 2.

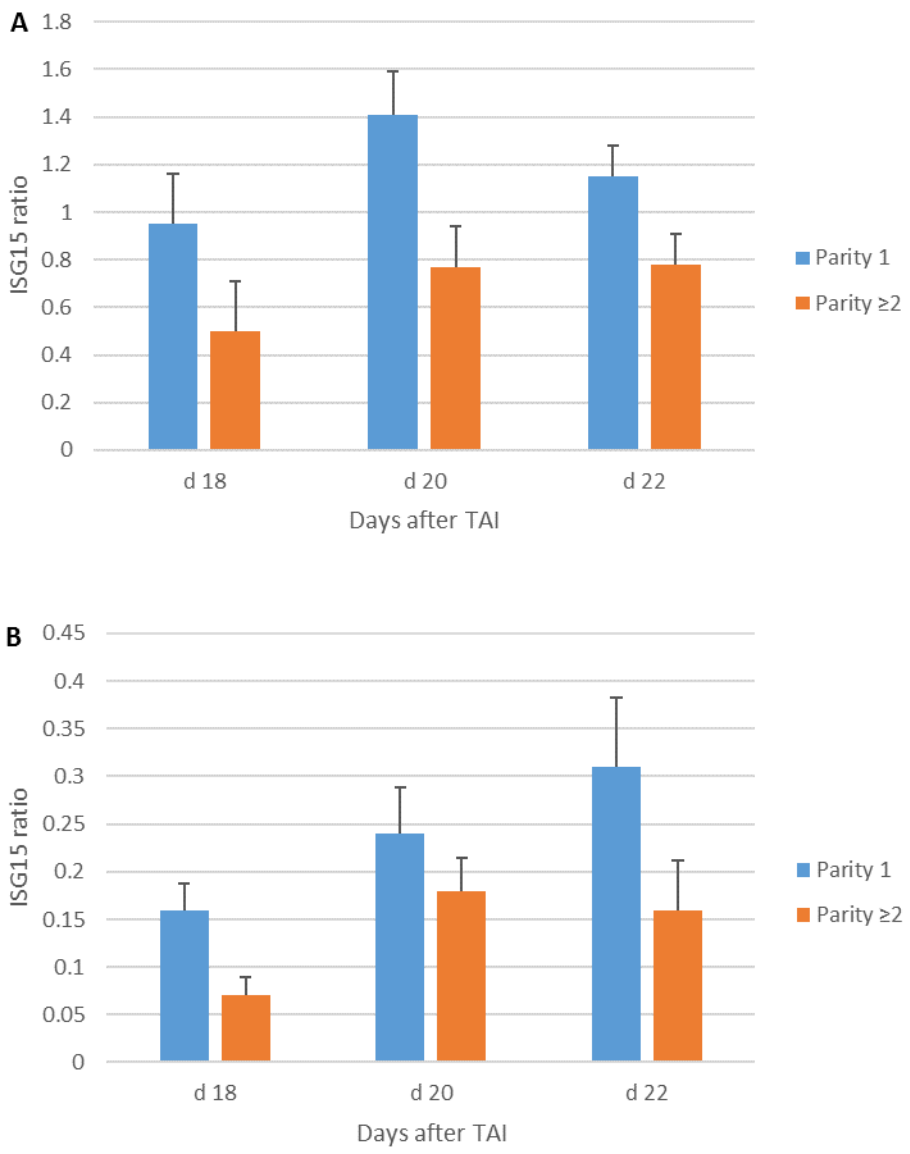

Figure 3-2. Ratio of ISG15 fold change to cyclophilin fold change by parity and day. A) Year 1, data from 49 cows (P = 0.1 for d 18, P = 0.03 for d 20 and P = 0.05 for d 22. B) Year 2, data from 50 cows (P = 0.01 for d 18, P = 0.3 for d 20 and P = 0.09 for d 22)

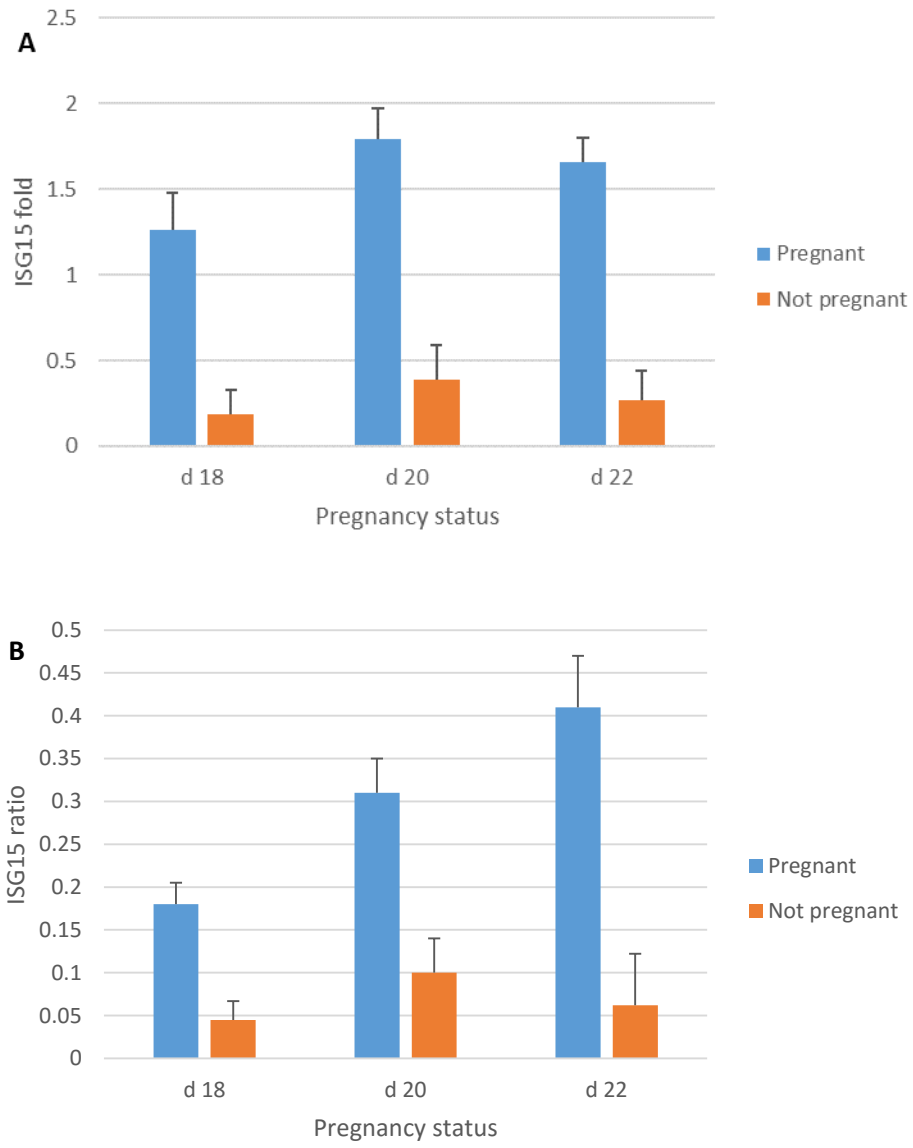

Figure 3-3. Ratio of ISG15 fold change to cyclophilin fold change by pregnancy status (Preg=pregnant). A) Year 1. B) Year 2. (The P values for every day, independently of the year were $P < 0.001$)

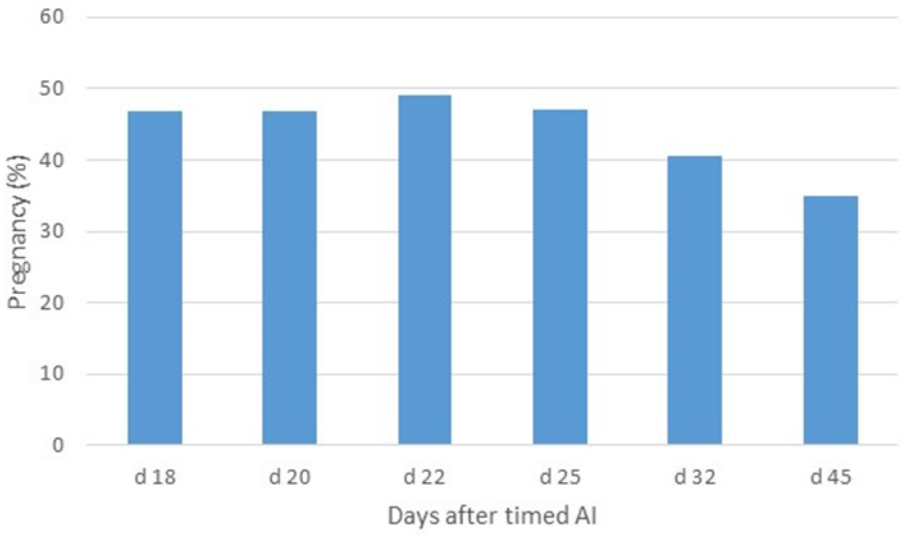

Figure 3-4. Overall percentage of pregnancy for the two years at different days after timed AI by using different methods of pregnancy detection. (d 18, 20 and 22, pregnancy detection by ISG15 expression; d 25 pregnancy detection by PAG test; d 32 and 45 pregnancy detection by ultrasound examination) Percentages are not different by day (Pr > Chi sq. 0.3) N for d 18, 20, 22, 25, 32 and 45 was 96, 94, 98, 102, 101 and 100 respectively.

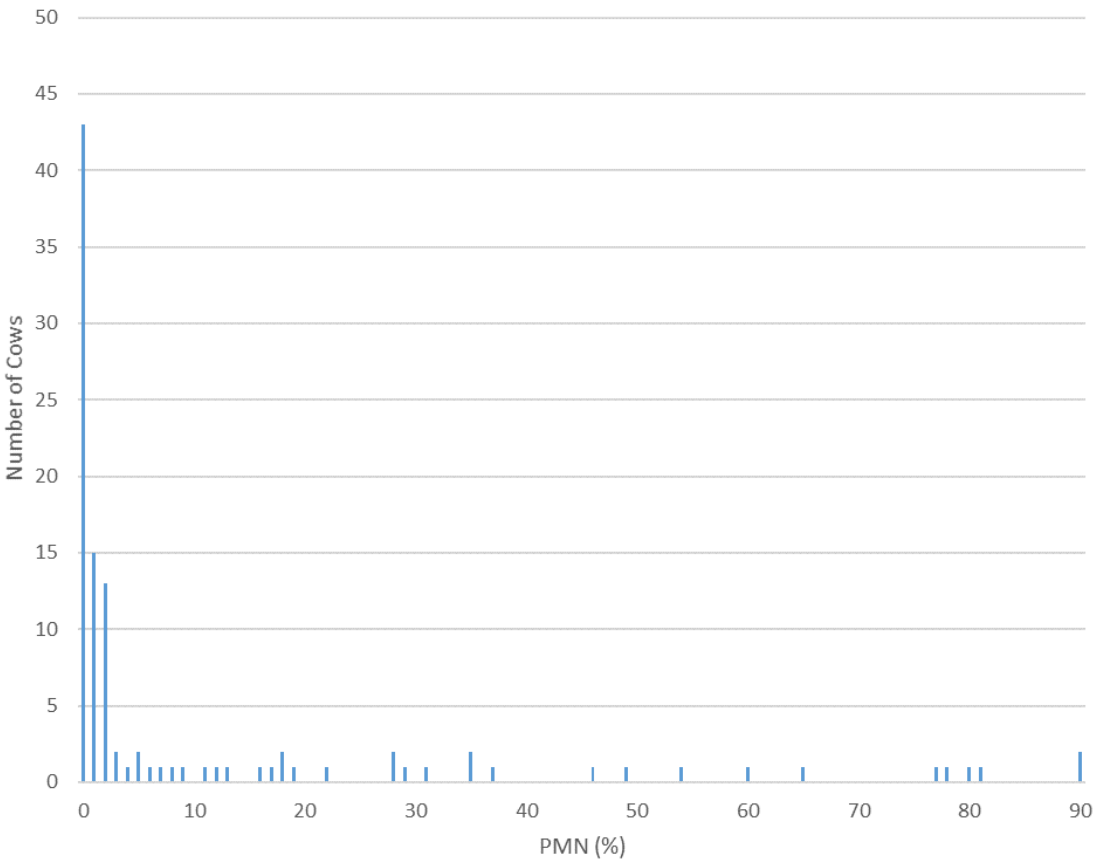

Figure 3-5. Distribution of cows by percentage of PMN based on microscope slide evaluation from cytobrush exam between 30 to 39 DIM (107 cows total)

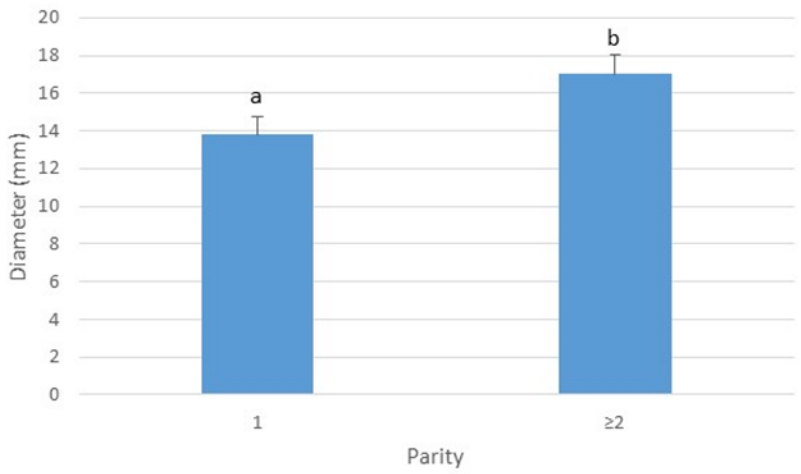

Figure 3-6. Diameter of the LargF at d 0 of the experiment by parity. Different letters on the bars indicates statistical difference between the groups (P = 0.03).

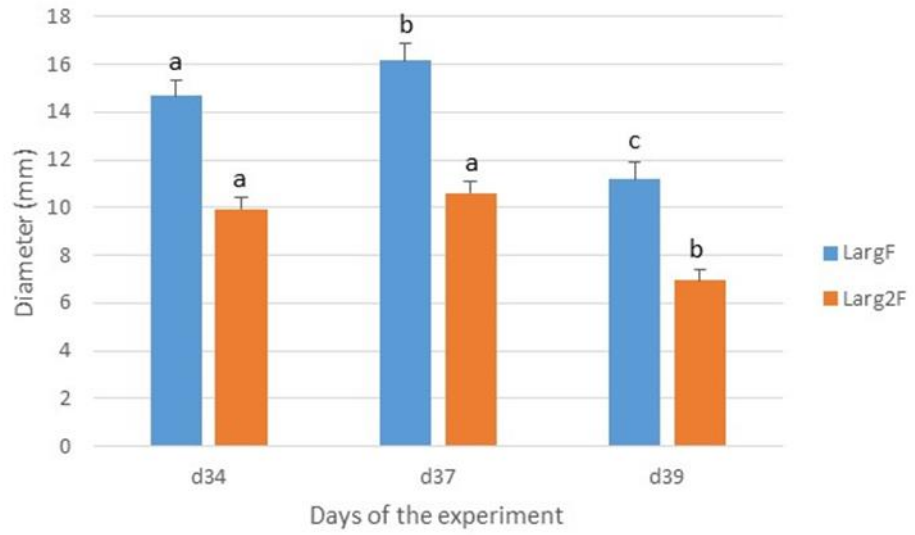

Figure 3-7. Diameter of the LargF and Larg2F on d 34, 37 and 39 of the experiment. Different letters on the bars indicates statistical difference between the groups within variables (LargF and Larg2F: P < 0.001).

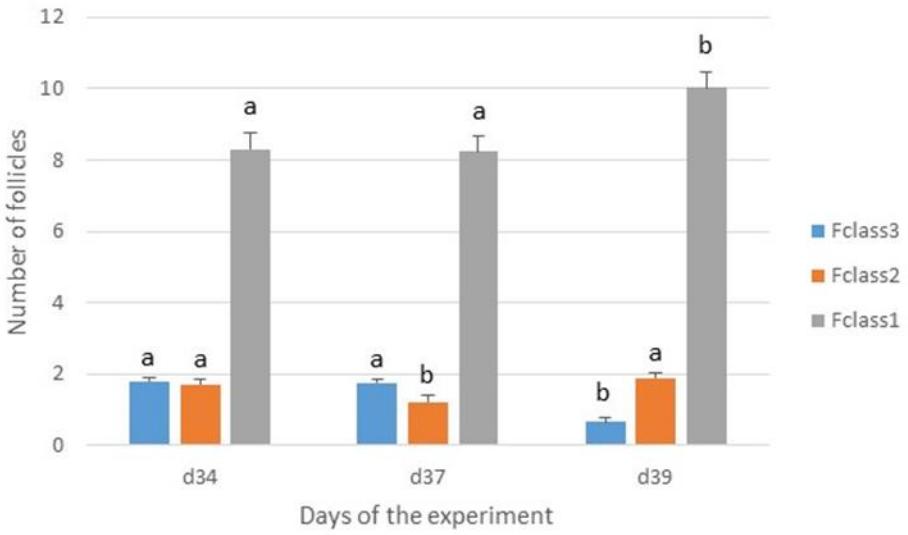

Figure 3-8. Number of follicles in each class (Fclass3, Fclass2 and Fclass1) at d 34, 37 and 39 of the experiment (Different letters on the bars indicates statistical difference between the classes within day) (Fclass3: $P < 0.001$; Fclass2: $P = 0.003$; Fclass1: $P < 0.001$).

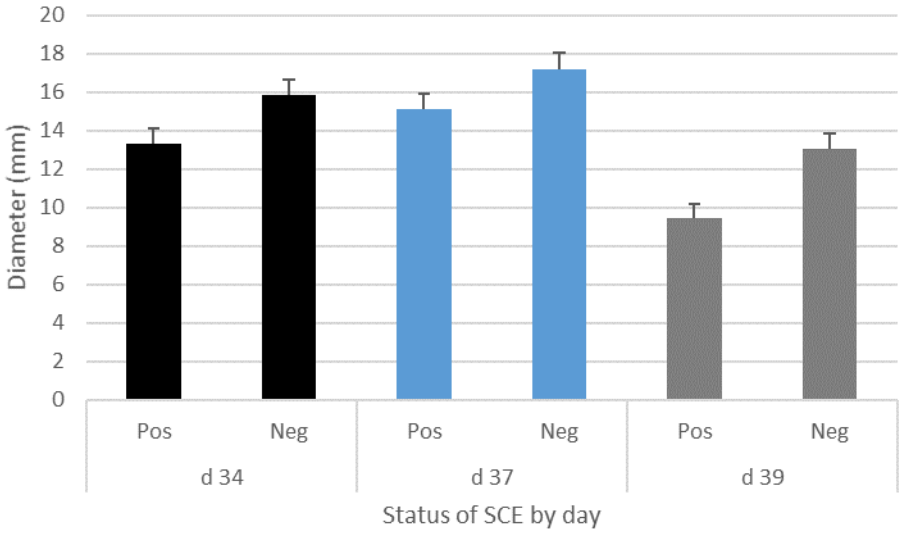

Figure 3-9. Diameter of the largest follicle on d 34, 37 and 39 by SCE status ($P = 0.04$). (Neg = negative for SCE; Pos = Positive for SCE).

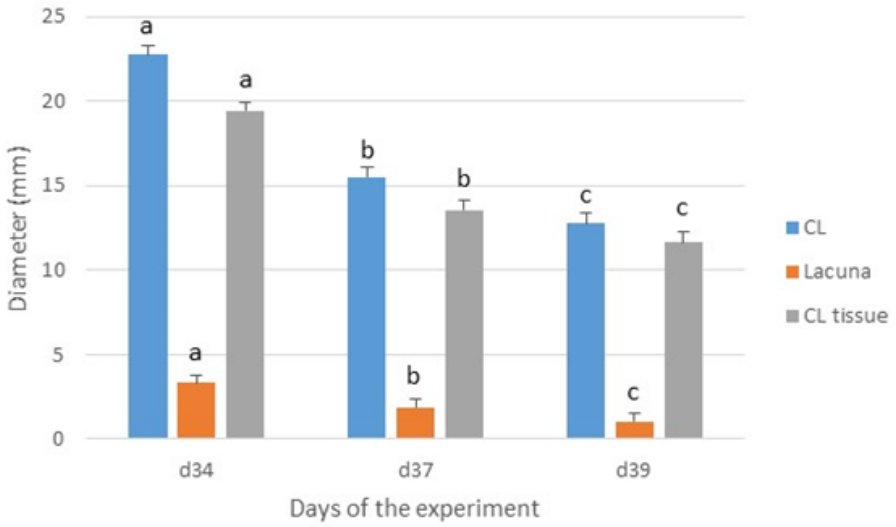

Figure 3-10. Diameter of the CL, lacuna and CL tissue (CL minus lacuna) on days 34, 37 and 39 of the experiment. Different letters on the bars indicates statistical difference between the days ($P < 0.001$).

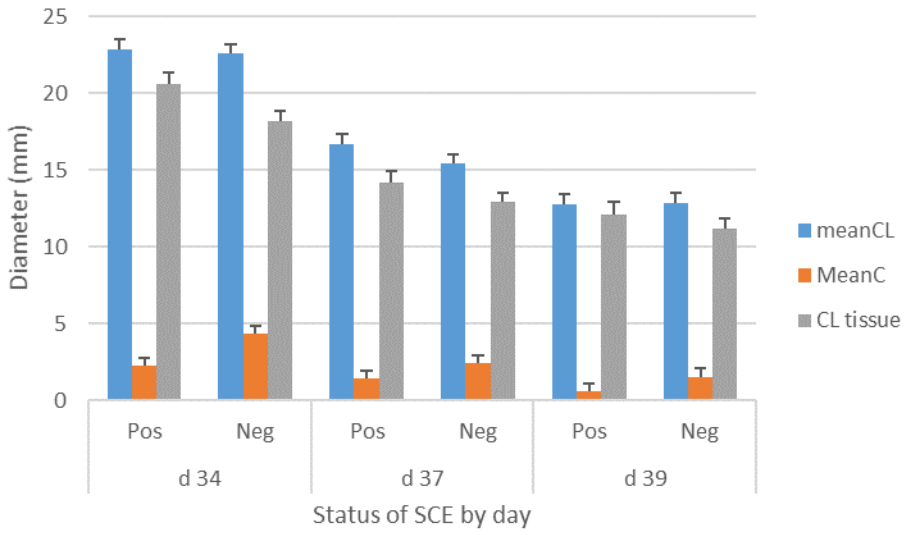

Figure 3-11. Diameter of the CL (P > 0.1), lacuna (P > 0.1) and CL tissue (P = 0.09) by SCE status at d 34, 37 and 39 of the experiment (Neg: SCE negative; Pos: SCE positive).

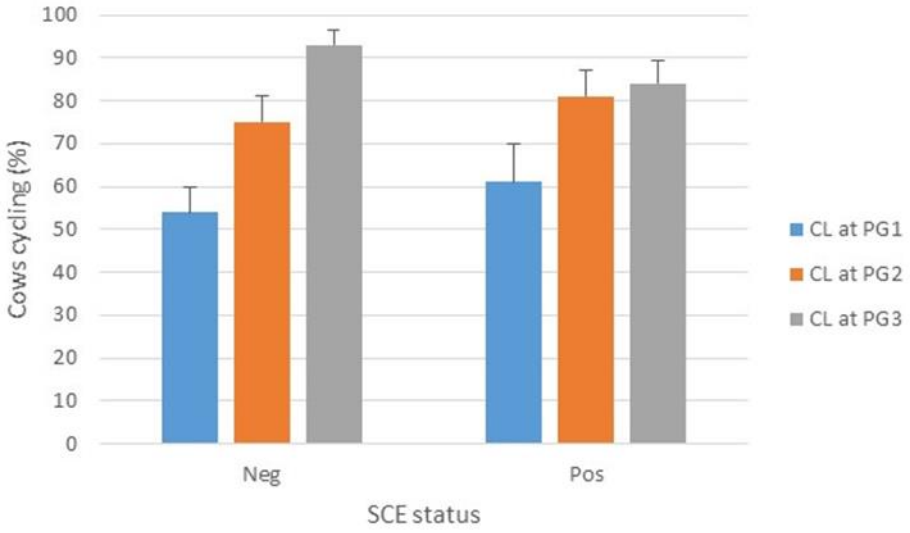

Figure 3-12. Percentage of cows cycling based on the presence of CL at the administration of prostaglandin (PG) by SCE status (P = 0.2 for CL at PG1, P = 0.5 for CL at PG2 and P = 0.6 for CL at PG3) (PG1 = d 0, PG2 = d14 and PG3 = d 34 of the experiment).

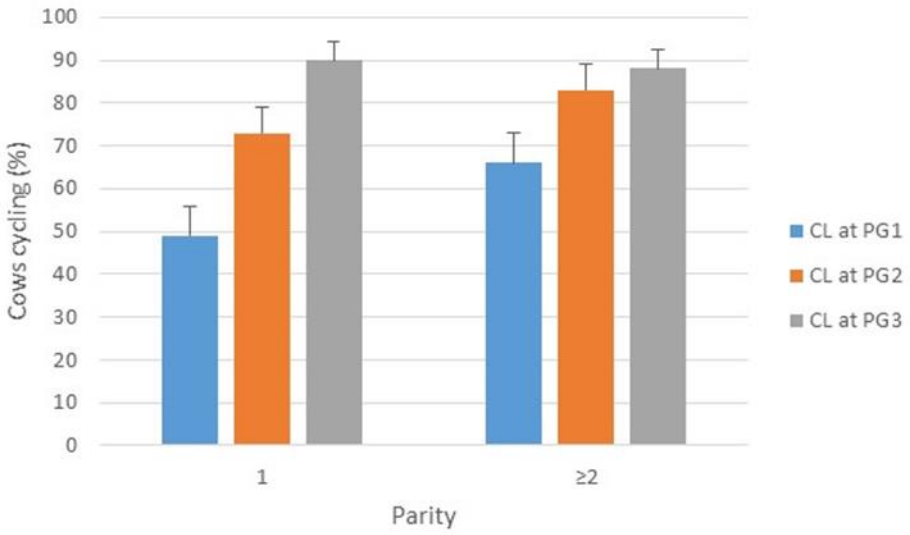

Figure 3-13. Percentage of cows cycling based on the presence of CL at the administration of prostaglandin (PG) by parity (P = 0.1 for CL at PG1, P = 0.3 for CL at PG2, P = 0.8 for CL at PG3) (PG1 = d 0, PG2 = d14 and PG3 = d 34 of the experiment).

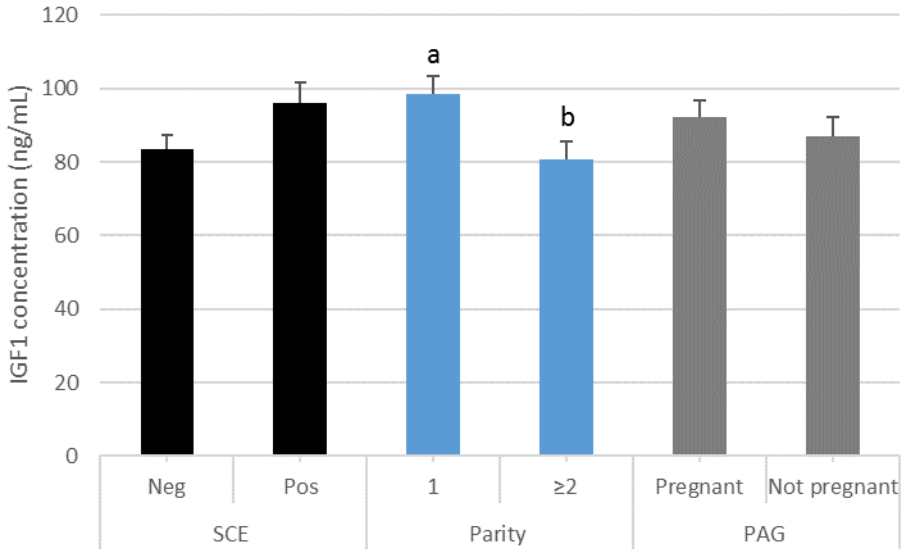

Figure 3-14. Plasma concentrations of IGF1 at d 0 of the experiment by SCE, Parity and pregnancy status based on PAG test after timed AI. Different letters on the bars indicates statistical difference between the groups within each variable (P = 0.07 for SCE, P = 0.01 for Parity and P = 0.5 for PAG).

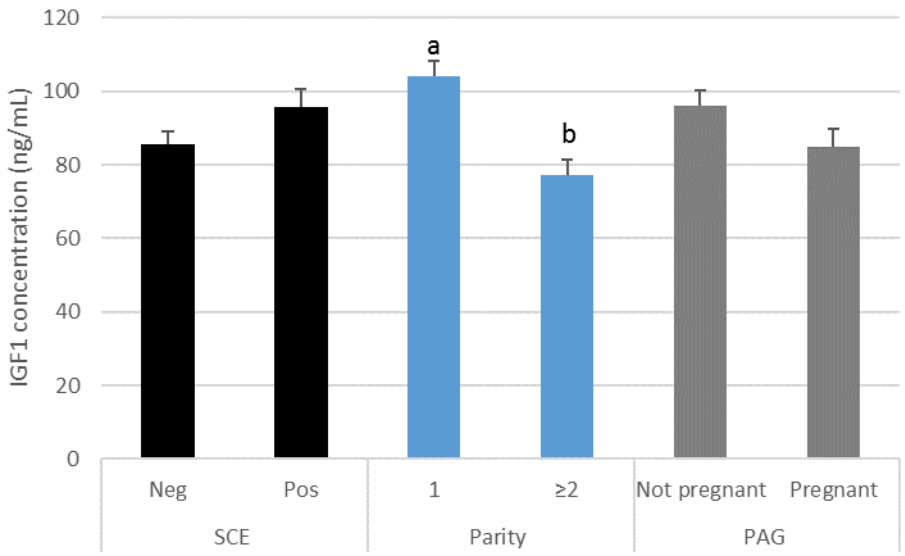

Figure 3-15. Plasma concentrations of IGF1 at d 14 of the experiment by SCE, Parity and pregnancy status by PAG test after timed AI. Different letters on the bars indicates statistical difference between the groups within each variable (P = 0.1 for SCE, P < 0.001 for Parity and P = 0.07 for PAG).

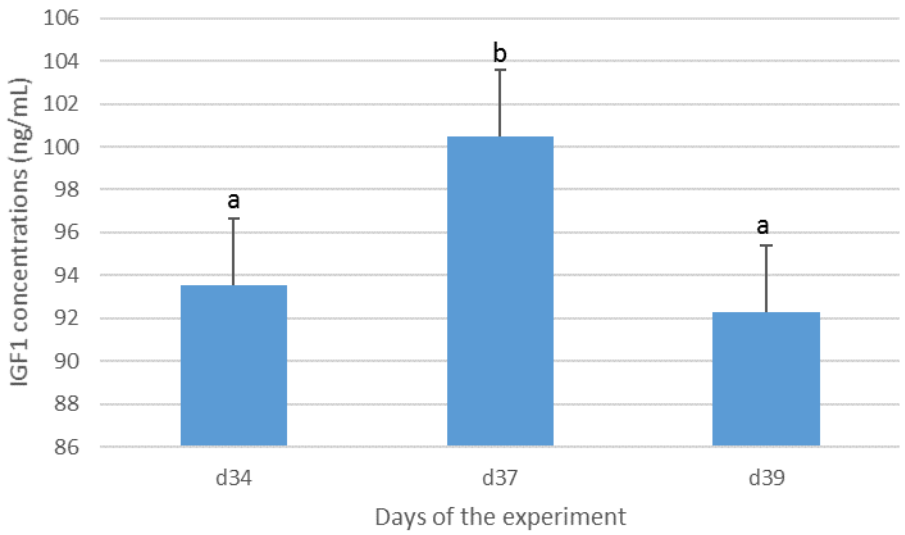

Figure 3-16. Concentrations of IGF1 at d 34, 37 and 39 of the experiment. Different letters on the bars indicates statistical difference between days (P < 0.001).

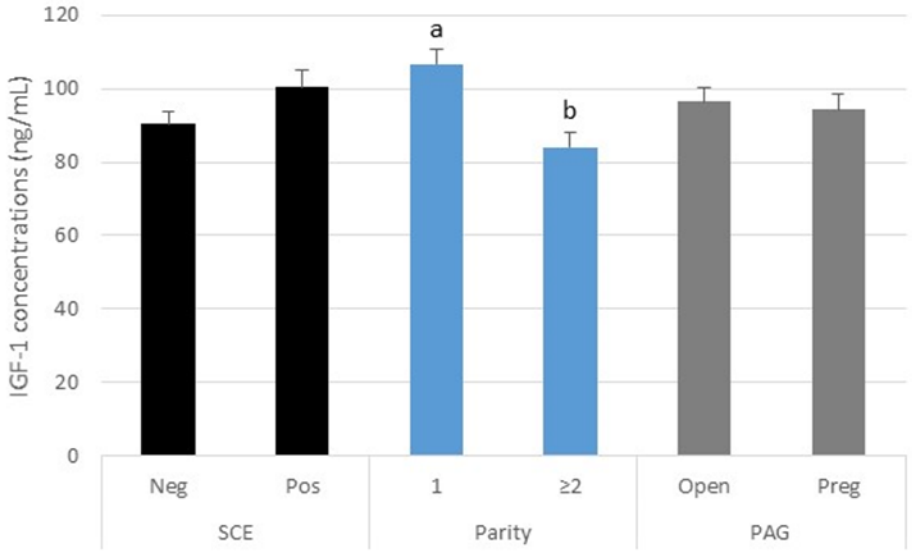

Figure 3-17. Concentrations of IGF1 at d 34, 37 and 39 for SCE, Parity and PAG status. Different letters on the bars indicates statistical differences between the groups within each variable (P = 0.08 for SCE, P < 0.001 for Parity and P = 0.7 for PAG).

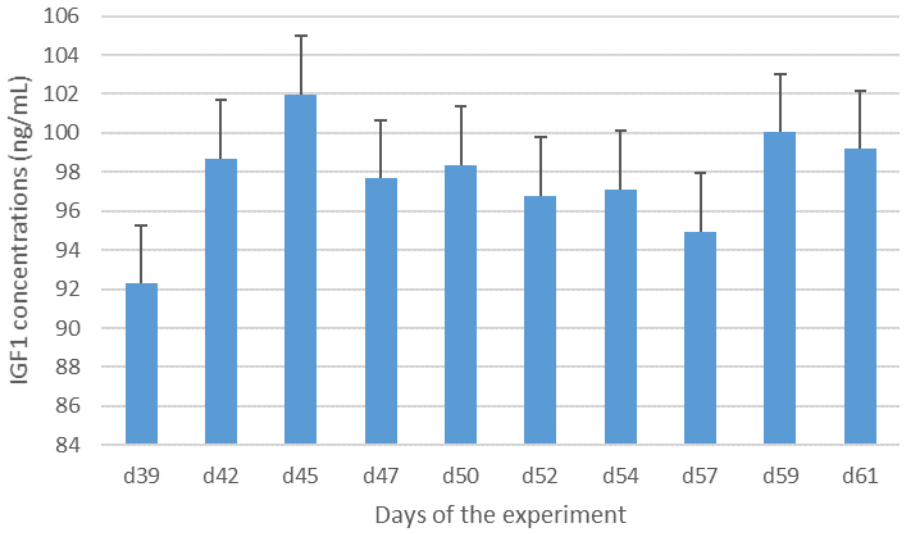

Figure 3-18. Plasma concentrations of IGF1 during the first cycle after timed AI (d 39 to 61) (P = 0.009).

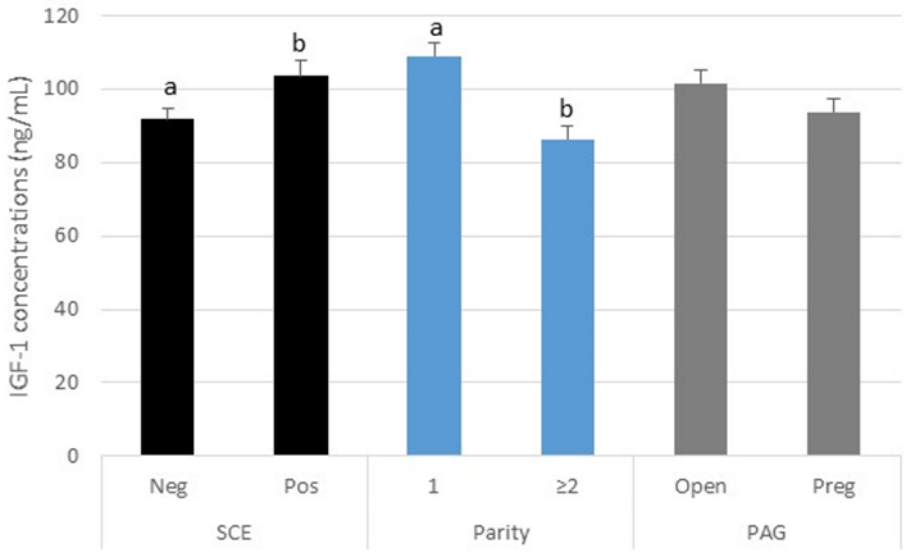

Figure 3-19. Plasma concentrations of IGF1 during the first cycle after timed AI (d39 to 61) for SCE, Parity and pregnancy status defined by PAG test after timed AI. Different letters on the bars indicates statistical difference between the groups within each variable (P = 0.02 for SCE, P < 0.001 for Parity and P = 0.1 for PAG).

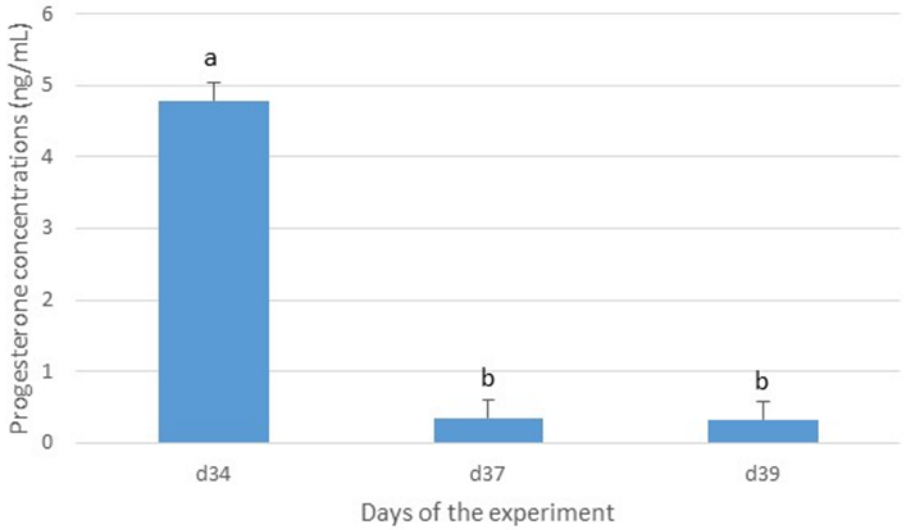

Figure 3-20. Plasma progesterone concentrations at d 34, 37 and 39. Different letters on the bars indicates statistical difference between days (P<0.001).

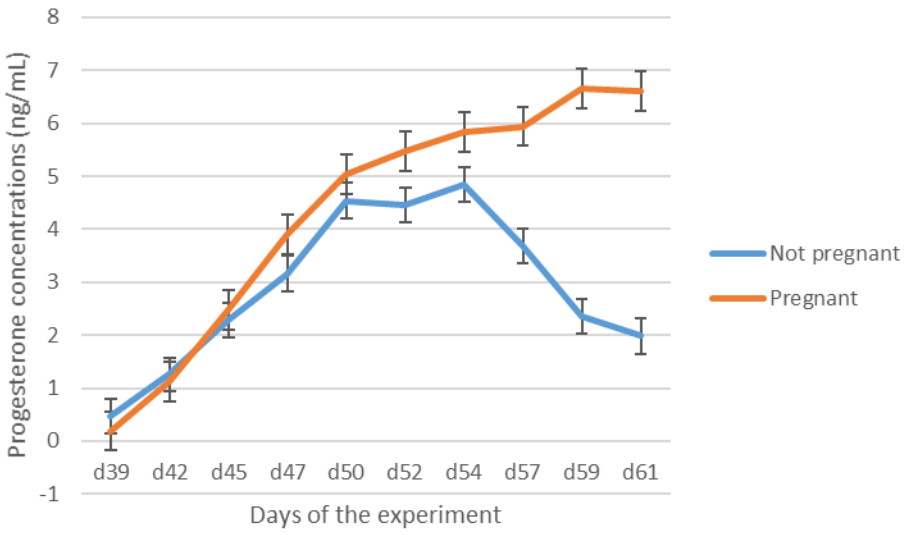

Figure 3-21. Plasma progesterone concentrations for the first cycle after timed AI on pregnant and not pregnant cows based on PAG test (d 39 to 61 of the experiment) (P < 0.001 for day * PAG interaction).

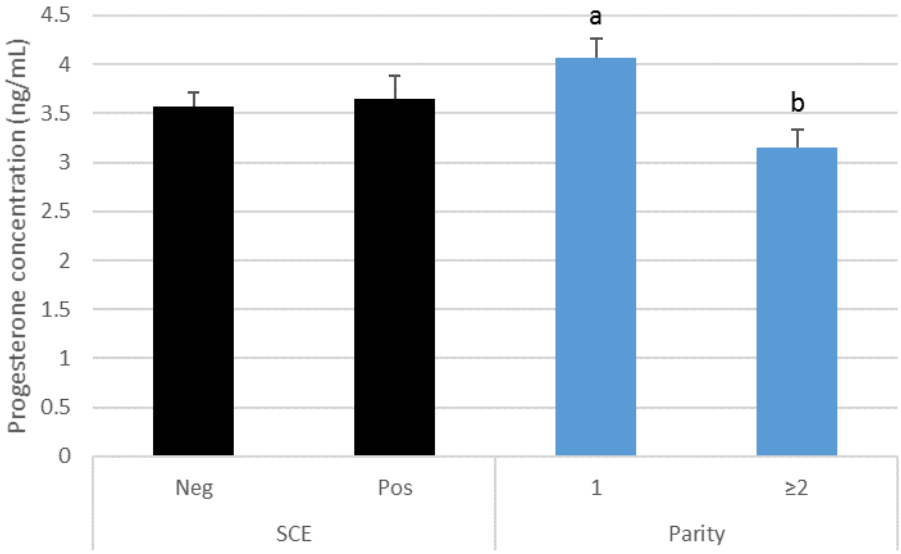

Figure 3-22. Plasma progesterone concentrations for the first cycle after timed AI for SCE and Parity status. Different letters on the bars indicates statistical difference between the groups within each variable (P = 0.8 for SCE and P = 0.001 for parity).

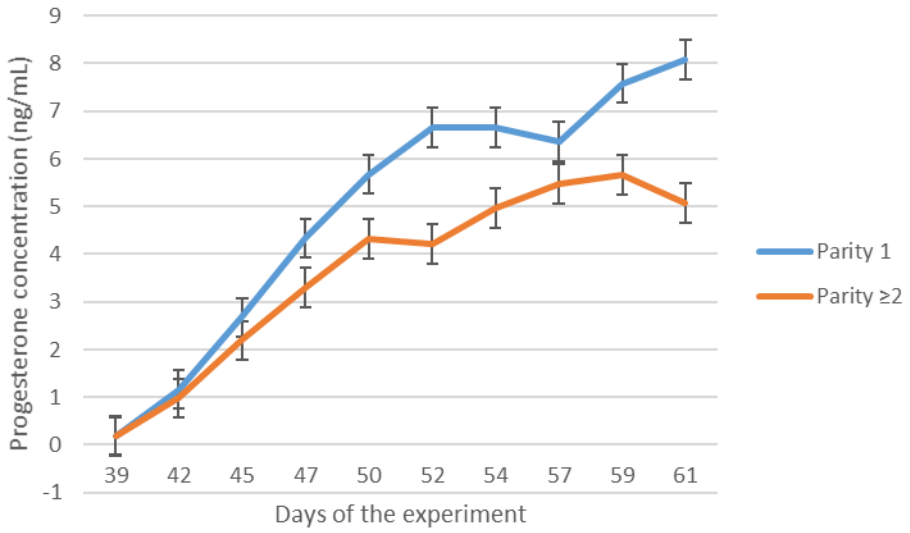

Figure 3-23. Progesterone concentrations in pregnant cows for the first cycle after timed AI by parity ($P < 0.001$).

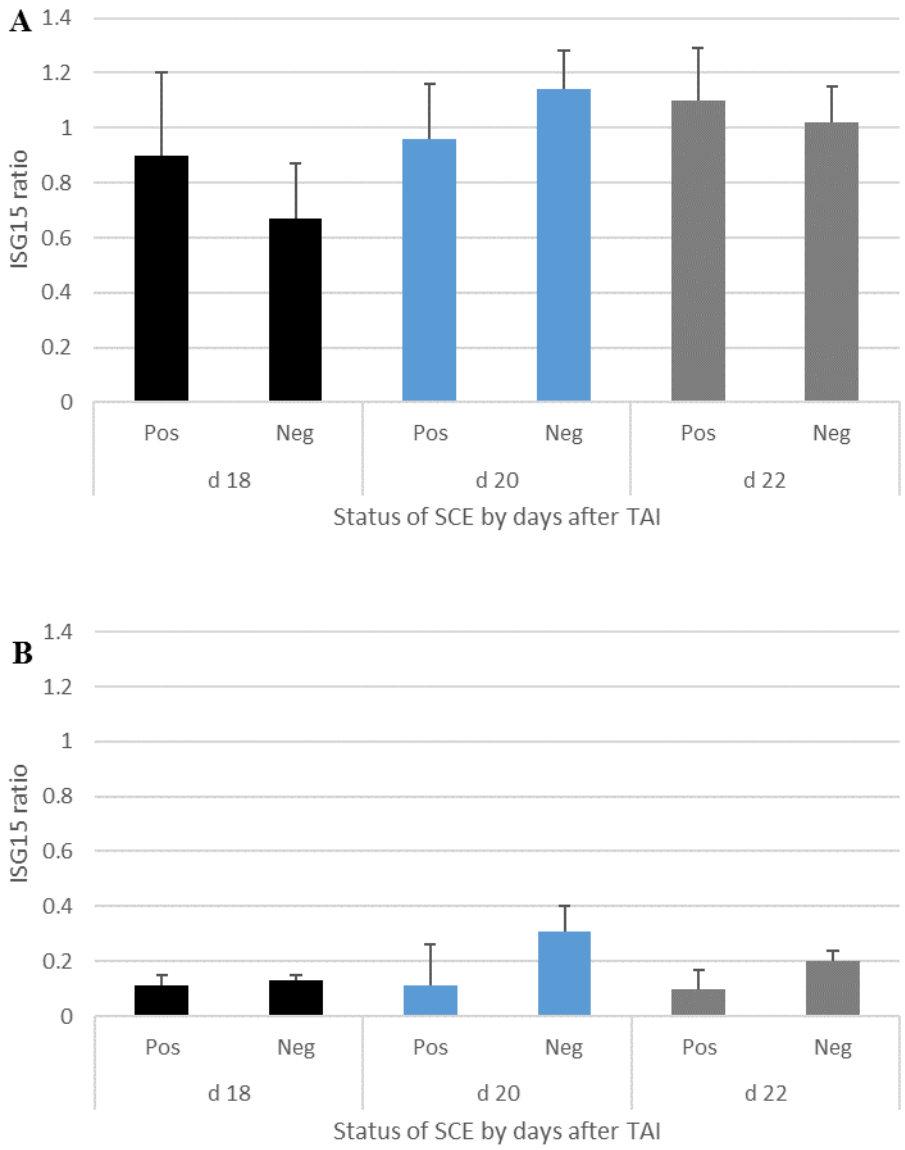

Figure 3-24. Ratio of ISG15 fold change to cyclophilin fold change at d 57, 59 and 61 of the experiment (d 18, 20 and 22 after timed AI respectively) by SCE. A) Pregnant cows (P = 0.5 for d 18; P = 0.5 for d 20; P = 0.7 for d22). B) Not pregnant cows (P = 0.7 for d 18; P = 0.3 for d 20; P = 0.3 for d 22).

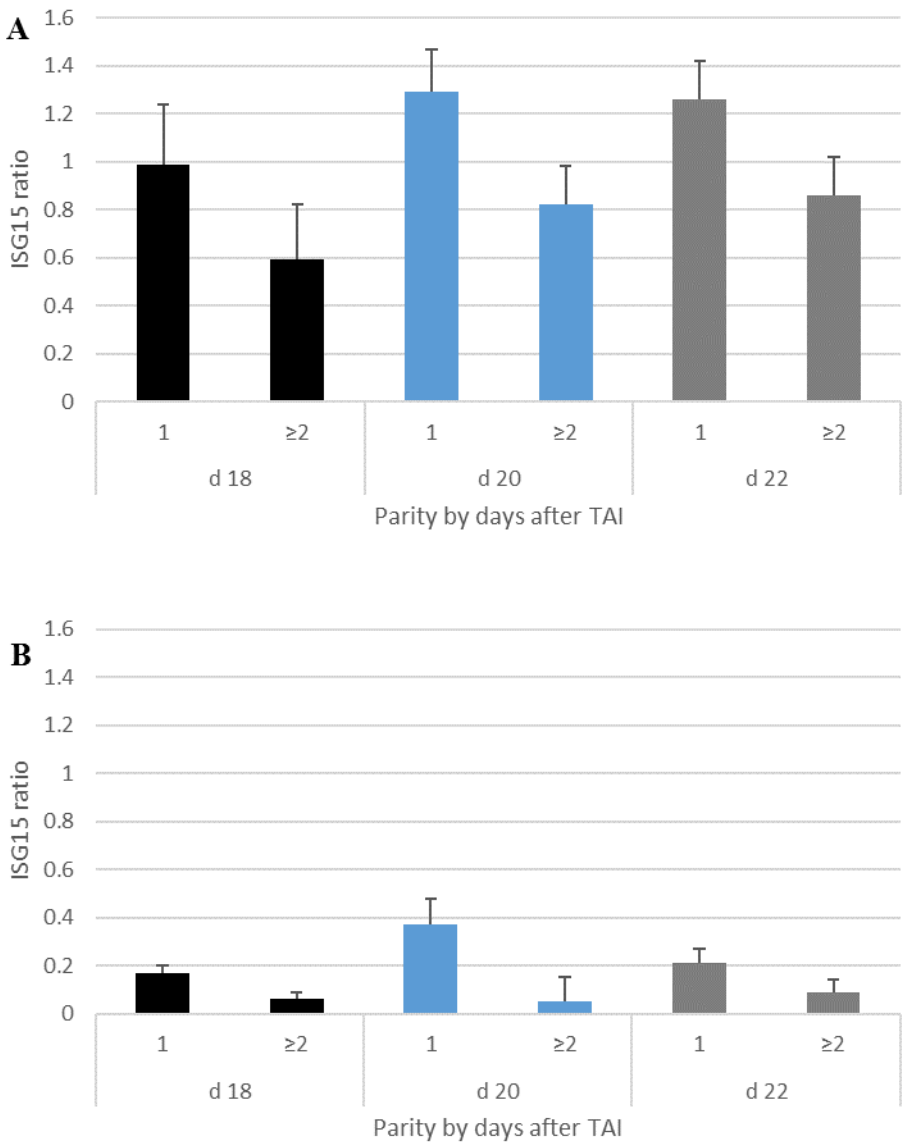

Figure 3-25. Ratio of ISG15 fold change to cyclophilin fold change at d 57, 59 and 61 of the experiment (d 18, 20 and 22 after timed AI respectively) by Parity. A) Pregnant cows (P = 0.2 for d 18; P = 0.06 for d 20; P = 0.07 for d 22). B) Not Pregnant cows (P = 0.01 for d 18, P = 0.03 for d 20 and P = 0.08 for d 22).

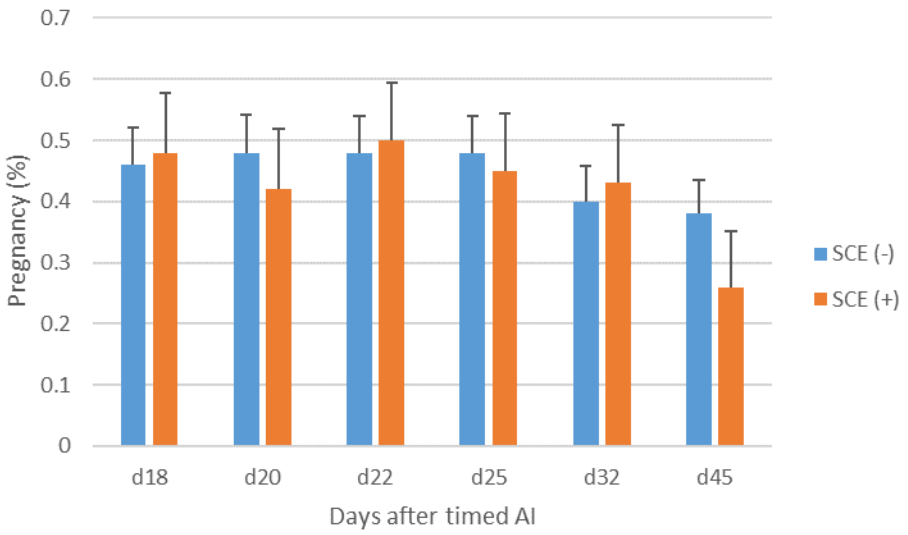

Figure 3-26. Percentages of pregnancy at days after timed AI by SCE status. Pregnacy detection at d 18, 20 and 22 by ISG15 expression. Pregnancy detection at d 25 by PAG test. Pregnancy detection at d 32 and 45 by ultrasound. The number of cows with SCE for d 18, 20, 22, 25, 32 and 45 were 27, 26, 28, 29, 28 and 27 respectively. The number of cows without SCE for d 18, 20, 22, 25, 32 and 45 were 69, 68, 70, 73, 73 and 73 respectively. Pregnancies did not differ per day [SCE (-) P = 0.7; SCE (+) P = 0.5].

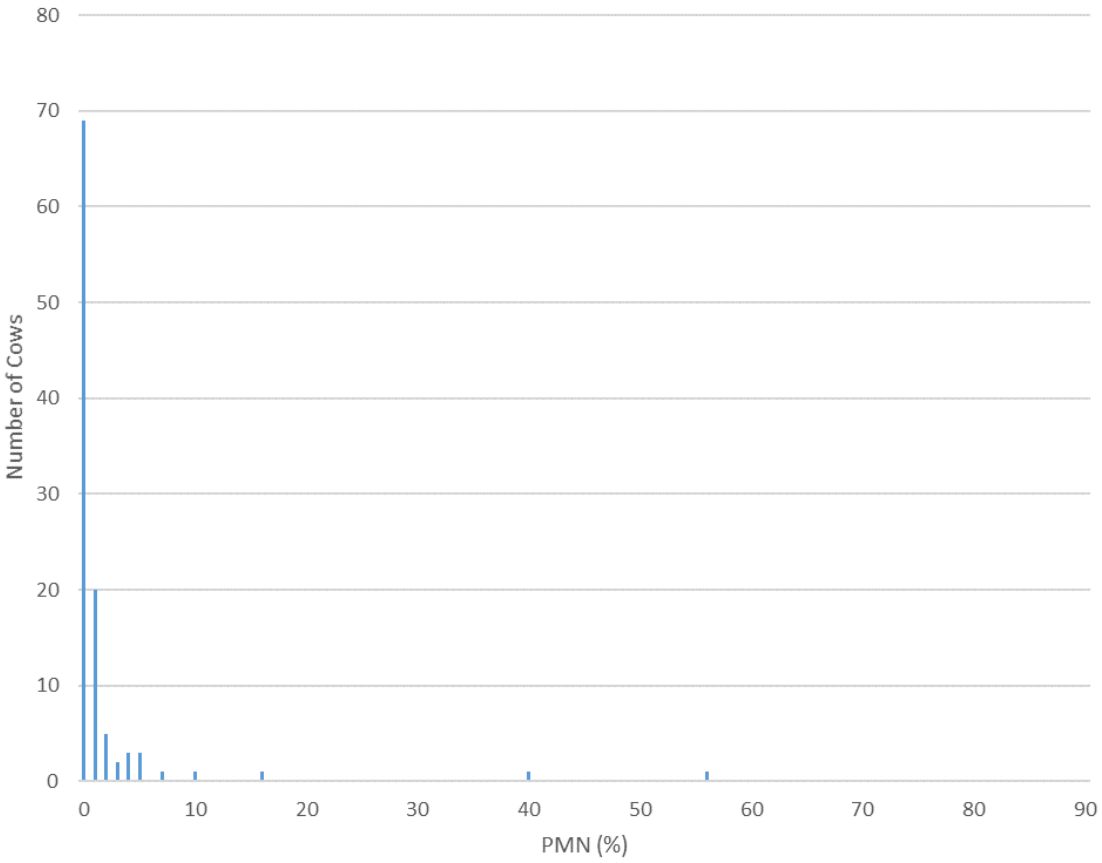

Figure 3-27. Distribution of cows by percentage of PMN based on microscope slide evaluation from cytobrush exam between 60 to 73 DIM (107 cows total).

4 Chapter

Discussion

This study evaluated the ovarian response to SCE within a timed AI. The cytobrush exam was used to assess SCE. The effects of SCE on uterine expression of ISG15 were also tested. It is known that SCE may cause failures in conception or embryonic survivability. This study was done to investigate associations between ovarian and uterine responses to SCE.

To our best knowledge, there are not many published papers about ROC curves for ISG15 ratio, however, Gifford et al. (2007) and Green et al. (2010) established the use of ISG15 ratio as an early pregnancy detection tool. This study clearly showed differential ISG15 ratio between pregnant and not pregnant cows. This supported other studies that more recently, also indicated that the expression of interferon stimulated genes is a useful tool for early pregnancy detection (Kizaki et al., 2013; Pugliesi et al., 2014; Meyerholz et al., 2016).

This study was the first one providing agreement on sensitivity, specificity and kappa values of ISG15 ratio with the PAG test. The PAG test was validated by (Green et al., 2009). Independently of the day and year evaluated, the sensitivity (true positive rate) and specificity (true negative rate) were very high and sensitivity or specificity were statistically similar between days confirming ISG15 ratio as a strong biomarker for pregnancy. The magnitude of the obtained kappa values are interpreted as almost perfect agreement between ISG15 ratio and PAG test (Flight and Julious, 2015).

The effect of parity on ISG15 expression obtained in this study was an effect previously shown by Green et al. (2010). For both years of this study the ISG15 ratio was

greater for first parity cows, however, it did not limit the use the ISG15 ratio as an early pregnancy diagnosis tool.

The number of cows on different percentages of PMN had a distribution skewed to the left which means that most of the cows were healthy (70.4 %) between 30 to 39 DIM. The range of percentages of PMN were from 0 to 90 %. The distribution obtained in this study is similar to the distribution reported by Priest et al. (2013) and Silper et al. (2016).

By using the cut point for PMN reported by Dubuc et al. (2010) ($\geq$ 6 % of PMN) 29.6 % of the cows were diagnosed with SCE, which is similar to the results of Denis-Robichaud and Dubuc (2015a) and also falling into the range of incidence of SCE reported by Pothmann et al. (2015).

Subclinical endometritis did not change the number or size of follicles during the timed AI protocol, except for the size of the largest follicle during days 34 to 39 of the experiment. Sheldon et al. (2009b) also reported uterine disease altering the largest follicle. No differences in follicles were found at d 0 and d 14 of the experiment. Maquivar et al. (2015) indicated a greater number of follicles in uterine healthy cows, which we did not find. The fact that follicles were similar was not surprising because at d 0 cows were not synchronized. Variation was probably due to cows at different stages of the cycle. Recently GobiKrushanth et al. (2016) showed no differences in follicles due to endometritis. Similar to d 0, d 14 did not show differences in follicles, again perhaps because follicular waves were not synchronized. This synchronization response to the timed AI protocol was also described by Galvao and Santos (2010).

The analysis of follicles between days 34 to 39 would be a more sensitive analysis of the effect of uterine disease on follicular response. Cows were pre-synchronized and started the Ovsynch56 a week before the evaluations, however, the Larg2F and classes of follicles were not affected by SCE. The LargF in the ovary controls growth of the rest of follicles, in spite of that, number of follicles in classes were not affected.

The differences in the size and number or follicles by day is explained by the stage of the synchronization protocol (Galvao and Santos, 2010). At d 0 and 14, sizes and number of follicles were similar because the cycles were not synchronized. At d 34 and 37 the dominant follicle was growing and the remaining follicles were regressing. At day 39 the dominant follicle had ovulated and a new cohort of small follicles must be emerging which is concise with the results of the study (Figures 3-7 and 3-8).

Parity affected the size of the LargF only during the stage of Presynch but not during the OvSynch. This indicates that the effect of synchronization may overcome effects of parity and probably the effects of SCE. It would be reason for the which, in this study, no major abnormalities of SCE on follicle response were found.

Corpora lutea was even less sensitive to SCE or parity effects than follicles. No significant effects on the size of the whole CL, lacuna or CL tissue were found at any of the evaluated stages within the timed AI synchronization protocol or 6 days after AI. These results are in agreement with Struve et al. (2013). In their study, the effect of uterine disease on the size of the CL were not clear because they were not constant during the experimental time. The differences in size of the corpora lutea during d 34, 37 and 39 were in response to the PG3 administered at day 35 of the experiment. After PG3 the CL was regressing.

The evaluation of cyclicity by ultrasound and progesterone at different points of the timed AI protocol gave similar results for cows with or without uterine disease and by parity. The percentage of cycling cows increased as the protocol progressed. The pre-ovulatory follicle diameter (approximately 15 mm) and ovulation at timed AI did not differ by SCE or parity (overall ovulatory rate approximately 92.5 %). No differences in ovulation were also found by GobiKrushanth et al. (2016). The overall ovulation rate was greater than the 80 % reported by Galvao and Santos (2010). Our results indicate that the estrous and ovulation synchronization is able to normalize the cow ovarian response independent of previous conditions such as uterine disease.

The relationship between SCE and IGF1 concentrations tended to be significant for d 0, 34, 37 and 39 and was statistically significant for the first cycle after timed AI. Cows with SCE had greater plasma concentrations of IGF1. The IGF1 concentrations are important during regenerative stages as the time after calving (uterine involution) and also, they depend on binding proteins and receptors for its functionality (Kawashima et al., 2007; Wathes et al., 2011; Clempson et al., 2012).

Parity is well known to affect IGF1 concentrations. In this study, first parity cows had greater IGF1 concentrations than cows in parity ≥ 2 which is in agreement with previous studies (Taylor et al., 2004; Grimard et al., 2013).

Fertility is positively correlated with IGF1 concentrations (Taylor et al., 2004; Patton et al., 2007; Velazquez et al., 2008), however, this study was unable to identify differences in IGF1 concentrations between cows that were pregnant or not pregnant after first AI.

Progesterone concentrations were not affected by SCE within the timed AI protocol. Struve et al. (2013) also was unable to detect progesterone differences by uterine disease. Sheldon et al. (2009b) indicated that progesterone secretion was affected. No differences in plasma progesterone or corpora lutea during the same period were found. The decrease in progesterone after PG3 was the same. Pregnant cows during the first cycle after timed AI began to differ in progesterone concentrations at approximately d 12 after AI compared with not pregnant cows. The period in which the differences appeared, coincided with the period of INFt release as the signal for pregnancy recognition (Roberts, 2007). It is also known that greater progesterone concentrations improve pregnancy development (Bazer et al., 2008). This physiological stage in pregnant cows and the normal course of the estrous cycle in not pregnant cows explain that difference in progesterone concentration and the pregnancy * day significant interaction. First parity cows had greater progesterone concentrations during this period than cows in parity 2 or greater. Similarly, Sartori et al. (2004) reported greater progesterone concentration in heifers than in adult cows.

Samples for evaluation of ISG15 were processed by the same person, by using the same reactants, procedure and equipment, however, the magnitude of the ISG15 expression was greater for the first year. This difference in magnitude did not appear to diminish the use of ISG15 as a method for pregnancy detection (section 3.1). For example, we were able to evaluate effects of uterine disease on ISG15 expression. No statistical difference or numerical trend was found for the effect of SCE on ISG15 expression in pregnant or not pregnant cows. In pregnant cows, parity did not have statistical differences, but cows in parity 1 tended to have greater ISG15 expression than

cows in parity ≥ 2 on d 20 and 22 after timed AI. In not pregnant cows, in spite of their negative pregnancy status. Parity affected the ISG15 expression on d 18 and 20 and tended to affect on d 22. Not pregnant cows in parity 1 had greater ISG15 expression than cows in parity ≥ 2. This indicates a greater threshold of ISG15 on primiparous than in multiparous cows.

The number of cows used for this study probably is not enough to make definitive conclusions about pregnancy (Lean et al., 2016), however, the early pregnancy detection by using ISG15 at d 18, 20 and 22 (Green et al., 2010; Pugliesi et al., 2014) and by using PAG test at d 25 (Green et al., 2009) plus the ultrasound scanning at d 32 and 45 after AI justify the analysis. There was evidence about uterine disease affecting the establishment of pregnancy (Vieira-Neto et al., 2014; Ribeiro et al., 2015; Toni et al., 2015; Bicalho et al., 2016), however, this study was unable to find significant differences in pregnancy in any of the days evaluated by different methods of pregnancy detection. Based on our findings, percentage of pregnancy was similar from d 18 to d 45 after AI. Ultrasound evaluations showed a numerical difference at d 45 where cows with SCE had lesser percentage of pregnancy. Overall, without statistical differences, it seems like SCE is causing embryonic losses after d 25 which was previously confirmed by Machado et al. (2015) and agrees with a recent paper from Lucy et al. (2016). Earlier embryonic losses (before d 18) are also possible based on fertilization rates reported by Diskin and Morris (2008).

The self-cure rate of SCE in this experiment was high. Subclinical endometritis decreased approximately 65 % with respect to the number of cows diagnosed with SCE at the first cytobrush exam. This self-cure rate was within the range reported by (Priest et

al., 2013). The number of cows in the experiment, the self-cure rate and the distribution of cows for parity and pregnancy did not allow an analysis for the second cytobrush exam as the analysis done for the first cytobrush exam, however, the eleven cows diagnosed with SCE based on the second cytobrush had in common the not pregnant status at d 45 of the experiment. Four of these cows lost the embryo very early after conception or never got pregnant. Six cows were diagnosed pregnant by ISG15 and/or PAG test but not pregnant by ultrasound which means that there were some embryonic losses detected by the use of these pregnancy methods. From one cow, no ISG15 information was available but this cow was diagnosed pregnant by PAG test and then not pregnant by ultrasound. The pregnancy fate of these individual cows indicate that SCE diagnosed during the same week of timed AI affected the establishment and maintenance of pregnancy which again agrees with Machado et al. (2015) and Lucy et al. (2016).

5 Chapter

Conclusions

The self-cure rate of SCE was high. Most of cows were not inseminated under uterine inflamed conditions.

There were no clear associations between SCE and ovarian structures. This study does not support the hypothesis that SCE impairs ovarian response to a timed AI program.

The timed AI protocol was able to overcome previous inflammatory conditions that affect fertility.

There were unexpected associations between IGF1 and SCE.

Plasma progesterone concentrations were not affected by SCE status.

The uterine expression of ISG15 was similar for cows with and without SCE. If SCE causes impairment of INFt secretion or ISG15 response, it is earlier or later than the evaluated period.

We did not observe a statistically significant effect of SCE on pregnancy. There appeared to be greater pregnancy loss after day 32 in SCE cows but this need to be re-evaluated in larger trials.

The second cytobrush demonstrated that the diagnose of SCE 4 days before timed AI was associated with 100% embryonic loss between day 25 and 45 of pregnancy.

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