The Relationship Between Postpartum Ovulation and Concentration of Nutritional and Inflammatory Blood Markers in Multiparous Dairy Cows

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AUTHORIZATION TO SUBMIT THESIS

This thesis of Ashalynn E. Bilton-Smith, submitted for the degree of Master of Science with a Major in Animal Science and titled "The Relationship Between Postpartum Ovulation and Concentration of Nutritional and Inflammatory Blood Markers in Multiparous Dairy Cows," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

Previous research has shown the association between negative energy balance, changes in blood metabolites, and reproductive performance. Cows that ovulate later in the early postpartum period exhibit an increased time to pregnancy. Thus, delayed ovarian cyclicity ultimately leads to longer calving intervals, multiple AI attempts, and loss of profit. Although relationships exist between inflammatory blood biomarkers and disease states, the relationships between these biomarkers, postpartum ovarian cyclicity, and fertility in dairy cows have not been thoroughly investigated. The objective of this study was to examine the relationship between body condition score (BCS), glucose (GLU), cholesterol (CHO), free cholesterol (FCHO), serum amyloid A (SAA), and resumption of ovarian activity during the early postpartum period in lactating dairy cows. Sixty-seven multiparous Holstein cows were monitored from 2 weeks prepartum to 8 weeks postpartum. Weekly blood samples, BCS, and ultrasonography were obtained to characterize inflammatory responses, energy status, ovarian structures, blood progesterone (P_4), and the approximate timing of ovulation. Cows were divided into two groups; early ovulation (EO < DIM; n=21), vs late ovulation (LO >28 DIM; n=46) and healthy (H; n=40), vs sick (S; n=27). As expected, BCS decreased over time for both groups ($P < 0.01$). However, BCS was greater for EO (2.8 ± 0.09 score) than LO (2.5 ± 0.06 score) ($P < 0.02$). Mean blood CHO concentrations were greater in EO when compared with LO in weeks 4,5 and 8 ($P < 0.03$). The rate of increase in CHO over 8 weeks tended to be greater for the EO group ($P < 0.08$). There was no difference between EO and LO groups regarding blood SAA, FCHO, or GLU concentrations. When comparing H and S groups, mean CHO concentrations were greater in H than S in weeks 4,7, and 8 ($P < 0.01$). The overall pattern of blood CHO concentrations during the postpartum period

increased over time and tended to be greater in H cows when compared to S ($P < 0.06$). The mean SAA concentrations in S cows were greater than H cows (138 ± 13.4 vs 76.1 ± 11.1 ug/mL) ($P < 0.01$). There was no difference between H and S groups regarding BCS, FCHO, or GLU concentrations. These results provide evidence that BCS and blood CHO concentrations during the early postpartum period are associated with the timing of the first postpartum ovulation in multiparous cows. Blood CHO and SAA concentrations are also associated with the presence of a disease state in early postpartum cows. Therefore, these markers may be used as a tool to identify late ovulating cows, or cows experiencing disease, and provide an opportunity for producers to better manage these cows.

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DEDICATION

To the people who raised me, and to those who have loved me. You have made a difference.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

The Relationship Between Postpartum Ovulation and Concentration of Nutritional and Inflammatory Blood Markers in Multiparous Dairy Cows

ABSTRACT

Previous research has shown the association between negative energy balance, changes in blood metabolites, and reproductive performance. Cows that ovulate later in the postpartum period have been shown to exhibit an increased time to pregnancy. Thus, delayed ovarian cyclicity ultimately leads to longer calving intervals, multiple AI attempts, and loss of profit to dairy farmers. Although relationships exist between inflammatory blood biomarkers and disease states, the relationships between these biomarkers, postpartum ovarian cyclicity, and fertility in dairy cows have not been thoroughly investigated. The objective of this study was to examine the relationship between serum amyloid A (SAA), glucose (GLU), cholesterol (CHO), free cholesterol (FCHO), body condition score (BCS), and resumption of ovarian activity during the early postpartum period in lactating dairy cows. Sixty-seven multiparous Holstein cows were monitored from 2 prepartum to 8 weeks postpartum. Weekly blood samples, body condition scores, and ultrasonography were obtained to characterize inflammatory responses, energy status, ovarian structures, blood progesterone, and time of ovulation. Cows were divided into two groups; early ovulation (EO < 28 DIM; n=21), vs late ovulation (LO >28 DIM; n=46) and healthy (H; n=40), vs sick (S; n=27). Biological markers were compared and analyzed between groups. When comparing EO vs LO BCS showed a tendency to be different ($P \leq 0.07$) in weeks 3 and 4 and were significantly different ($P \leq 0.02$) in weeks 5,6 and 8. As expected, BCS decreased

over time for both groups ($P < 0.01$). Mean blood CHO concentrations were greater in EO when compared with LO in weeks 4,5 and 8 ($P < 0.03$). In weeks 3 and 7, mean blood CHO concentrations tended to be greater in EO than LO ($P \leq 0.07$). The overall pattern of CHO increase over time was different between EO and LO groups ($P < 0.01$) where the rate of increase CHO over 8 weeks tended to be greater for the EO group ($P < 0.08$). There was no difference between EO and LO groups regarding blood SAA, FCHO, or GLU concentrations. When comparing H and S groups there was an effect of the week by health status interaction on blood CHO concentrations ($P = 0.04$). Mean CHO concentrations were greater in H when compared to S in weeks 4, 7, and 8 ($P < 0.01$) and tended to be different in weeks 2, 3, 5, and 6 ($0.07< P < 0.1$). Regression analysis showed the overall increase pattern of blood CHO concentrations over time and was different between H and S groups ($P < 0.01$). Overall, the mean SAA concentrations in S cows were greater than H cows (138 ± 13.4 vs 76.1 ± 11.1 ug/mL) ($P < 0.01$). There was an effect of health status by week interaction on SAA ($P < 0.05$). Sick cows showed a decreasing concentration in SAA between weeks 1 and 2 while SAA concentrations in H cows stayed constant. There was no difference between H and S groups regarding blood BCS, FCHO, or GLU concentrations. These results provide evidence that BCS and blood CHO concentrations during the early postpartum period may be associated with the timing of the first postpartum ovulation in multiparous cows. Blood CHO and SAA concentrations may be associated with the presence of a disease state in early postpartum cows. Therefore, these markers may be used as a tool to identify late ovulating cows, or cows experiencing disease and provide an opportunity for producers to manage the care of these cows.

INTRODUCTION

Over the past few decades, selection for genetics that favor increased lactational output and metabolic efficiency has improved the milk yield of a dairy cow. Along with the selection for high production, a concern about declining fertility has arisen. There is evidence to suggest a greater incidence of delayed postpartum ovarian cycles during the voluntary waiting period in high-producing dairy cows (Stevenson et al., 1983; Staples et al. 1990; Shrestha et al., 2004). In high producing dairy cows, the incidence of abnormal postpartum ovarian cycles was reported to be 55.5% inclusive of inactive ovaries, ovarian cysts, and nonfunctional (low progesterone producing) corpora lutea (Shrestha et al., 2004). The prevalence of anovulation between 50 and 60 DIM has been reported to affect 20% to 40% of cows in a herd (Moreira et.al., 2001; Cerri et.al., 2004; El Zarkouny et. Al., 2004). Galvão, et al. (2010) reported increasing days between parturition and time of first ovulation, correlated with an increased time to pregnancy. Increased abnormal ovarian cyclicity (and anovulatory anestrus) during the early postpartum period may negatively impact subsequent reproductive performance, including increased days postpartum to first AI, increased submission rates to AI, reduced pregnancy rates, and ultimately increasing the calving interval (Lucy et al. 1992; Shrestha et al., 2004).

Negative energy balance (NEB) is a natural and expected metabolic state, which dairy cows go through during the early postpartum period. Negative energy balance is the result of insufficient dry matter intake (energy intake) to support the nutritional demands of lactation (energy output). Many blood metabolites are altered during the time when lactating cows go through the NEB state and blood metabolites have been implicated in playing a potential role in influencing early postpartum ovarian activity (Nebel and Mc Gillard, 1993;

Butler, 1998; Rajala-Schultz et al., 1999; Royal et al., 2000; Shrestha et al., 2004). Efficient utilization of GLU by immune cells is critical for maintaining cellular functions, as well as supporting an optimal response to invading microorganisms and aiding in repair processes (Ingvartsen and Moyes, 2013). Two of the major energetic fuels currently known to be used by immune cells are GLU and glutamine (Ingvartsen and Moyes, 2013). Greater concentrations of blood GLU and CHO have been associated with fewer days open (Reist et al., 2003). Unfortunately, GLU levels are lowest during the early postpartum period. In certain metabolic states, such as NEB, alteration in the availability and utilization of GLU and CHO may contribute to immunosuppression during the transition period and negatively affect postpartum ovulation.

Wathes et al. (2009) provided evidence that cows in severe NEB were still undergoing active uterine inflammatory responses during the first 2 weeks postpartum, while white blood cell count and lymphocyte numbers were reduced. The authors hypothesized that severe NEB may prevent cows from mounting an effective immune response to the microbial challenge expressed after calving, prolonging the time required for uterine recovery and compromising subsequent reproductive performance.

Acute-phase proteins (APPs) are a class protein measured in plasma that either increase or decrease in response to an inflammatory response, and in literature are often called acute phase reactants (APR). Serum amyloid A (SAA) is an acute-phase protein that is expressed in normal, healthy tissues (Berg et al., 2011). An increase in SAA has been recorded in cows exhibiting signs of clinical disease (Abuajamieh et al., 2016; Biswal et al., 2014; Chan et al., 2010; Dervishi 2016; Nazifi et al., 2010; Gozho et al., 2005; Gozho et al., 2007; Guzelbektes et al., 2010; Ksenija et al., 2019; Sadek et al., 2017; Szczubiał et al.,

2012). In the days following parturition, an increase in SAA has also been observed in healthy cows (Alsemgeest et al., 1993). Also, an increase in the number of APPs has been used as a potential method of quantifying clinical disease, as well as detecting subclinical disease through the measurement of the inflammatory response.

Serum Amyloid A (SAA) elevated levels have been associated with the presence of endometritis (Biswal et al., 2014; Chan et al., 2010; Dervishi 2016; Nazifi et al., 2010), mastitis (Sadek et al., 2017; Szczubiał et al., 2012.), abomasal displacement (Guzelbektes \et al., 2010), retained placenta, ketosis (Abuajamieh et al., 2016), acidotic states (Gozho et al., 2005; Gozho et al., 2007), laminitis, and hoof ulcers (Ksenija et al., 2019) as well as being elevated in the first days following parturition (Alsemgeest et al., 1993). Collectively, studies show that nutrition and nutritional status play a pivotal role in the immune response, and the nutritional effect may be through nutrients or indirectly through blood metabolites.

Although previous studies have examined the association between immune markers, energy balance, and overall reproductive health, these studies did not investigate the relationship between blood inflammation markers, nutrition markers, and the resumption of ovarian activity in early postpartum dairy cattle. Observing and quantifying blood metabolites in the early postpartum period may help identify the earliest realistic opportunity for better reproductive management.

HYPOTHESIS

As the interval between parturition to first postpartum ovulation increases, the time to pregnancy increases (Galvão et al., 2010). Delayed postpartum ovarian cyclicity

ultimately leads to delay in conception, multiple AI attempts, longer calving intervals, and loss of profit to dairy farmers.

Although studies have established relationships between many blood-borne biomarkers, disease states, and reproductive performance, the relationships between SAA, CHO, FCHO, GLU, and postpartum ovarian status have not been thoroughly investigated. Evaluating the relationship between these metabolite markers and their association with the resumption of ovarian activity will open more avenues to investigate factors influencing reproductive inefficiency. We hypothesize that there is a difference in BCS, blood GLU, blood CHO, blood FCHO, and blood SAA concentrations in cows exhibiting a longer interval from parturition to first ovulation when compared to cows with a shorter interval.

OBJECTIVES

To examine the relationship between BCS, blood GLU, blood CHO, blood FCHO, and blood SAA concentrations, and postpartum resumption of ovarian cyclicity in multiparous dairy cows.

Specific goals:

1) Observe and evaluate:
    a. Time of first ovulation postpartum
        i. Conducting transrectal ultrasonography to observe ovarian structures
        ii. Characterizing serum progesterone concentrations.
    b. Presence or absence of a clinical disease state.
    c. Blood concentrations of nutritional metabolites:

i. Characterizing plasma GLU concentrations.

ii. Characterizing serum CHO concentrations.

iii. Characterizing serum-free-CHO concentrations.

d. Blood concentrations of inflammatory metabolites:

i. Characterizing plasma SAA concentrations.

MATERIALS and METHODS

ANIMALS

This study was conducted continuously from October 2017 to April 2019 at the University of Idaho Dairy Research and Education Center located in Moscow, Idaho. All animal treatment protocols and handling procedures were approved prior to the initiation of the experiment by the University of Idaho Institutional Animal Care and Use Committee (IACUC # 2018-58). Sixty-seven multiparous dairy cows, from approximately 2 weeks prepartum through 8 weeks post-partum, were included in this study. All cows were pregnant and healthy at the initiation of the study. Cows received ad libitum access to a total mixed ration (TMR) and water for close-up dry cows and lactating cows provided by the University of Idaho Dairy Center. Dry cows were housed in straw bedded and dirt lots, were moved to a heated maternity barn immediately after calving, and within 24 hours were turned into a loafing area with either free stall or tie stall housing. Cows were milked four times daily for the first 8 weeks postpartum.

CHARACTERISTICS OF RESEARCH COWS

Approximately 2 weeks before the expected parturition date, cows were enrolled in the study. Initially, 73 cows were enrolled in the study but 6 were removed because of illness and death. One cow died from hypocalcemia shortly after calving, and another suffered from a left displaced abomasum resulting in her removal from the study. Another fell, resulting in a fractured pelvis, one exhibited hemorrhagic bowel, and two others became non-ambulatory because of unknown causes: all resulting in humane euthanasia under the approved University of Idaho IACUC protocol for humane euthanasia of dairy cattle. The data were collected from 67 cows in the experiment.

EXPERIMENTAL PROCEDURES

Two weeks before the anticipated calving date, all animals were confirmed pregnant, blood samples were taken, and initial body condition scores were recorded. On the day of calving (6-12 hours postpartum), a blood sample was collected, and farm protocols were followed in the movement and processing of the dam. Cows were moved from the close-up pen at the onset of parturition and placed in a heated maternity barn. All cows then received a calcium bolus and ad libitum alfalfa, TMR, and water after calving. Cows remained in the maternity barn for approximately 2-6 hours before being separated from their calves and moved into free-stall housing or placed back in the tie stalls. After being placed in the lactating herd, cows started receiving TMR formulated to meet or exceed the nutritional requirements for high-producing cows (NRC, 2001). Cows also had ad libitum access to water and >20 h access to TMR. Transrectal ultrasonography was performed, and blood samples were obtained every 7 days postpartum until 56 DIM. Additionally, body condition

scores (BCS), expression of estrus, and health status were recorded at enrollment into the study (-2 weeks postpartum) and weekly thereafter until the end of the study (Figure 1.1). Health conditions were monitored and recorded by research staff as well as by farm personal during the experimental period. Both milk yield and parity data were provided using farm records via Dairycomp 305 software (Valley Agricultural Software, Tulare, CA). Body condition scoring was conducted by 2 individuals using a 5-point scale with 1 = very thin and 5 = obese (Edmonson et al., 1989). The mean of the 2 scores was recorded.

BLOOD COLLECTION

Blood sampling was performed to characterize progesterone, blood-based markers, and their association with the resumption of ovarian cyclicity and early postpartum estrous behavior. Two blood samples were collected approximately 14 days before parturition, on the day of parturition, and every 7 days (+/- 1 day) for 56 days after calving (Figure 1.1). Cows were restrained in headlocks, and samples were taken via coccygeal venipuncture using 18G, 1 ½" Vacutainer® needles, and 10ml BD, Vacutainers® (Becton and Dickinson, Franklin, NJ). All samples were placed on ice immediately and transported to the lab within 3 hours of collection. Blood for plasma collection was placed into tubes containing sodium heparin, centrifuged at 2100 x *g* at 4˚C for 20 mins within 3 hours of collection. Blood samples for serum collection were collected into empty tubes without an anticlotting agent, stored at 4˚C for 24 hours before being centrifuged at 2100 x *g* at 4˚C for 20 mins. All harvested plasma and sera were stored at -20˚C until assayed.

ULTRASONOGRAPHY AND CHARACTERIZATION OF OVULATION

Transrectal ultrasonography (Aloka SSD-500 V, Aloka, Tokyo, Japan), was conducted on days 7, 14, 21, 28, 35, 42, 49, 56 (+/-1d) postpartum using a 7.5-MHz transrectal linear probe (Figure 1.1). Ovarian structures were visualized to determine the presence of follicles and corpora lutea (CL). The location, number of follicles, and CL were recorded, and the approximate timing of the first postpartum ovulation was established.

Occurrence of the first ovulation postpartum was determined by analyses of plasma progesterone (P_4; ≥1 ng/mL) from blood samples collected weekly (Lopez et al., 2005). Evidence of cyclicity beyond the first postpartum ovulation was determined from two blood samples collected in consecutive weeks, where one or both samples were greater than 1 ng/mL of progesterone. Retrospectively, occurrence of the first postpartum ovulation was verified by the comparison of progesterone concentration to the visualization of a corpus luteum by ultrasonography. Ovulation was defined as the disappearance of any follicle >10mm in diameter and the formation of a CL in the same location (Sellars et al., 2006).

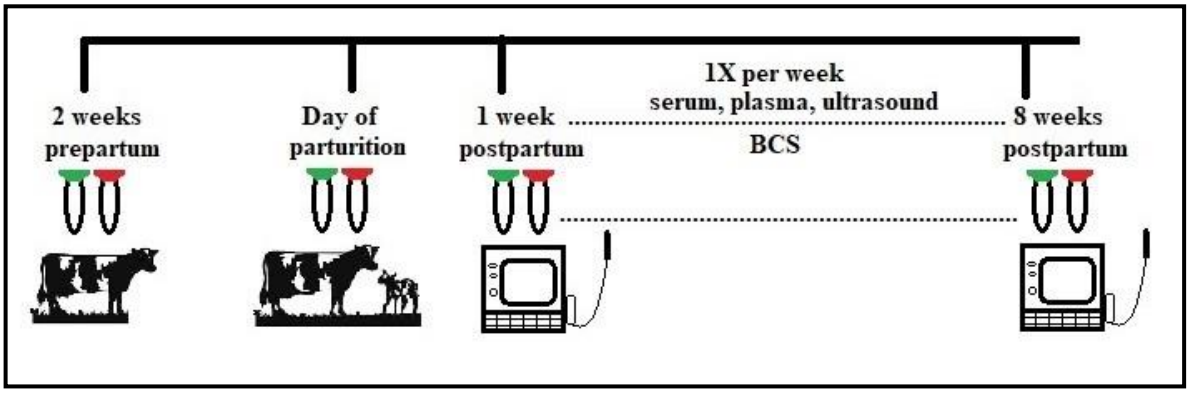

Figure 1.1 Experimental protocol in multiparous Holstein cows. Coccygeal blood samples were collected weekly from 2 weeks prepartum to 8 weeks postpartum, including the day of calving. Progesterone, GLU, and CHO concentrations were measured for all weeks, FCHO concentrations were measured weeks 1-4, and SAA concentrations were measured in weeks 1 and 2. Body condition score was also monitored weekly and weekly ultrasounds were performed after calving to visually monitor ovarian activity in conjunction with P_4 concentrations.

CHARACTERIZATION OF EARLY AND LATE OVULATORY GROUPS

Based on the estimated time of first postpartum ovulation cows were retrospectively separated into two groups. Cows exhibiting first ovulation postpartum before 28 days were categorized as the early ovulatory group (EO) and cows that ovulated after 28 days postpartum were categorized as the late ovulatory group (LO). The EO group consisted of 21 cows and the LO group consisted of 46 cows. Unless stated to the contrary, LO cows included cows that did not ovulate for the first time by the conclusion of the study at 56 days. There were 29 cows that did not ovulate by 56 days.

CHARACTERIZATION OF HEALTHY AND SICK COWS

Health events were recorded over the duration of the experimental trial by researchers and University of Idaho Dairy staff. Incidence of any clinical disease symptoms or cows undergoing treatment for disease symptoms were considered ‘sick’ cows. Diseases encountered included mastitis, lameness, retained placenta, ketosis, metritis, and hemorrhagic diarrhea. Although a wide variety and severity of diseases were observed, there was not one disease that was more prevalent than another. For the first 9 days postpartum the University of Idaho monitored rectal temperatures on cows. Elevated body temperature was considered a sign of disease and cows were given an aspirin bolus as per farm protocol. Cows with an elevated body temperature were included in the sick category. Cows with clinical mastitis received antibiotic treatment, cows with lameness/swelling received flunixin meglumine, and ketotic cows were given dexamethasone, dextrose, and propylene glycol as outlined by U of I farm protocol. All cows with no incidence of disease were placed in the healthy cohort.

BLOOD CONCENTRATION MEASUREMENTS

PROGESTERONE

Progesterone concentrations were quantified using a double-antibody solid-phase radioimmunoassay (ImmuChem Double Antibody, P4- ^{125}I RIA; ICN Biomedicals, Inc., Costa Mesa, CA) in an equilibrium condition. Samples were run in duplicate with a 7-point standard curve from 0.0 to 25 ng/ml. Briefly, 100 ul of serum samples, P_4 standards, and controls were aliquoted into 5 ml tubes. Using a repeat pipettor, 500 ul of primary antiprogesterone antibody and 200 ul of Iodinated P_4 were aliquoted into sample tubes. All tubes were then vigorously shaken and incubated at 37 °C for 120 minutes. After incubation, 500 ul of precipitant solution (second antibody) was added, the samples were vigorously shaken again and centrifuged at 1000 x *g* for 20 minutes at 4 °C. To separate bound from free P_4, supernatants were decanted into radiological waste containers. After allowing the tubes to dry, the pellets (bound P_4) in sample tubes were counted in the gamma counter (Packard Cobra Gamma Counter 5010) for 1 minute.

Samples (n=35) that were found to be uncharacteristically high or low were again tested for P_4 using a solid phase single antibody-coated tube RIA coated tube radioimmunoassay in an equilibrium condition (ImmuChem Coated Tube P_4-^{125}I RIA; ICN Biomedicals, Inc., Costa Mesa, CA). These coated tubes were coated in antiserum generated in a rabbit using 11α-hydroxy- P_4-11α-hemisuccinate-BSA as an antigen. This assay involved adding 100ul serum into coated tubes, adding 1.0 ml of iodinated P_4 to all tubes, and incubating them for 120 minutes at 37 °C. After incubation, the tubes were decanted and read by the gamma counter to quantify the level of P_4 (Packard Cobra Gamma Counter 5010).

Assay standards were modified per the procedure developed by Scarpa et al. (2019). Briefly, P_4 (minimum 99%, SIGMA-ALDRICH, Inc., St. Louis, MO) was dissolved in absolute ethanol for a working P_4 stock solution (50 ug/mL; VWR Seradigm LIFE SCIENCE®, VWR International, LLC, Randor, PA)). Progesterone standards were prepared by serial dilution of the P_4 stock solution in fetal bovine serum (Sigma-Aldrich, St. Louis, MS). Fetal bovine serum was used as a diluent to account for matrix effects observed in previous assays using original standards (with human serum as the diluent) provided by the manufacturer. The intra-assay coefficient of variation (CV) between the 3 assays was 3.65% and the inter-assay CV was 8.70%.

GLUCOSE

Glucose was measured in plasma using an enzymatic, colorimetric method (FUJIFILM Wako Pure Chemical Corporation, Chuo-Hu Osaka, Japan). The manufacturer's microtiter procedure was followed, and plates were read using the SpectraMax i3 Multi-Mode Microplate Reader with the SoftMax Pro 6.5 1' software. The 96 well Corning Costar microplates were incubated and read at 505-600 nm wavelengths. The 6-point standard curve was set at 0.0 to 160 mg/dL using one thaw cycle plasma. All weekly samples from cows were measured from 2 weeks prepartum to 8 weeks postpartum in duplicate. The intra-assay coefficient of variation (CV) between 19 plates was 3.97% and the inter-assay CV was 3.70%.

TOTAL CHOLESTEROL

Total CHO was measured in serum using an enzymatic, colorimetric method (FUJIFILM Wako Pure Chemical Corporation, Chuo-Hu Osaka, Japan). The manufacturer's microtiter procedure was followed, and plates were read using the SpectraMax i3 Multi-Mode Microplate Reader with the SoftMax Pro 6.5 1' software. Ninety-six well Corning Costar microplates were incubated and read at 600-700nm wavelengths. The 6-point standard curve was set at 0.0 to 300 mg/dL using one thaw cycle serum. All weekly samples from cows were measured from 2 weeks prepartum to 8 weeks postpartum in duplicate. The intra-assay coefficient of variation (CV) between 22 plates was 7.68% and the inter-assay CV was 3.51%.

FREE CHOLESTEROL

Free CHO was measured in serum using an enzymatic, colorimetric method (FUJIFILM Wako Pure Chemical Corporation, Chuo-Hu Osaka, Japan). The manufacturer's microtiter procedure was followed, and plates were read using the SpectraMax i3 Multi-Mode Microplate Reader with the SoftMax Pro 6.5 1' software. Ninety-six well Corning Costar microplates were incubated and read at 600-700nm wavelengths. The 6-point standard curve was set at 0.0 to 100 mg/dL using first thaw serum. Weekly samples from cows were measured from the day of calving to 4 weeks postpartum in duplicate. The intra-assay coefficient of variation (CV) between 10 plates was 2.30% and the inter-assay CV was 2.29%.

SERUM AMYLOID A

Serum amyloid A was measured in plasma using a multispecies enzyme-linked immunosorbent assay (Tridelta Development Limited, Maynooth, Ireland). The manufacturer's procedure was followed, and plates were read using the SpectraMax i3 Multi-Mode Microplate Reader with the SoftMax Pro 6.5 1’ software. A monoclonal antibody specific for SAA was coated onto a manufacturer-provided 96 well microplate which was incubated and read at 450nm wavelengths with 630nm as reference. The 6-point standard curve was set at 0.0 to 300 ng/mL using first thaw plasma. Week one and week two postpartum samples from all cows were measured after being diluted at 1:500 or 1:1000. Dilutions were performed using the kit-provided dilution solution and at the direction of the Tridelta instructions for the bovine species. The intra-assay coefficient of variation (CV) between 4 plates was 12.6% and the inter-assay CV was 14.7%.

STATISTICAL ANALYSIS

ANALYSIS OF DEPENDENT VARIABLE OVULATION STATUS

The difference between groups (EO vs LO) for descriptive data, including average days to ovulation, parity, milk production, body condition score (BCS), and SAA were analyzed by ANOVA using the GLM procedure of SAS version 9.4. The model included the main effect of EO/LO treatment, parity, and treatment by parity interaction. The mixed model (PROC MIX) procedure of SAS was used to conduct an analysis of repeated measures on GLU, CHO, and FCHO. The statistical model included treatment (EO or LO), the repeated factor of time (weeks), and treatment × time interaction. Cows were considered the random effect. Regression analysis was also conducted to analyze the changes in blood

GLU, CHO, and FCHO over time using the PROC REG function in SAS. The overall pattern of the regression line, slope (the rate of change over time), and intercept between the groups were compared using contrast statements. All means are expressed as least square means (LSM) ± standard error of the mean (SEM). A probability of 0.05 or less was considered significant, and a probability between 0.05, and 0.10 were considered as a tendency.

ANALYSIS OF DEPENDENT VARIABLE HEALTH STATUS

The difference between groups (healthy vs. sick) for descriptive data including average days to ovulation, parity, milk production, body condition score (BCS), and serum amyloid A were analyzed by ANOVA using the GLM procedure of SAS version 9.4. The model included the main effect of health status (H or S), parity, and treatment × parity interaction. The mixed model (PROC MIX) procedure of SAS was used to conduct an analysis of repeated measures on GLU, CHO, and FCHO. The statistical model included treatment (healthy/ sick group), the repeated factor of time (weeks), and treatment × time interaction. Cows were considered the random effect. Regression analysis was also conducted to analyze the changes in blood GLU, CHO, and FCHO over time using the PROC REG function in SAS. The overall pattern of the regression line, slope (the rate of change over time), and intercept between the groups were compared using contrast statements. All means are expressed as least square means. A probability of 0.05 or less was considered significant, and a probability between 0.05, and 0.10 were considered as a tendency.

ANALYSIS OF PROBABILITY OF OVULATION

Predicted probabilities of pregnancy were computed using the LOGISTIC procedure in SAS and the effect of BCS or CHO at weeks three postpartum on the incidence of ovulation by day 28 postpartum was determined. The logistic regression equation for the prediction model for the probability of pregnancy took the form of:

ln [P / 1 - P] = β_0 + β_1x1; x = BCS or blood CHO;β_0 = intercept; β_1 = slope

REVIEW OF LITERATURE

Reproductive efficiency strongly affects the economic wellbeing of dairy producers, therefore planning and management are essential for maximizing profit. Nutrition, genetics, housing, and disease prevention must all align to optimize and secure livestock health. The reproductive efficiency of lactating dairy cows is not optimal, even after the implementation of transition and fresh cow management protocols on many dairies in the past 10-15 years. The suboptimal reproductive efficiency may be related, in part, to a lack of understanding about the resumption of ovarian cyclicity, early postpartum estrous behavior, and nutritional status prior to the end of the voluntary waiting period (day 50 to 70 postpartum).

The effects of abnormal postpartum ovarian cycles on reproductive performance, and the mechanisms involved, have not been well established. Moreover, the relationship between the resumption of ovarian cyclicity, nutritional markers, and inflammatory biomarkers such as GLU, CHO, FCHO, and serum amyloid A is not well defined. There is strong evidence that the incidence of abnormal postpartum ovarian cycles during the pre-service period is increased in high-producing dairy cows (Stevenson et al., 1983; Staples et

al., 1990; Shrestha et al., 2004). Shrestha et al. (2004) reported a high incidence (55.5%) of abnormal ovarian cycles postpartum in high-producing dairy cows, in part, due to inactive ovaries, ovarian cysts, and non-functional corpora lutea (luteal cysts). The incidence of a prolonged luteal phase (33.6%) was also high. Furthermore, it has been shown that anovulation between 50 and 60 DIM affects approximately 20-40% of lactating dairy cows (Cerri et al., 2004; Chebel et al., 2006; El-Zarkouny et al., 2004; Moreira et al., 2001; Santos et al., 2009).

Increased abnormal ovarian activity (and anovulatory anestrus) during the early postpartum will negatively impact subsequent reproductive performance, pregnancy rates, and ultimately the calving interval (Lucy et al., 1992). Investigation of practical markers to estimate the timing of ovulation is not well understood and would prove useful to dairy producers to facilitate the implementation of alternative management practices.

TRANSITION PERIOD

LATE GESTATION

During the late gestational period, dairy cows are on a positive nutritional plane, utilizing dietary surplus beyond maintenance towards the growth of the fetus and adipose deposition. With the initiation of parturition and the start of lactation, a wide variety of physiological changes transpire close together. Plane of nutrition, utilization of nutrients, and reproductive status shift dramatically during this period (Lucy et al., 1992). These dramatic shifts lead dairy cows to be exceptionally vulnerable to metabolic and reproductive diseases. The efficiency by which the cow handles and overcomes each biological alteration

ultimately determines the further capacity for the animal to resume reproductive activity (Lucy, 2015).

PARTURITION

As the fetus grows, fetal stress also increases, and fetal adrenocorticotropic hormone (ACTH) is released resulting in the release of fetal cortisol. Fetal cortisol triggers an increase in gene expression to support Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and estradiol 17β (E2) release. Prostaglandin $F_{2\alpha}$ affects the CL and placenta directly by inhibiting progesterone synthesis and secretion. Prostaglandin $F_{2\alpha}$ also signals relaxin release from the placenta to support pelvic ligament relaxation, and with increased E2, signals for the end of uterine quiescence and initiates myometrial contractions (Senger, 2012). Cervical stimulation as a result of physical pressure encourages the release of oxytocin from the neurohypophysis. The cycle of myometrial contractions increases pressure and signals further oxytocin release for additional and more frequent contractions until the expulsion of the fetus and placental membranes are completed (Senger, 2012). During this period, prolactin is also stimulated for secretion from the anterior pituitary to support lactation (Norman and Henry, 2014).

From late pregnancy through the first few weeks of lactation, plasma IGF-I is reduced despite an increase in feed intake to support lactation (Rhodes et al., 2004). During parturition, dairy cows can experience up to a 70% decrease in plasma IGF-I which is in part due to the downregulation of growth hormone (GH) receptors in hepatic tissues. Thus, GH and GH receptor binding is limited resulting in compromised hepatic synthesis of IGF-I. (Kim et al., 2004). However, systemic GH is elevated because of the decreased negative feedback elicited by lower IGF-I concentrations (Kim et al., 2004) and this favors a

catabolic state that supports high milk production in early lactation (Lucy et al., 2004). It is undetermined if the GH-IGF-I axis is also altered in the reproductive tissues if IGF-I is synthesized locally in the ovarian tissues (Rhodes et al., 2004; Lucy et al., 2011), it is likely that the metabolic consequences of the IGF-I/GH interaction have indirect effects on reproductive tissues (Lucy et al., 2011).

Zulu et al. (2002) argue that IGF-I has an endocrine role on the reproductive axis. IGF-I influences gonadotropin-induced folliculogenesis, ovarian steroidogenesis, and luteal function. It also modulates pituitary and hypothalamus functions. According to these authors, severe undernutrition decreases blood IGF-I concentrations. In early postpartum and during the critical period of NEB, in high-producing dairy cows, plasma IGF-I concentrations are low. The IGF-I levels are positively correlated to energy status and reproductive function during this period.

UTERINE INVOLUTION

Immediately after calving, the reproductive tract undergoes a series of physiological adjustments to reduce the size of the uterus and alter ovarian status to restore reproductive capacity. Complete involution takes place between 20 and 42 days postpartum, but many factors contribute to the length of time this process takes (Elmetwally, 2018). Environmental microorganism contamination exists in the uterus of about 90% of cows during the first 2 weeks of the puerperium period (Sheldon, 2008). The presence of puerperal disease is the most crucial factor determining the duration of uterine involution. Diseases such as mastitis, metritis, retained placenta, and metabolic disorders all have negative effects on uterine involution (Oresnik, 1995). Immune cells and PGF_{2a} play a significant role in the

remodeling of uterine tissues, as well as being the critical endocrinological component responsible for the regression of luteal cells (Dolezel and Kudlac, 1992). However, increased duration of PGF_{2a} secretion is correlated with increased days to complete uterine involution and longer intervals between parturition and the resumption of ovarian cyclicity (Kindahl et al.,1982; Madij et al., 1984). Successful involution coincides with a decrease in positive acute-phase proteins (APP), with prolonged levels of APPs indicating possible complications in the healing process (Sheldon et al., 2001; Chan et al., 2010). During this time, lactational demand, GLU utilization, ability to handle stress, and immune response to disease are all factors that affect uterine involution and subsequent reproductive performance.

POSTPARTUM OVARIAN ACTIVITY

Before parturition, PGF_{2a} initiates luteal regression. After the regression of the CL and removal of the inhibitory action of P_4 on the hypothalamic-pituitary axis, follicular growth is less impeded which results in an increase in systemic E2 (Senger, 2012). The resumption of ovarian cyclicity following parturition is highly dependent on the reestablishment of the interplay between the hypothalamus, pituitary, gonads, neurohypophysis, and adenohypophysis. The long exposure to high concentrations of P_4 inhibits GnRH secretion, which negatively affects the synthesis of LH leading to a gradual depletion of pituitary LH reserves (Ambrose et al., 2021). The reestablishment of ovarian cyclicity happens in three phases. The first phase involves the anterior pituitary replenishing its store of luteinizing hormone, which has been cited to be a limiting factor in reproductive resumption in the postpartum period (Elmetwally, 2018; Nett et al., 1988). The second step

is increasing the sensitivity of the hypothalamic-pituitary axis to E2. Preovulatory follicles produce E2, which in turn upregulate the GnRH receptor mRNA in gonadotrophs, thereby increasing the sensitivity of the anterior pituitary to GnRH (Schoenemann et al., 1985; Rispoli and Nett, 2005). Estradiol 17β is also directly responsible for stimulating the hypothalamic release of GnRH from the surge center causing the pre-ovulatory surge of LH. The last stage involves overcoming the effect of lactation. Cows milked more frequently (3 times per day) exhibit later resumption of ovarian cyclicity when compared with cows milked with less frequency (1 time per day) (Patton et al., 2006).

In addition to the above-mentioned factors, it is also believed that increased LH pulsatile secretion and resumption of ovarian activity are related to the energy status of the lactating cows. Before and even after the first postpartum ovulation, there are divergences from normal patterns of follicular behavior which include anovulatory but active ovaries (dominant follicles develop but do not ovulate) and the development of follicular and luteal cysts (Lucy et al.,1992a). It is hypothesized that follicular dynamics are altered significantly during the postpartum period because of NEB and nutrient partitioning (Lucy et al.,1992a). Canfield and Butler (1990) hypothesized that pulsatile LH secretion is suppressed until the NEB nadir is reached, at which time LH pulse frequency increases stimulating first postpartum ovulation (this phenomenon will be discussed in more detail in a later section).

There is an association between a short interval to the first postpartum ovulation and an increased rate of pregnancy (Galvão et al., 2010). Cows exhibiting their first ovulation later than 53 DIM were 1.6 times more likely to have an increased period between calving and first estrus when compared with cows that ovulated before 21 DIM (Westwood et al., 2002). Other considerations for ovarian resumption are the effects of high milk production,

faster metabolic rate, and increased dry matter intake (DMI) on hepatic steroid metabolism (Sangsritavong et al., 2002; Lopez et al., 2004). This state of high blood filtration by the liver can lead to lower circulating estradiol from the preovulatory follicles, ultimately leading to limited feedback to the hypothalamic-pituitary axis and associated GnRH/LH actions. Seasonality and temperature-induced stress can also affect postpartum ovulation (Bulman and Lamming, 1978; Montgomery et al., 1980; Westwood et al., 2002).

OVULATION

As E2 increases in circulation, it acts as positive feedback to the hypothalamic surge center where it signals for a surge of GnRH. Following a surge of GnRH, a surge of LH from the anterior pituitary occurs. The LH surge also causes a shift from E2 to P_4 synthesis of the theca interna cells of the pre-ovulatory follicle(s). The theca interna cells are also the site for CHO side-chain cleavage enzyme activity and are the cells that express LH receptors when the follicle reaches class III maturation (Norman and Henry, 0000; Lucy et al., 1992a). Progesterone then stimulates the synthesis of collagenase enzyme which degrades the connective tissues of the tunica albuginea of the ovulatory follicle. Simultaneously an increase in follicular volume is occurring resulting in a structural apex of the ovary, called the stigma (Senger, 2012). The surge of LH also causes the increase in prostaglandin E_2 (PGE_2) and PGF_{2a} and the formation of the macula pellucida by which the egg and cumulus mass exit the follicle (Williams and Erickson, 2012). Prostaglandin F_{2a} signals for smooth muscle contractions of muscles surrounding the ovary, ultimately causing an increase of pressure on ovarian components. It also stimulates lysosomes in the follicular granulosa cells to release lysosomal enzymes to degrade connective tissues of the follicular wall

(Senger, 2012). The overall increase in follicular pressure and weakening of the follicular wall allows for the burst of the oocyte from the Graffian follicle which is the defining moment of ovulation. Prostaglandin E_2 increases blood flow to the ovary and activates plasminogen/plasmin which dissolves to coagulation of the corpus hemorragicum caused by the trauma of ovulation and helps remodel the theca and granulosa cells into the luteal cells and development of corpus luteum (Senger, 2012).

First postpartum ovulation in healthy cows has been reported to range from approximately 17 to 42 days postpartum (Butler, 1981). It is generally accepted that "early ovulators" experience ovulation between 7-28 DIM. "Typical ovulators" and "late ovulators" experience ovulation between 29-49 DIM and 50+ DIM, respectively (Beam and Butler 1997, 1998; Darwash et al., 1997; McCoy et al., 2006; Galvão et al., 2010). Cows classified as late ovulation groups are often considered anovulatory cows. Anovulation between 50 and 60 DIM affects approximately 20-40% of lactating dairy cows (Cerri et al., 2004; Chebel et al., 2006; El-Zarkouny et al., 2004; Moreira et al., 2001; Santos et al., 2009). Many factors may influence the interval from parturition to first postpartum ovulation, including but not limited to parity, milk yield, breed, nutritional status, energy balance (EB), and prevalence of postpartum diseases environment and management (Ambrose et al., 2021). Nevertheless, the absence of pulsatile GnRH secretion from the hypothalamus is what appears to negatively impact LH production. An increase in the frequency of LH pulsatile secretions is the necessary event for ovulation.

NUTRITIONAL METABOLITES

NEGATIVE ENERGY BALANCE

Energy balance in the postpartum cow is the dietary energy consumed, minus the required energy for maintenance and milk synthesis. During the prepartum period, EB is zero or positive, even with the consideration of gestational demands. In the postpartum transitional phase, EB shifts drastically; cows go through a negative energy balance (NEB) because of the high caloric demand for lactation and the inability to ingest energy to support that demand (Butler et al. 1981; Butler et al., 2003). Negative EB persists for approximately 50 days of the lactational period, and the degree of energy deficit often correlates with dry matter intake and milk yield (Butler et al. 1981). The length and duration of the NEB during the postpartum phase influence resumption of ovarian cyclicity (Beam, 1999), presumably because of the relationship between LH secretion and the timing of nadir of NEB (Canfield and Butler, 1990). Canfield and Butler (1990) proposed that an increase in pulsatile LH secretion does not occur until the negative EB nadir is reached, at which time LH pulse frequency increases stimulating first postpartum ovulation. The authors hypothesized that many metabolites including non-esterified fatty acids (NEFA) and EB are directly related. Thus, NEFA may serve as a peripheral signal of EB to the central nervous system for an increase in LH pulsatile secretion.

Follicular recruitment in lactating cows is different from nonlactating cows in that nonlactating cows demonstrate greater numbers of class 1 (3-5 mm), class 2 (6-9 mm), and class 3 (10-15 mm) follicles during synchronized estrus (De La Sota et al., 1993). Cows in a NEB develop similar numbers of class 1 and class 2 follicles but exhibit fewer dominant follicles (class 3) when compared with cows with a more positive energy balance between 0-

26 DIM (Lucy et al., 1991). Body condition scores (BCS) have been a long-time standard in assessing the change in energy balance over time. Beam (1999) showed that cows having a more significant loss in BCS during the first 30 days postpartum go through a longer interval between calving and the first ovulation postpartum. Although the correlations between the degree of NEB, BCS, and the resumption of ovarian cyclicity are cited many times in the literature, the number of days to first ovulation using these measures has been inconsistent (Beam, 1999). This may be related to the weak correlation between BCS and EB. While NEB observed over time can provide useful information, it cannot exclusively determine a tight range for reproductive recrudescence.

Oxidative stress is also prevalent in the periparturient period, especially in dairy cows (Abuelo et al., 2019; Castillo et al., 2005) and is directly associated with EB (Castañeda-Gutiérrez). States of oxidative stress (high levels of BHBA and NEFA) reduce the functionality of immune cells and increase susceptibility to disease (Sordillo and Aitken, 2009). There appears to be a link between oxidative states and immunological function, and studies show that high levels of BHBA and NEFA reduce odds of pregnancy in early lactation (Van Knefsel et al., 2005; Ospina et al., 2010; Turk et al., 2015).

GLUCOSE

GLUCOSE is a necessary fuel for maintenance and production, which is provided through the processes of glycogenolysis, yielding GLU from glycogen stores, or through dietarily fermented volatile fatty acids and subsequent hepatic gluconeogenesis (Hill et al., 2012). The need for GLU increases drastically as cows progress through early to mid-lactation. Seventy-two grams of GLU are required to produce one kg of milk (Bell, 1995). In

the early postpartum period, GLU is characteristically low because of the high demand for milk synthesis, despite the series of homeorhetic mechanisms that cows display to elevate GLU supply (Bauman and Currie, 1980). Shortly after parturition, and as DMI increases, cows primarily increase GLU production by increasing liver gluconeogenesis, which utilizes substrates, propionate derived from rumen fermentation, and catabolic processes affecting muscle and adipose tissues. Cows also go through a metabolic adaptation to prevent GLU to glycogen conversion. Low GLU gives rise to low insulin and IGF1, effectively keeping them in a catabolic state, allowing circulating GLU to remain available for production demands (Bauman and Elliott, 1983).

Circulating blood GLU levels at, or shortly following, calving is well studied and defined. However, the mechanisms by which ruminants partition GLU (e.g., for maintenance, production, immune system, and reproduction) is less understood. The individual differences in GLU concentrations during the early postpartum period may simply reflect the ability of an individual to store glycogen in the dry period and mobilize it quickly postpartum (Lucy et al., 2013). Later in lactation, a difference may reflect the cows' overall ability to adapt to lactation, synthesize GLU within liver tissue, and their level of insulin sensitivity. How cows adapt to GLU demands in the early postpartum period directly affects an individual's metabolites and metabolic hormones, which may have further impact on reproductive performance (Lucy et al., 2013).

The relationship between blood GLU concentrations and pregnancy has been seen in both confinement operations as well as in pasture-based systems (Moore et al. 2014). Most studies investigate early postpartum GLU levels and later associated pregnancy rates. Cows that become pregnant following first AI attempts had a greater blood GLU level on the day

of calving and for the three weeks following parturition when compared with cows that did not become pregnant (Garverick et al., 2013). As indicated earlier, blood GLU concentrations are lowest during the early postpartum period and studies have shown that greater concentrations of blood GLU were associated with fewer days open (Reist et al. 2003; Moallem et al., 1997) and shorter interval from calving to first behavioral estrus (Ahmadzadeh et al. 2012; Moallem et al., 1997).

Artificial insemination attempts and resulting pregnancy rates occur months after calving and initial differences in GLU levels. This suggests that some nutritional metabolites, including GLU, during the early postpartum period may be predictive of later reproductive performance. It should be noted that not all studies showed a relationship between GLU and fertility in dairy cows. For example, Kappel et al., (1984) showed that blood GLU concentrations were not related to days-to-conception in both summer and winter calving seasons.

Velazquez et al. (2008) showed there is a positive association between IGF1, insulin, and the day in which a cow begins to cycle. Leroy et al. (2008) determined that GnRH secretions in the postpartum dairy cow were affected by insulin and GLU levels. Leroy and authors (2008) report that greater levels of GLU also initiate greater levels of insulin and IGF1, which positively affects the hypothalamus, GnRH pulsatility, and subsequent LH release. Thus, cows with greater postpartum blood GLU concentrations may exhibit a shorter time to cyclicity resumption by affecting the capacity for ovarian cells to respond to gonadotropin.

There is evidence that GLU may affect ovarian steroid synthesis and secretion. In vitro studies in cattle report the promotion of steroidogenesis by GLU (Lynn et al., 1965)

and suggest that GLU may promote CHO uptake into the ovarian cells (Rabiee and Lean, 2000). Chase and co-workers (1992) showed that the uptake and rate of metabolism of GLU were greater in growing bovine CL than immature or regressing CL. This may indicate that the requirements for steroid hormone precursors are met by sufficient availability of GLU.

Two of the major energetic fuels currently known to be used by immune cells are GLU and glutamine (Ingvartsen and Moyes, 2013). In certain metabolic states, such as NEB, the shifts in the utilization of these nutrients may contribute to immunosuppression during the transition period and affect postpartum ovulatory response. An efficient GLU uptake by immune cells is critical for maintaining cellular functions and eliciting an optimal host response to invading microorganisms (Ingvartsen and Moyes, 2013). Both glutamine and blood GLU concentrations are lowest in cows during early lactation and low GLU availability may affect immune function and ultimately the resumption of ovarian cyclicity.

Collectively, it can be hypothesized that in certain metabolic states such as NEB, alteration in the utilization of nutrients such as GLU may contribute to alteration in GnRH, gonadotropin secretion, and steroidogenesis, as well as alteration of immune function during the transition period and affect postpartum ovulatory response.

CHOLESTEROL

Cholesterol is the sterol backbone of all steroid hormones and vitamin D3. It is also an essential component in milk composition (Bitman and Wood, 1990), partially responsible for membrane fluidity, critical in bile salt formation, and essential in cellular repair processes (Tall and Yvan-Charvet, 2015). Cells obtain CHO through interaction with the environment or by *de novo* synthesis, starting with acetyl-CoA in the liver or intestines.

During the transitional period from a nonlactating state to a lactating state, the shift in nutrient stores is not limited to GLU but involves lipid metabolism to meet increased nutritional demands as well (Bauman et al., 1988; Drackley, 1999; Lucy et al., 1992; Lucy et al., 2013; Rowlands et al., 1980).

The onset of lactation triggers an increase in hepatic gene expression in enzymes key to the CHO biosynthesis pathway (Kessler et al., 2014). Total CHO (TC), FCHO (FC), and CHO esters (CE) in plasma have been shown to fall immediately post parturition with concentrations reaching the lowest values approximately one week postpartum and reestablishing prepartum levels approximately 3-5 weeks post parturition (Kessler et al., 2014; Gross et al., 2015). Inversely related is the concentration of TC in milk (Kessler et al., 2014). Although there is both an increase in enzyme expression and an increase in milk concentration, it is unclear as to whether lactational demand or insufficiency of hepatic gene upregulation ultimately causes the low plasma levels of TC (Kessler et al., 2014).

Plasma CHO levels, DMI, NEB, and illness are interrelated, although a clear cause-and-effect relationship between any of these factors is difficult to determine. Low CHO concentrations postpartum are closely associated with postpartum health disorders (metritis), and low CHO levels are exacerbated in the face of multiple clinical disease events (Sepúlveda-Varas et al., 2015). Blood CHO levels are closely associated with DMI in transitioning dairy cattle, and several studies cite a decrease in DMI when disease states are present (Huzzey et al., 2006; Goldhawk et al., 2009; González et al., 2008). Lower DMI worsens the severity of NEB and results in lower blood CHO levels in the postpartum period (Kim and Suh, 2003; Rugg et al., 1992).

Cholesterol efflux from macrophages is unidirectionally transported onto the HDL structure, which initiates the process of reverse CHO uptake (Tall and Yvan-Charvet, 2015). FCHO in HDL is esterified, giving rise to CE. Cholesterol can then be recycled to form very-low-density lipoproteins or excreted into the bile. During proinflammatory states, CHO efflux decreases as the change in protein messengers alter the behavior of HDL favoring inflammatory actions that do not suppress the adhesion of monocytes to the endothelium (Tall and Yvan-Charvet, 2015). Reverse CHO transport by HDL is impaired during inflammatory states at multiple points; there is speculation that this impairment leads to enhancement of inflammatory response and CHO accumulation by macrophages (Tall and Yvan-Charvet, 2015).

Cholesterol changes in the quantity or type of CHO present in blood could play a significant role in regulating steroid hormone biosynthesis by the ovary (Henderson et al., 1981), and therefore it may play a role in postpartum ovulation. It is known that both LDL and HDL are sources of CHO for steroidogenesis. Previous studies have suggested a role for CHO in conception, possibly as a precursor for steroid hormones (Wehrman et al., 1991). In the case of *in vitro* bovine luteal cells, LDL and HDL increased P_4 production. In contrast, reducing the CHO fraction and increasing $PGF_{2\alpha}$ inhibits lipoprotein-induced steroidogenesis responsible for P_4 (Pate and Condon, 1989). This CHO-induced shift in steroidogenesis may explain the interaction between CHO and reproduction. Some in vivo studies may not support this hypothesis. Cholesterol concentrations in both plasma and milk have not been predictive of days to first or second postpartum ovulation in one study (Francisco et al., 2003). However, greater concentrations of plasma CHO are positively associated with the likelihood of conception, estrus expression at first ovulation, and a

shorter interval from calving to conception (Westwood et al., 2002). Cows with plasma CHO concentrations above 1.8 mmol/L during the first ten weeks of lactation were 2.7 times more likely to become pregnant by 150 DIM. In contrast, cows with plasma concentrations of ≤0.9 mmol/L were 1.4 times more likely to experience delayed calving to conception (Westwood et al. 2002).

High concentrations of blood CHO have been associated with shorter days open. (Reist et al., 2003). Moallem et al. (1997) also reported a negative correlation between days open and plasma CHO concentrations. The authors also reported that the number of days from calving to first behavioral estrus were also negatively correlated with plasma CHO concentrations. In contrast, Rowlands et al. (2009) show plasma CHO concentrations increased 2½-fold during the first 8 weeks of lactation but there was no relationship between conception rate and CHO concentration in lactating Holstein cows.

GLUCOSE AND CHOLESTEROL RELATIONSHIP

Both CHO and GLU are utilized by the ovary for ovarian functions, including specifically for the processes of estrus (Rabiee et al., 1997) and the development of growing CL tissues (Chase et al., 1992). Studies suggest that GLU may promote the direct transport of blood CHO to ovarian structures (Rabiee and Lean, 2000). A study conducted *in vitro* showed the promotion of steroidogenesis in CL tissues by GLU (Lynn et al., 1965). The study conducted by Reist et al. (2003) showed that high levels of blood GLU and high levels of blood CHO were associated with a short interval from parturition to conception in cows. This study agreed with Moallem et al. (1997) who demonstrated a negative correlation between blood GLU, blood CHO, and days open. Thus, blood GLU and blood CHO may be

involved directly with ovarian function, luteal development, and steroidogenesis, which could subsequently affect the timing between parturition and conception (Reist et al. 2003).

INFLAMMATORY METABOLITES

SERUM AMYLOID A

Serum Amyloid A (SAA) is a small protein comprised of 104 amino acids, which plays a critical role in the control and propagation of an acute phase response (APR) (Sack, 2018). The APR results in the induction of an inflammatory mediator cascade resulting in multiorgan physiological changes, as a consequence of trauma, infection, inflammation, toxins, cancer, and other events. This inflammatory cascade occurs initially through activated plasma monocytes and tissue macrophages at the site of inflammation (Uhlar and Whitehead, 1999). Following the activation of macrophages, the cytokines (i.e., interleukin-1, interleukin-6, and tumor necrosis factor) are released (Uhlar and Whitehead, 1999). These cytokines directly influence the expression of SAA. In addition to many physiological changes caused by the APR, SAA increases by a thousand-fold or even more in response to trauma or inflammation. Serum Amyloid A is found in varying levels in healthy tissues, depending on the species, and has multiple polymorphisms (Berg et al., 2011; De Buck et al.,.2016). Serum amyloid A is primarily produced by hepatocytes (as much as 2.5% of the total production of the liver) and released into the blood circulation (van Der Westhuyzen et al., 2005).

There are several SAA isoforms. The SAA Isoforms 1,2, and 4 function as proteins that bind to lipids (also called an apolipoprotein) and are partitioned into HDL particles and secreted into the systemic blood circulation (Sacke, 2018). The formation of lipoprotein is

necessary for the homogeneous transportation of SAA in blood. Serum Amyloid A1 and SAA2, structurally and functionally, are closely related. Both SAA1 and 2 are expressed predominantly in the liver in response to proinflammatory stimuli (Sacke, 2018). The SAA3 isoform is synthesized and secreted from adipose tissue and has little to no interaction with HDL particles, which may indicate that it comprises a small fraction, if any, of the plasma SAA concentration (Chiba et al., 2009). The SAA3 isoform is the predominant isoform in mammary epithelial cells of cattle, and its expression is increased dramatically in the presence of bacteria (Saremi et al., 2013). In humans, SAA4 is moderately expressed in response to inflammatory stimuli, and although a similar isoform exists in the mouse, it is unclear as to whether cattle have this isoform (Saremi et al., 2013).

SERUM AMYLOID A AND LIPID METABOLISM

Lipid metabolism is altered by SAA involvement through actions on CHO efflux pathways (Cai Lei et al., 2005; van Der Westhuyzen, 2005). Cholesterol efflux to plasma occurs by two mechanisms; passive diffusion from the plasma membrane or by active transport mediated by the ABCA-1 pathway to plasma lipoproteins (Rothblat et al., 1999). Serum Amyloid A binds and replaces the apoA-I tag protein on HDL altering its behavior by a) promoting HDL binding to macrophages (Banka et al., 1995; Artl et al., 2000), b) promoting HDL CHO uptake from cells (Banka et al., 1995; Liang and Sipe 1995; Artl et al., 2000), and c) slowing the reverse CHO transport process (Cai Lei et al., 2005). Some hypotheses suggest that SAA mediates the delivery of CHO to lipid-poor regenerating cells at the sites of inflammation and tissue repair (Feingold et al., 2010). Even independently of these receptors, SAA still results in some CHO efflux (Stonik et al., 2004; van Der

Westhuyzen, 2005). Thus, another hypothesis on the role of SAA is being a mediator of lipid export from apoptotic and necrotic cells (Stonik et al., 2004). Those cells cannot support the active and energy-dependent (ABCA-1) pathway necessary for CHO efflux, thus SAA may assist in the process of lipid export in dying cells (Stonik et al., 2004).

SERUM AMYLOID A, A POTENTIAL BIOMARKER

During the transition period and lactational onset, dairy cattle are especially susceptible to diseases that can negatively affect the animal's ability to shift from non-lactating to lactating status. The cost of transition period diseases goes beyond the associated milk loss, increased culling rate, and treatment costs. Early postpartum diseases can have harmful effects on the reproductive efficiency of dairy cows (Ahmadzadeh et al., 2009).

The utilization of SAA as a biomarker for human disease has been studied for decades. For example, elevated SAA has been shown to have associations with pulmonary hypertension (Sadushi-Kolici et al., 2015) and atherosclerosis (Vallon et al., 2012). In recent years, the classification and measurement of SAA as an APP have been promising in both farm and companion animal medicine (Eckersall and Bell, 2010). Not all inflammatory states are disease states, and APR reactions can arise and remediate in a natural and expected way; such is the case with the rise and fall of APPs in early postpartum (Chan et al., 2010; Nazifi et al., 2010). However, it is an extended period of inflammation that is considered a disease state. Disease states then lead to a decrease in overall fitness which may explicitly result in a change in reproductive fitness by reducing conception rates by 4%-20% following the first service (Fourichon et al., 2000). This decrease in conception rate

translates to an additional 4 to 41 days to successful conception depending on the severity of the disease (Fourichon et al., 2000).

Although SAA had been commonly associated with the APR and is often described exclusively as an APP, studies investigating chronic inflammation have found that SAA exists in elevated levels outside of a strictly acute-phase reaction (Uhlar and Whitehead, 1999). Specifically, elevated SAA levels in humans have been linked to pulmonary hypertension (Sadushi-Kolici et al., 2015; Salobir et al., 2015), atherosclerosis (Targonska-Stepniak and Maidan, 2012; Vallon et al., 2012), and amyloidosis, (Targonska-Stepniak and Maidan, 2012). In cattle specifically, increased SAA concentrations have been observed in the presence of left and right displaced abomasum (Guzelbektes et al., 2010), acute laminitis (Ksenija et al., 2019), sole hoof ulcers (Ksenija et al., 2019), and ketosis (Abuajamieh et al., 2016). In addition, there is evidence that elevated SAA may be involved in acute ruminal acidosis, although the findings are contradictory (Gozho et al., 2007; Cannizzo et al., 2012). Regarding cattle reproduction, increased SAA concentrations have been observed in cows with metritis, especially acute puerperal metritis (Chan et al., 2010: Nazifi et al., 2010; Dervishi 2016). Chan and associates (2010) reported that the SSA concentration of healthy dairy cows peaked at day 3 postpartum and decreased following the first week after calving. In cows experiencing metritis, SAA peaked 4-7 days postpartum, at a level 1.6-fold higher than healthy cows, and did not decrease until 4 weeks postpartum. Brodzki et al. (2019) reported that cows at 60 days after parturition with follicular cysts had greater (~52 ug/mL) SAA concentrations when compared with cows with no cysts (~8 ug/mL) in the follicular phase. In addition, cows with luteal cysts had greater (37ug/ml) SAA concentrations when compared with cows with no cysts (~10 ug/mL) in the luteal phase. Collectively, the above

information provides evidence that elevated SAA can be indicative of some acute or chronic states.

The use of SAA and other APPs as an indicator of disease is promising; however, the implications of elevated SAA in the context of fertility are much less studied. In women attempting to conceive, a strong positive linear correlation between SAA in follicular fluid and blood plasma has been observed (Urieli-Shoval, 2013). Elevated SAA levels in these IVF patients were indicative of lower pregnancy rates (by up to 50%) and indicative of "significantly poorer reproductive potential" (Urieli-Shoval, 2013). Lucy (2000) hypothesized that the combined effect of a reduction in IGF-I and LH may compromise ovarian follicular growth and development during the early postpartum. It was suggested (Shrestha et al., 2004) that the aforementioned hormonal changes may lead to an increased incidence of inactive ovaries, ovarian cysts, and non-functional corpora lutea in postpartum cows, resulting in a prolonged interval to first ovulation after calving. Given the positive associations between elevated SAA and ovarian cysts in cattle (Brodzki et al., 2019), SAA may be used as a biomarker to potentially identify cows with reproductive issues during the early postpartum.

RESULTS

DESCRIPTIVE DATA FOR EARLY VS LATE OVULATORY GROUPS

Sixty-seven multiparous lactating dairy cows were used in this study. Retrospectively, cows were divided into two ovulation groups depending on the timing of their first ovulation postpartum. Cows were considered to be an early ovulator when the first ovulation occurred before 28 days postpartum (EO; n=21) and a late ovulator when the first

ovulation occurred after 28 days (LO; n=46). The LO group included cows that did not ovulate by the conclusion of the trial at 56 days postpartum. There was no difference between EO and LO groups regarding milk production (36.3 ± 2.4 vs 40 ± 1.6 kg/d) and parity (2.7 ± 0.3 vs 3.2 ± 0.2 lactations). As expected, there was a difference ($P < 0.01$) between groups in the average days to ovulation (EO= 20.6 ± 1.3 and LO = 37.5 ± 1.4 days). It should be noted that the 29 cows that did not ovulate (NO) by 56 days were not included in the days to ovulation analysis because there was no data for the date of ovulation. In the LO group, mean days to ovulation tended to be greater ($P = 0.06$) for 2nd lactation cows compared with cows in 3rd lactation and greater (30.9 ± 1.3 days vs 27.2 ± 1.4 days).

DEPENDENT VARIABLES FOR EARLY AND LATE OVULATION

BODY CONDITION SCORE

Overall BCS was greater for EO (2.8 ± 0.09 score) compared to LO (2.5 ± 0.06 score) ($P < 0.02$). Body condition score (BCS) was not different between groups in weeks -2 through 2; however, BCS showed a tendency to be different ($P \leq 0.07$) in weeks 3 and 4 and were significantly different ($P \leq 0.02$) in weeks 5,6 and 8 (Figure 1.2 and Table 1.1). As expected, BCS decreased over time for both groups ($P < 0.01$).

Further regression analysis showed that the rate of BCS change (BCS loss) over time was greater in LO cows when compared with EO cows ($P < 0.03$) and the intercept at 2 weeks before calving was also different ($P < 0.05$) (Figure 1.3). It should be noted, that the mean BCS at 2 weeks before calving (intercept) for EO and LO was 3.3 ± 0.08 and 3.2 ± 0.13 scores, respectively. This difference may not be biologically relevant. There was also

more variation in BCS during weeks 2 and 3 compared with the later weeks (5-8) (Figure 1.3).

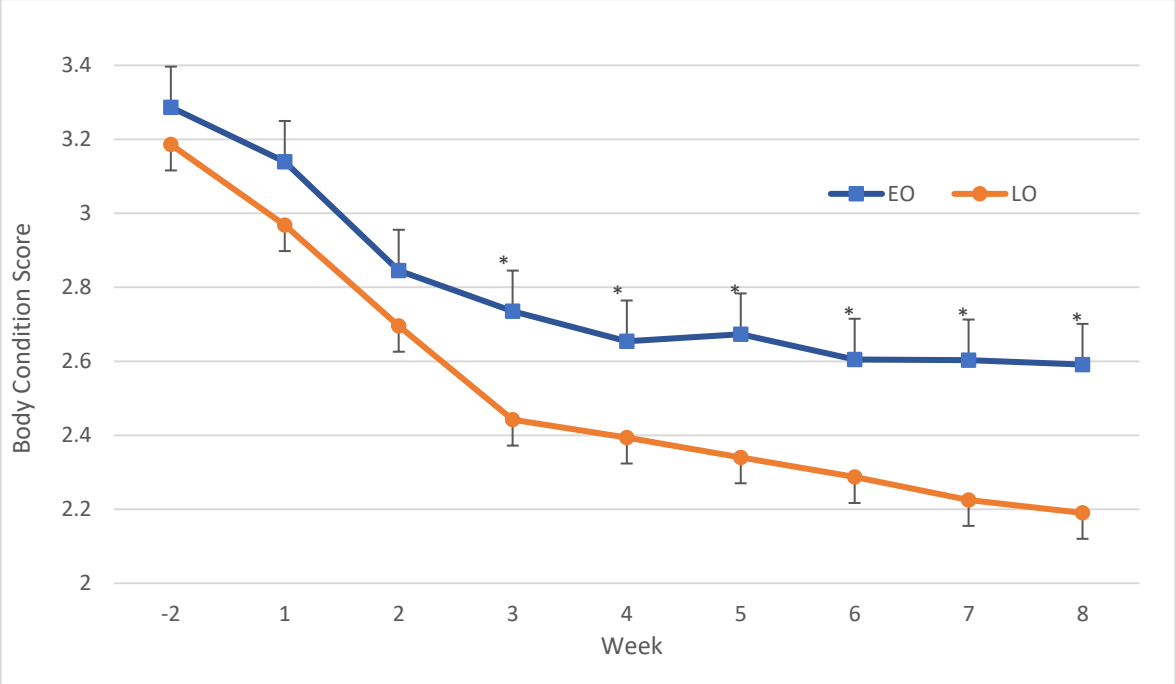

Figure 1.2 Body condition score (mean score ± SE) in early (EO) and late ovulating (LO) multiparous Holstein cows before and after calving.
Early ovulators (n=21) ovulated < 28 days postpartum, and LO (n=46) ovulated > 28 days postpartum. Body condition score was measured using a scale of 1 to 5 in 0.25 increments (1=emaciated; 5=obese).
* Means differ between EO and LO groups ($P < 0.05$).

Table 1.1 Mean Body Condition Score (BCS) in Early (EO) and Late Ovulating (LO) Postpartum Cows (Week)

Week	Ovulation Group	BCS[#]	P-value
3	EO	2.70 ± 0.11	0.02*
	LO	2.45 ± 0.07	
4	EO	2.65 ± 0.11	0.04*
	LO	2.40 ± 0.06	
5	EO	2.67 ± 0.11	0.01*
	LO	2.33 ± 0.06	
6	EO	2.60 ± 0.11	0.02*
	LO	2.29 ± 0.06	
7	EO	2.60 ± 0.11	0.01*
	LO	2.22 ± 0.06	
8	EO	2.60 ± 0.11	0.01*
	LO	2.20 ± 0.06	

*Means differ between EO and LO groups ($P < 0.05$).
[+] Means tend to differ between EO and LO groups ($P > 0.05$; $P < 0.1$).
[#] BCS was measured using a scale of 1 to 5 in 0.25 increments (1=emaciated; 5=obese)

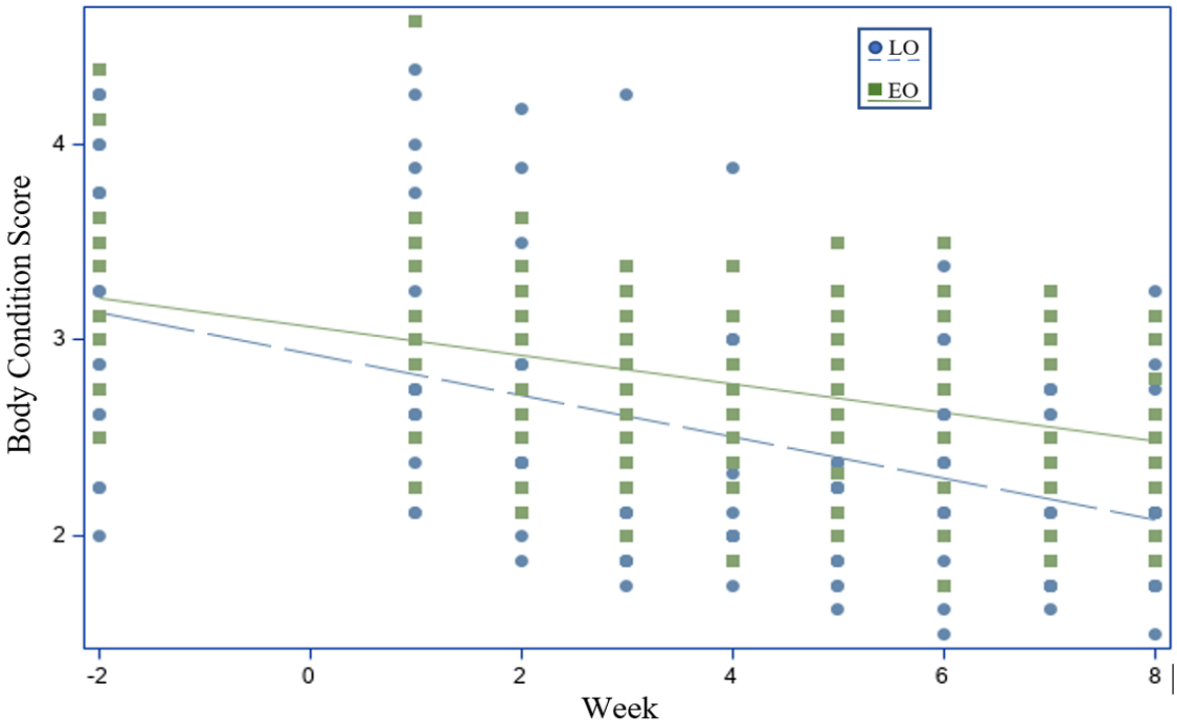

Figure 1.3 Regression lines for body condition scores (BCS) in early (EO) and late ovulating (LO) multiparous Holstein cows before and after calving (week 0 = calving). Early ovulators (n=21) ovulated < 28 days postpartum, and LO (n=46) ovulated > 28 days postpartum.
BCS was measured using a scale of 1 to 5 in 0.25 increments (1=emaciated; 5=obese). The overall pattern of BCS loss over time as well as the rate of BCS decline over time was different between EO and LO ($P \leq 0.01$).

GLUCOSE

Analysis of repeated measures for blood GLU concentrations determined that there was an effect of the week on GLU concentrations ($P < 0.05$), but there were no effects of ovulation status or week by ovulation status on GLU concentrations. Regardless of ovulation status, blood GLU significantly declined after parturition and remained low until week 4. Glucose concentrations were different between LO and EO in week 0 only (EO= 57 ± 2.0 mg/dL: LO= 62 ± 1.4 mg/dL) ($P < 0.04$) (Figure 1.4). Blood GLU then gradually increased from week 1 through 8 but was never different between EO and LO groups (Figure 1.4). Regression analysis showed no detectable difference in pattern or change in GLU

concentration over time between EO and LO groups. When data from H cows alone were analyzed, analysis of repeated measures showed no difference in concentration of blood GLU over time between EO and LO cows (data not shown).

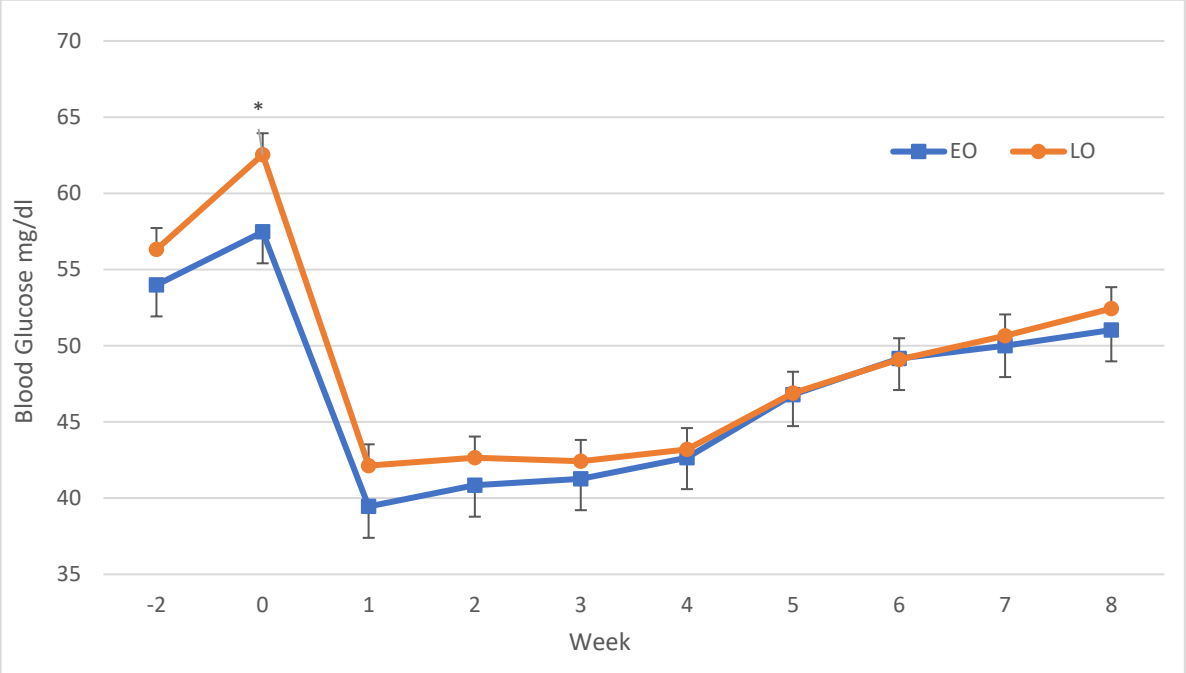

Figure 1.4 Mean blood GLU concentrations (LS means ± SE) in early (EO) and late ovulating (LO) multiparous Holstein cows before and after calving (week 0 = calving). Early ovulators (n=21) ovulated < 28 days postpartum, LO (n=46) ovulated > 28 days postpartum.
*Means differ between EO and LO groups ($P < 0.05$).

TOTAL CHOLESTEROL

Mean blood concentrations of CHO showed differences between ovulation status ($P < 0.05$), weeks ($P < 0.01$), and week by ovulation status interaction ($P < 0.02$) indicating that the change in blood CHO concentrations over time was not consistent between the groups. Mean blood CHO concentrations were greater in EO when compared with LO in weeks 4,5 and 8 ($P < 0.03$). In weeks 3 and 7, mean blood CHO concentrations tended to be greater in EO than LO ($P \leq 0.07$) (Figure 1.5). The overall pattern of CHO increases over time was different between EO and LO groups ($P < 0.01$). Specifically, the rate of increase in serum

CHO concentrations over 8 weeks tended to be greater for the EO group than LO ($P < 0.08$) (Figure 1.6).

When data from only H cows were analyzed, analysis of repeated measures showed no overall difference in concentration of blood concentration over time between EO and LO cows. However, there was an ovulation status by week interaction on blood CHO concentrations in that the mean blood CHO concentrations were greater in weeks 4 and 8 in EO when compared with LO (data not shown).

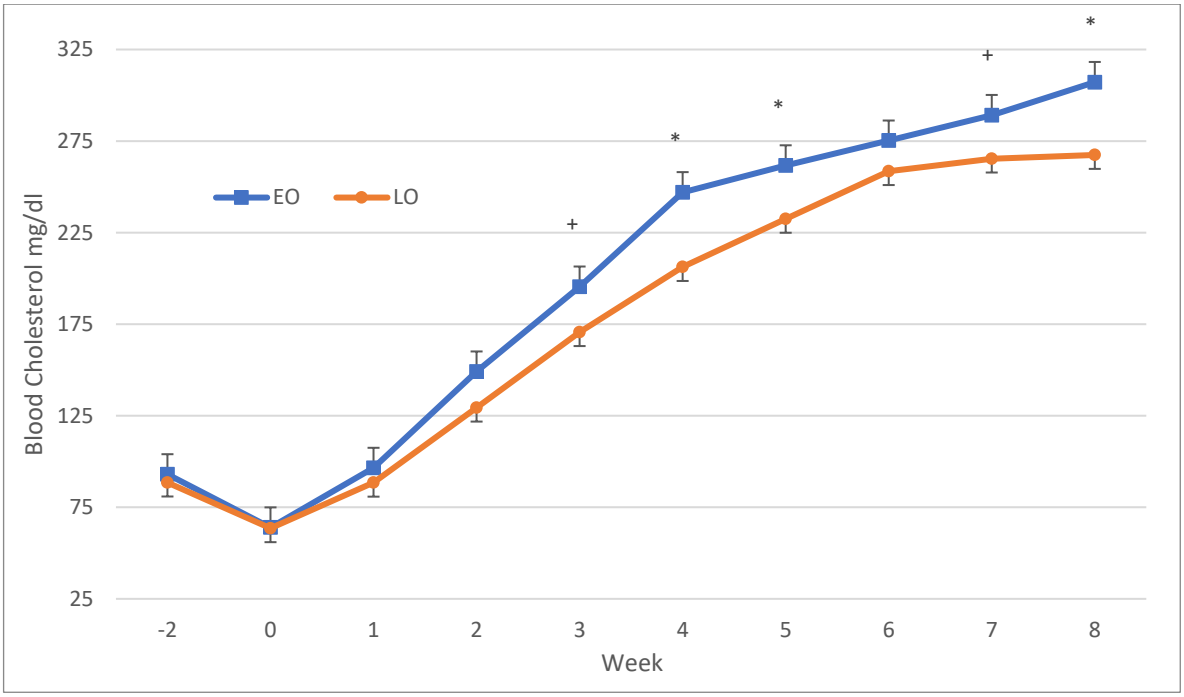

Figure 1.5 Mean blood CHO concentrations (LS means ± SE) in early (EO) and late ovulating (LO) multiparous Holstein cows before and after calving (week 0 = calving). Early ovulators (n=21) ovulated < 28 days postpartum, LO (n=46) ovulated > 28 days postpartum.
* Means differ between EO and LO groups ($P < 0.05$).
$^{+}$ Means differ between EO and LO groups ($P > 0.05$; $P < 0.1$).

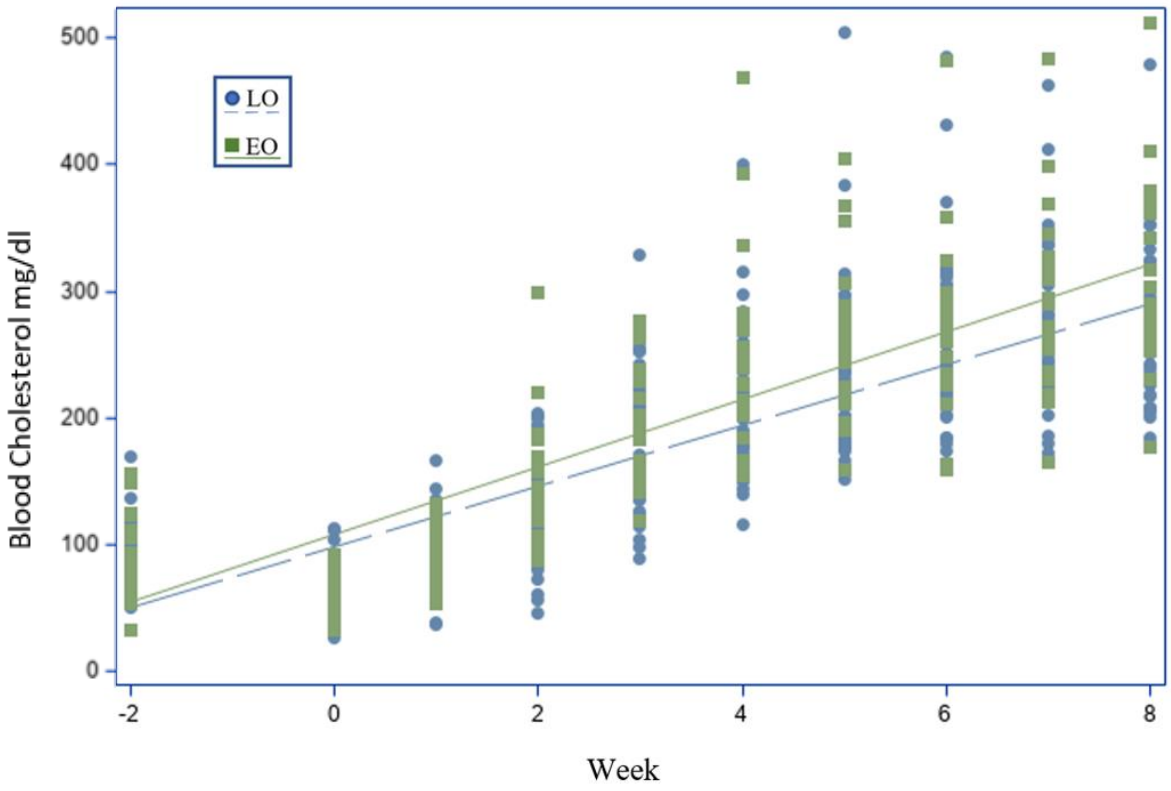

Figure 1.6 Regression lines for blood CHO concentrations in early (EO) and late ovulating (LO) multiparous Holstein cows before and after calving (week 0 = calving).
Early ovulators (n=21) ovulated < 28 days postpartum, and LO (n=46) ovulated > 28 days postpartum.
The overall pattern of CHO change over time was different between EO and LO groups ($P < 0.01$) where the rate of increase in CHO (slope) over 8 weeks tended to be greater for the EO group ($P < 0.08$).

FREE CHOLESTEROL

Blood FCHO was measured from calving (week 0) to week 4 postpartum. Mean concentrations of free blood CHO were not different between EO and LO groups ($P = 0.39$). There was no ovulation status by week interaction on blood FCHO, indicating that the change in FCHO concentrations over time was not different between groups from weeks 0 to 4 ($P = 0.60$). There was an effect of the week on mean blood FCHO concentrations ($P < 0.01$). Regardless of ovulation status, mean blood FCHO increased from week 0 (calving) to week 4 postpartum (Figure 1.7).

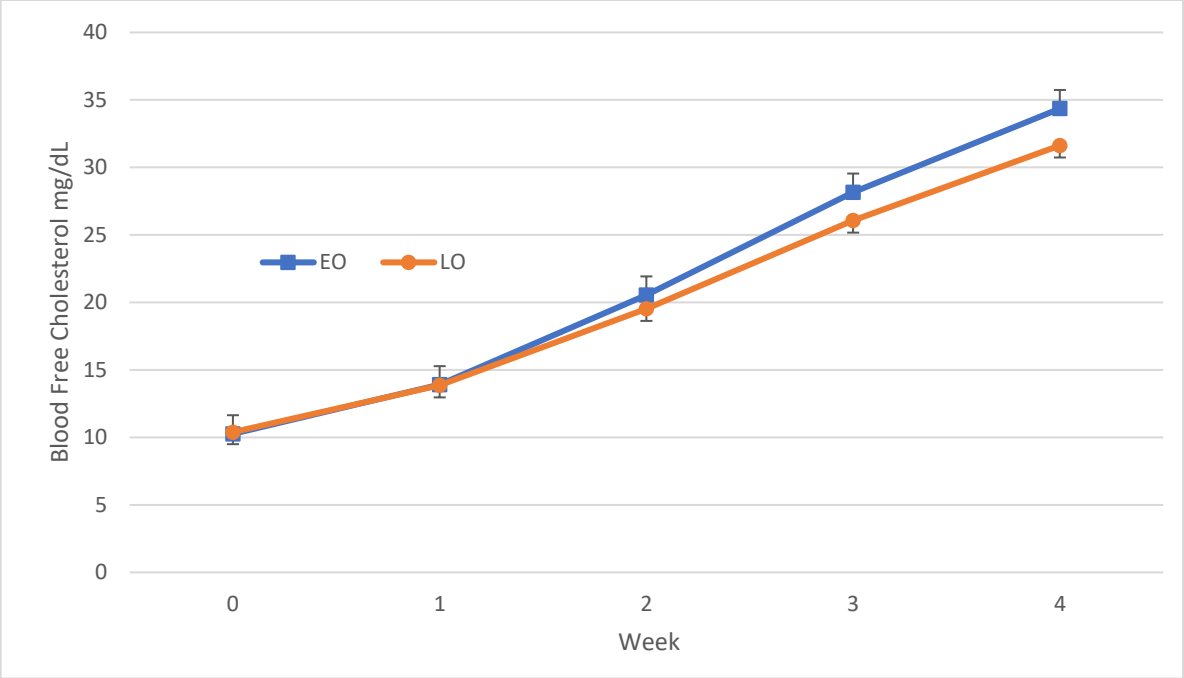

Figure 1.7 Mean blood FCHO concentrations (LS means ± SE) in early (EO) and late ovulating (LO) multiparous Holstein cows before and after calving (week 0 = calving). Early ovulators (n=21) ovulated < 28 days postpartum, and LO (n=46) ovulated > 28 days postpartum.

SERUM AMYLOID A

Analysis of variance showed there was no effect of ovulation status (P = 0.76), week (P =0.35), or week by ovulation status interaction (P= 0.31) on SAA. There was no effect of parity on SAA concentrations (P= 0.30) (Figure 1.8). Mean SAA declined from week 1 to 2 in LO whereas it stayed steady in EO. It should be noted that SAA showed a large variation in both groups.

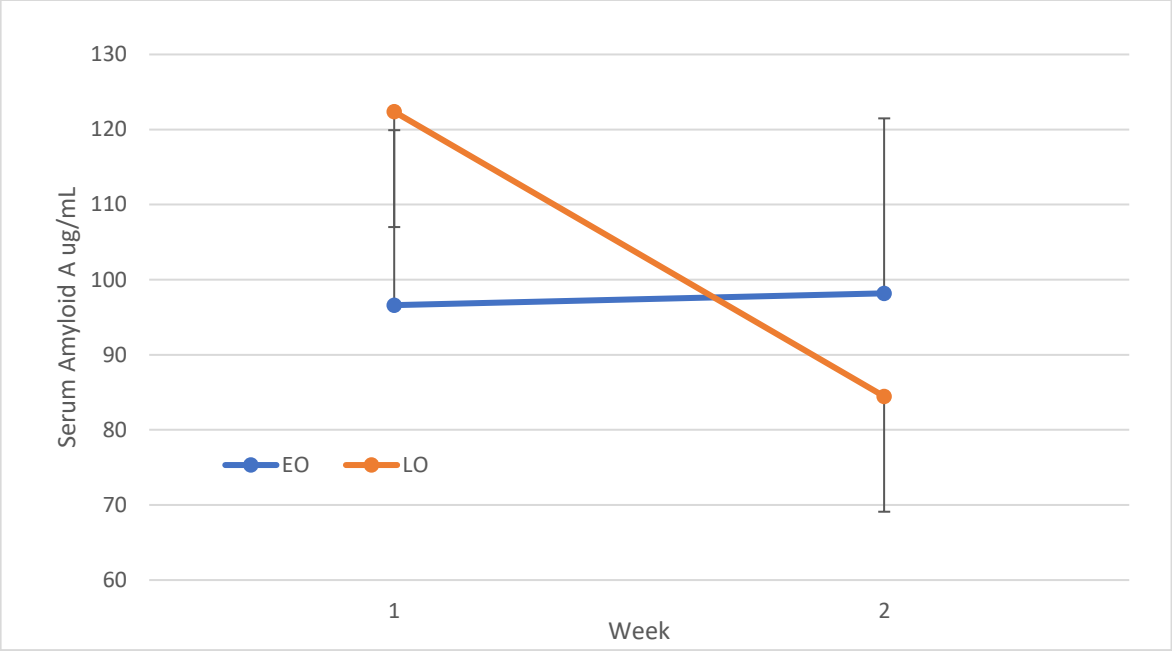

Figure 1.8 Mean serum amyloid A (SAA) concentrations (LS means ± SE) in early (EO) and late ovulating (LO) multiparous Holstein cows in weeks 1 and 2 postpartum. Early ovulators (n=21) ovulated < 28 days postpartum, and LO (n=46) ovulated > 28 days postpartum.

DESCRIPTIVE DATA FOR HEALTHY VS SICK GROUPS

Cows were also retrospectively divided into health status groups depending on whether they exhibited clinical signs of sickness (S; n=27), or not (H; n=40) (Table 1.2, Table 1.3). The means for days to ovulation were different ($P < 0.04$) between H and S (27.8 ± 1.1 vs 32.4 ± 1.8 days). In addition, the mean lactation number of cows was greater ($P = 0.04$) in S than H cows (3.5 ± 0.27 vs 2.8 ± 0.23 lactation). There was no difference ($P = 0.19$) between H and S groups regarding milk yield (37.7 ± 1.7 vs 34.2 ± 2.0 kg/d).

DEPENDENT VARIABLES OF HEALTHY VS SICK COWS

BODY CONDITION SCORE

Overall, H cows had similar BCS when compared to sick cows (2.99 ± 0.04 vs 2.93 ± 0.05 scores) ($P = 0.24$). Regardless of health status BCS decreased over time ($P = 0.01$).

There was no effect of the week-by-health status interaction on BCS, indicating that the change in BCS was similar between H and S groups through the experimental period. Regression analysis shows that the rate of decrease in BCS over time was also not different between healthy and sick cows (Figure 1.9).

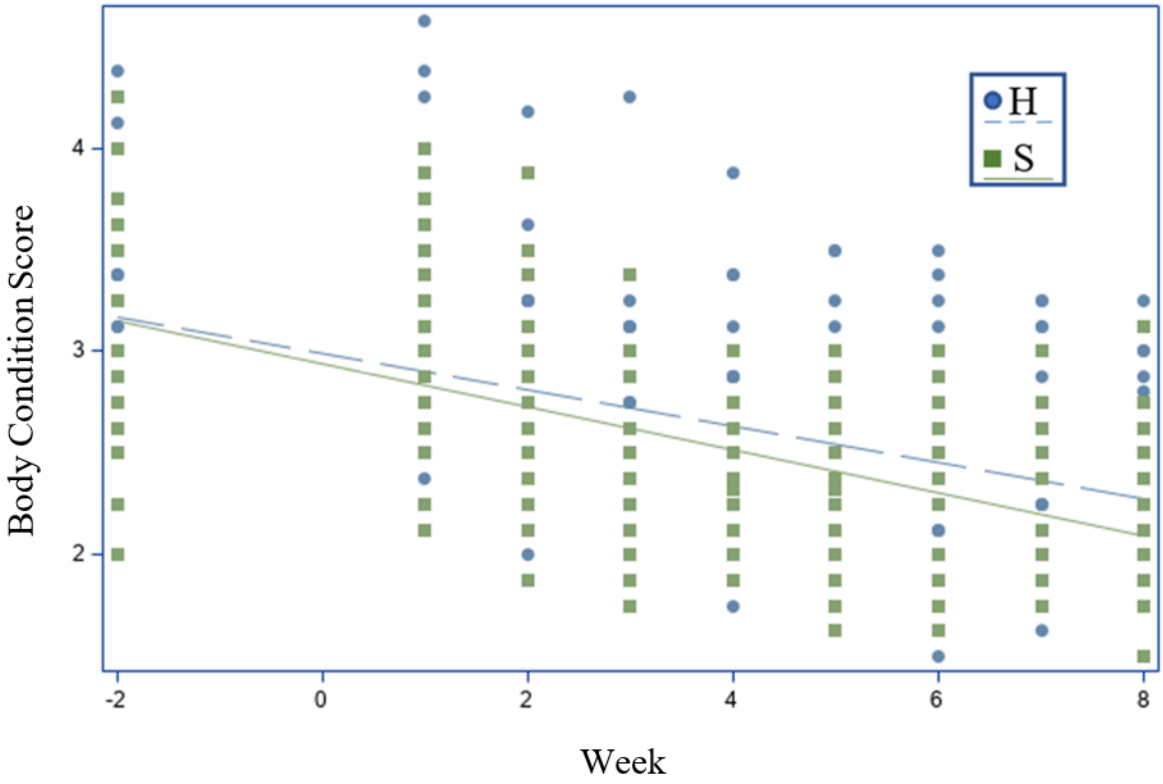

Figure 1.9 Regression lines for body condition scores in sick (S) and healthy (H) multiparous Holstein cows before and after calving (week 0 = calving).
Cows exhibiting clinical signs of disease were determined to be sick (n=27), while others were determined to be healthy (n=40). BCS was measured using a scale of 1 to 5 in 0.25 increments (1=emaciated; 5=obese).

GLUCOSE

There was no difference between S and H groups (P = 0.31) mean serum GLU concentrations and no health group by week interaction was detected (P = 0.33) (Figure 1.10). Regardless of health status, there was an effect of the week (P< 0.01). The data

between the H and S groups follow the same pattern where blood GLU concentrations drop by a large margin immediately following calvingGlucose concentrations then trend upwards and continue a gradual increase over the following 8 weeks postpartum regardless of health status.

Regression analysis showed no detectable pattern or change in GLU concentration over time between H and S groups.

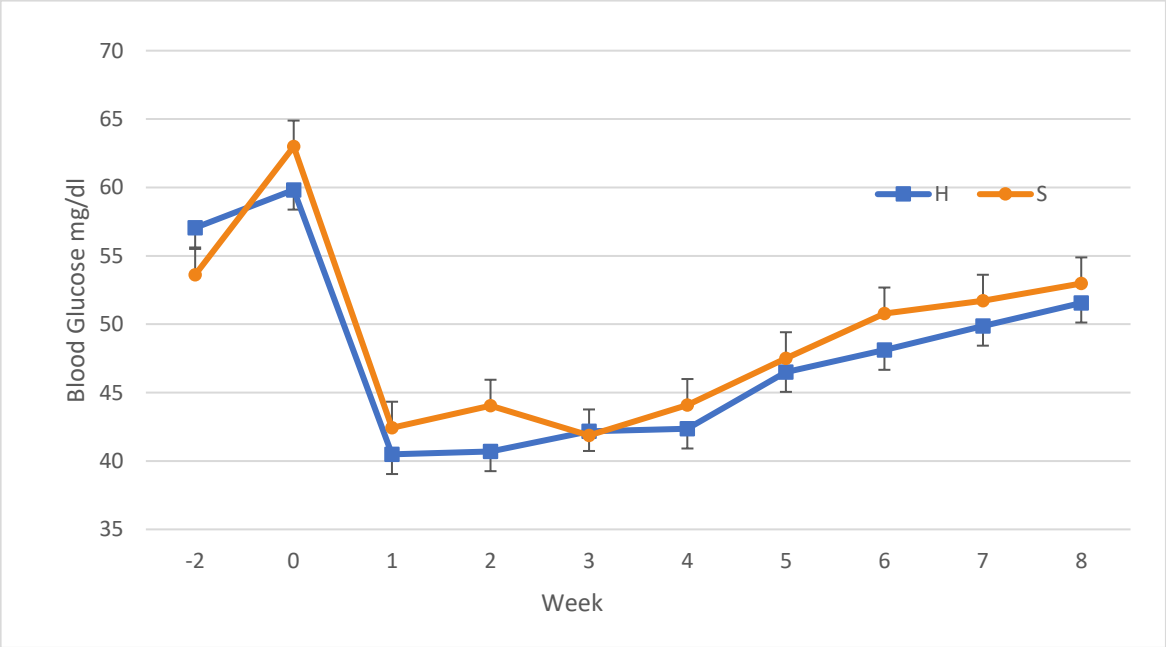

Figure 1.10 Mean blood GLU concentrations (LS means ± SE) in sick (S) and healthy (H) multiparous Holstein cows before and after calving (week 0 = calving).
Cows exhibiting clinical signs of disease were determined sick (n=27), while others were determined healthy (n=40)

TOTAL CHOLESTEROL

Regardless of health status blood CHO increased ($P < 0.01$) during the 8 weeks postpartum (Figure 1.11 and 1.12). Overall, there was a difference in mean blood CHO concentrations in H compared with S (191 ± 6.36 vs 170 ± 7.96 mg/dL). There was an effect of the week-by-health status interaction on blood CHO concentrations ($P = 0.04$). Mean

CHO concentrations were greater in H than S in weeks 4,7, and 8 ($P < 0.01$) and tended to be different in weeks 2, 3, 5, and 6 ($0.07 < P < 0.1$) (Figure 1.11). Regression analysis showed an overall increased pattern of blood CHO concentrations over time and was different between H and S groups ($P < 0.01$). Further, the rate of increase in CHO over 8 weeks tended to be greater for the H group than the S group ($P < 0.06$) (Figure 1.12).

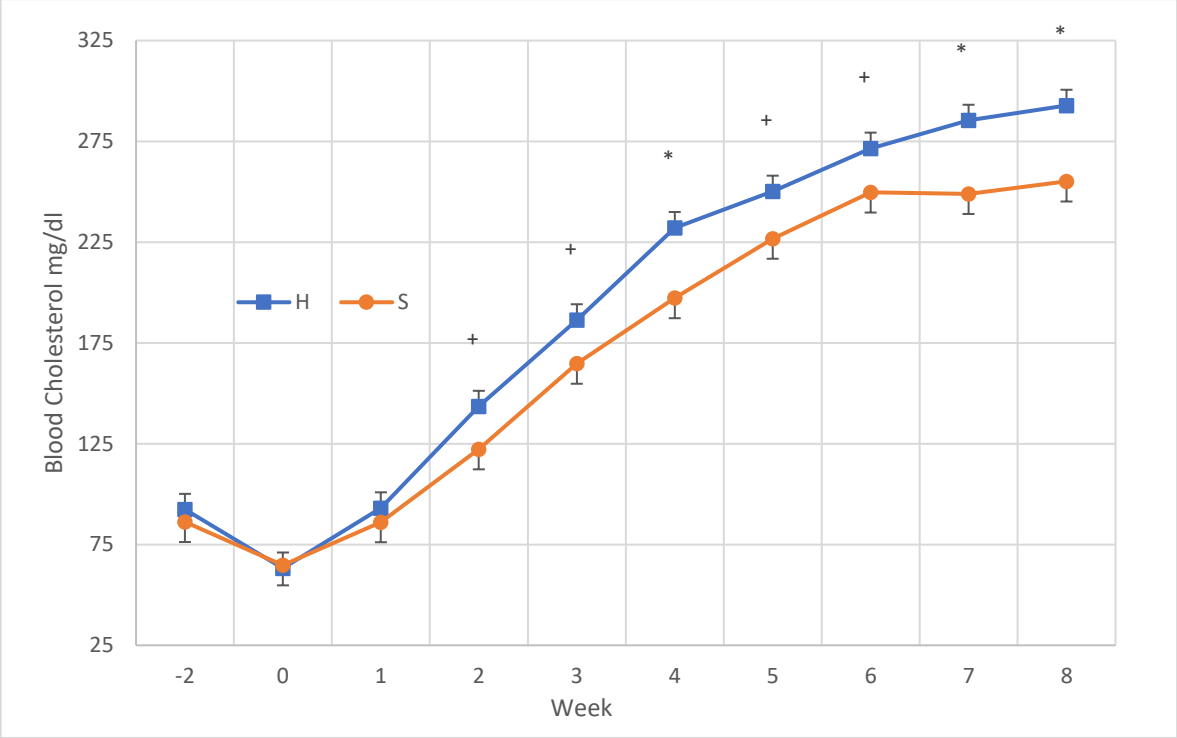

Figure 1.11 Mean blood CHO concentrations (LS means ± SE) in sick (S) and healthy (H) multiparous Holstein cows before and after calving (week 0 = calving).
Cows exhibiting clinical signs of disease were determined to be sick (n=27), while others were determined to be healthy (n=40). BCS was measured using a scale of 1 to 5 in 0.25 increments (1=emaciated; 5=obese).
*Means differ ($P < 0.05$) and $^{+}$ tended to differ ($P > 0.05; < 0.1$) between H and S groups.

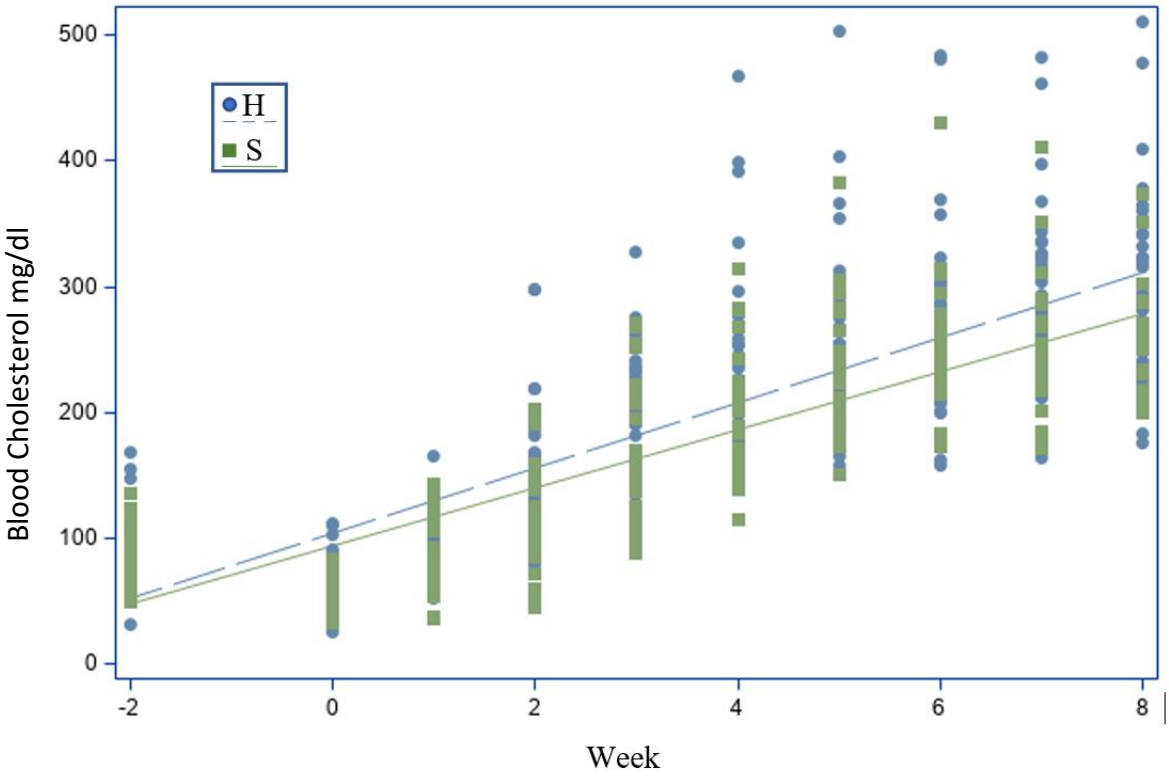

Figure 1.12 Regression lines for blood CHO concentrations in sick (S) and healthy (H) multiparous Holstein cows before and after calving (week 0 = calving).
Cows exhibiting clinical signs of disease were determined to be sick (n=27), while others were determined to be healthy (n=40).
The pattern of blood CHO concentration increase over time was different between H and S groups ($P < 0.01$) where the rate of increase in CHO over 8 weeks tended to be greater for the H group than the S group ($P < 0.06$).

FREE CHOLESTEROL

Analysis of repeated measures showed that there was no difference in FCHO concentrations between H and S cows ($P = 0.86$). Additionally, there was no health status by week interaction indicating that the pattern of change in FCHO for weeks 0 to 4 was not different between the health groups ($P = 0.33$). However, there was an effect of the week on mean blood FCHO concentrations ($P < 0.01$). Overall and regardless of health status, mean blood FCHO increased from week 0 (calving) to week 4 postpartum. Mean serum FCHO was approximately 3-fold greater ($P < 0.01$) in week 4 compared with the day of parturition.

Also, mean FCHO concentrations were different ($P < 0.01$) between 2nd lactation cows and 3rd and greater lactation cows (22.15 vs 19.19 ± 0.83 mg/dL).

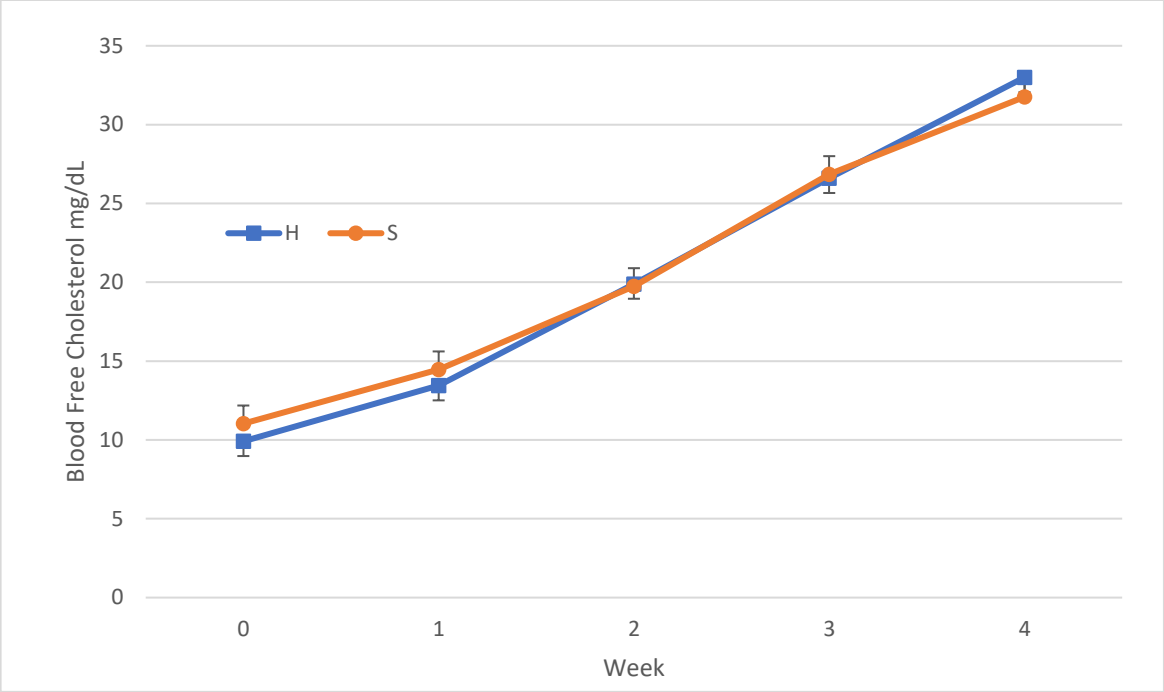

Figure 1.13 Blood FCHO concentrations (LS means ± SE) in sick (S) and healthy (H) multiparous Holstein cows before and after calving (week 0 = calving). Cows exhibiting clinical signs of disease were determined to be sick (n=27), while others were determined to be healthy (n=40).

SERUM AMYLOID A

Overall, the mean SAA concentrations in S cows were greater than H cows (138 ± 13.4 vs 76.1 ± 11.1 ug/mL) ($P < 0.01$). There was an effect of health status by week interaction on SAA ($P < 0.05$). Sick cows showed a decreasing concentration in SAA between weeks 1 and 2 while H cows' SAA concentrations stayed constant. There was no difference seen between parities in mean SAA concentrations.

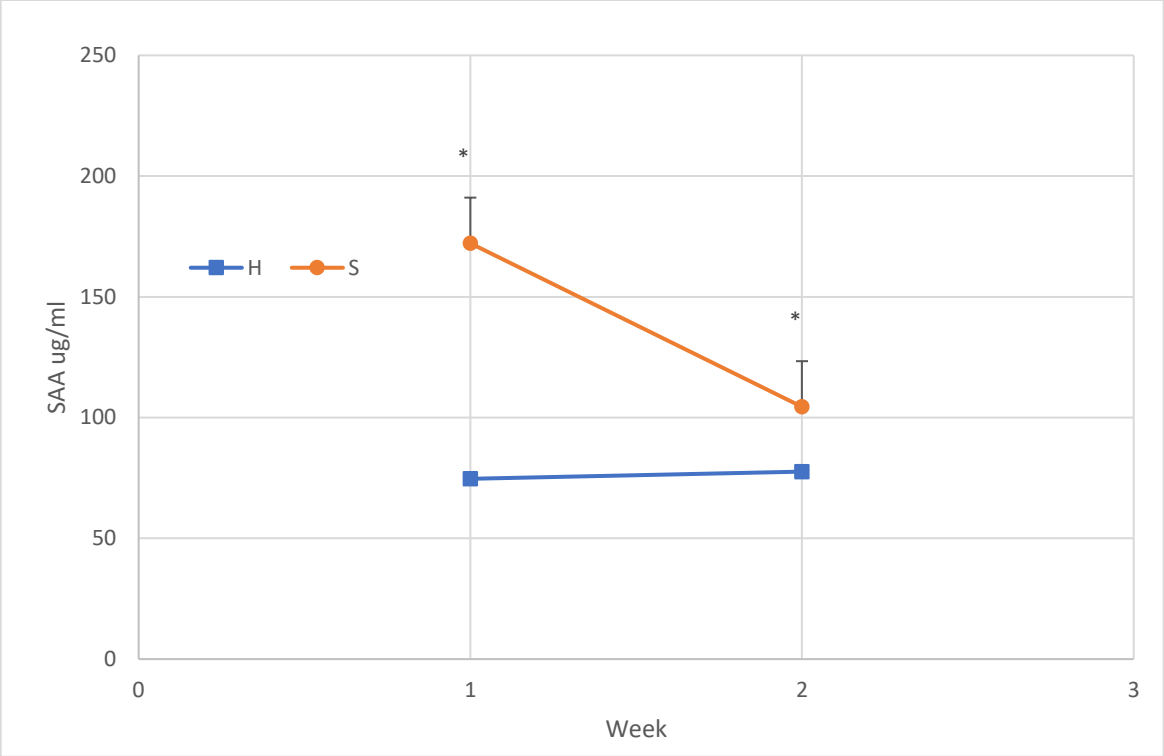

Figure 1.14 Serum amyloid A (SAA) concentrations (LS means ± SE) in sick (S) and healthy (H) multiparous Holstein cows before and after calving (week 0 = calving). Cows exhibiting clinical signs of disease were determined to be sick (n=27), while others were determined to be healthy (n=40).
*Means differ between H and S groups ($P < 0.01$).

Table 1.2 Clinical Disease Presentation and Number of Cows Afflicted
Cows.

Disease Symptom	Number of Cows Affected
Lameness	5
Mastitis	6
Purulent Vaginal Discharge	6
Milk Fever	1
Follicular or Luteal Cysts	2
Ketosis	1
Hemorrhagic Diarrhea	2
Displaced Abomasum	1
Retained Placenta	3
Elevated Body Temperature	10
Unknown Hypo-coagulative Disorder	1

Cows exhibiting clinical signs of disease were determined to be sick (S; n=27). Some cows exhibited more than 1 clinical symptom.

Table 1.3 Number of Clinical Disease Presentations in the Same Cow and Number of Cows Afflicted

Number of Disease Symptoms	Number of Cows
0	40
1	19
2	7
3	1

Cows exhibiting clinical signs of disease were determined to be sick (S; n=27). Some cows exhibited more than 1 clinical symptom. Cow exhibiting no signs of clinical disease were considered healthy (H; 40).

DISCUSSION

Identifying, and overcoming reproductive inefficiency in dairy cows is a major focus of the dairy industry. Studies show that increased time between calving and first postpartum ovulation results in lower reproductive success in the early breeding window (Galvão et al., 2013; Stevenson et al., 1983; Staples et al. 1990; Shrestha et al., 2004). The main objective of this study was to examine relationships among certain metabolites and a blood immune factor and interval to first postpartum ovulation. A secondary objective was to observe if cows' health status influences interval to first postpartum ovulation and if health status has a relationship to the blood metabolites and an immune factor. By estimating the timing of first postpartum ovulation, monitoring health characteristics, and determining blood metabolite profiles between these groups, we were able to observe differences between these groups.

The descriptive data analysis for EO and LO groups provided evidence that there was a biological difference between these two groups. The results showed there was no difference between milk production or parity but there was a difference in average days to ovulation (EO= 20.6 ± 1.3 (n=26) and LO = 37.5 ± 1.4 days (n=17); $P < 0.01$). This was expected and by design; however, it should be noted that cows that did not ovulate by the end of the study (n=29) at 56 DIM were classified as LO but were not included in the LO average for days to ovulation because the data for the day of ovulation did not exist. In reality, the average days to ovulation for LO would be significantly greater than 37.5 days. For all other variables, the LO group consisted of 46 cows.

Using the dairy record system (Dairy COMP), milk production was recorded and averaged for the 56 days from the day of calving to the termination of the study. According to recorded data provided by the farm, mean milk yield was not different between EO and

LO or H and S groups. Some of the daily milk records were not available (milk discarded because of drug withdrawal or symptomatic disease reasons). The exclusion of that milk yield data could have altered statistical analysis.

Overall BCS and change in BCS during the postpartum were different between EO and LO in that EO appeared to have a lesser decrease in BCS from prepartum to 3 weeks postpartum. Cows in the EO group maintained a greater average BCS for the rest of the experiment when compared with the LO group (Figure 1.2). Thus, a greater rate of BCS loss was associated with a delay in postpartum ovulation. Our observation on BCS in this study is similar to previous findings in Holstein cows. Beam and Butler (1999) showed cows who exhibited a greater loss in BCS in the first 30 days postpartum resumed ovulation later. Cows that lost 0.5 of a BCS ovulated 29.7 days postpartum whereas cows that exhibited greater than 1 BCS loss ovulated at 49.9 days postpartum (Beam and Butler, 1999). Similarly, Kadivar et al. (2014) showed cows that ovulated after 45 days postpartum had an overall lower BCS during the transition period and lost more BCS than cows that ovulated sooner. Shrestha et al. (2005) reported a loss of one BCS (or more) between calving and 49 days postpartum increased the occurrence of delayed first postpartum ovulation.

Our study showed that at week 4 and week 5 postpartum BCS for EO was > 2.6 and greater than the LO group (BCS ≤ 2.4). These findings support the previous finding by Yamada et al. (2003). The authors showed that the proportion of cows resuming ovarian cyclicity before 55 days postpartum that had a BCS of 2.75-3.25 at 30 days postpartum were significantly greater than in cows with a BCS ≤2.5 during the same time frame. Additionally, Yamada et al. (2003) demonstrated that cows with a BCS of 2.75-3.25 at 30 days postpartum were significantly more likely to establish ovarian cyclicity before 55 DIM

when compared to cows with BCS <2.5. Staples et al. (1990) monitored BCS immediately postpartum and reported that greater energy deficits during the early lactation period delayed the first detected estrus. Lucy et al. (1991) reported fewer class III follicles (10-15mm) recruited in cows with a more severe negative energy balance at 25 days after calving. Markusfeld et al. (1997) also hypothesized cows with greater body condition loss during the dry period were more likely to have inactive ovaries in the early postpartum period. Late active/inactive ovaries happen for a variety of potential reasons: the suppression of pulsatile LH secretion and reduction in ovarian responsiveness to LH stimulation in negative energy balance condition (Royal et al. 2002; Le Roy et al., 2008), and a reduced secretion of progesterone due to caloric deficiency (Villa-Godoy et al. 1988).

Data from this study, along with others provide evidence that overcoming negative energy balance (NEB) is an important prerequisite for the resumption of ovarian activity. Body condition scoring is an easy and reliable way to determine the energy status and perhaps the severity of the NEB. By monitoring the gain or loss of BCS over time, the approximate use of adipose deposits to support lactation can be estimated. Utilizing a BCS monitoring system may be a tool to help producers identify animals that will face reproductive difficulty in the postpartum period by signaling that ovarian activity may be delayed. Producers may then proceed to enact management strategies to assist cows that have lost BCS over a duration of time or by a magnitude that could signal reproductive difficulty.

The total loss in body condition and the rate of recovery during the postpartum period can be related to the reproductive performance and the occurrence of postpartum diseases in dairy cattle (Markusfeld et al., 1997). Although we did not observe any

significant change in BCS between healthy and sick cows, the days to first postpartum ovulation were longer for the sick cows. Previous studies have shown that the incidence of postpartum diseases was associated with delayed first postpartum ovulation. Kadivar et al. (2014) showed that first postpartum ovulation occurred later in cows with clinical endometritis compared to normal cows and occurrence of clinical endometritis had a negative correlation with BCS. In this study H and S, animals were categorized based on visible signs of clinical disease (Table 1.2).

The relationship between CHO concentrations and ovarian activity has been studied mainly in animals in which high serum CHO concentrations were induced by a dietary fat supplement. The present study was conducted to investigate this relationship in Holstein cows receiving a traditional lactating ration without any fat supplementation. Total CHO measurements showed an overall greater and increasing concentration in EO when compared with LO. The greater CHO concentrations in EO started in week 3 and continued through to the end of the experimental period (with exception of week 6) (Figure 1.5). Data from this experiment agrees with other literature that found in beef cows, serum CHO values were greater from 2 weeks before to 4 weeks after calving in cows that resumed ovarian cyclicity early (4 to 6 week postpartum) compared to those that resumed cyclicity in the mid-range (7 to 10 weeks postpartum) or late (after 11 weeks postpartum) (Guedon et al., 1999). Jeong et al. (2015) showed total CHO concentration was lower in noncyclic cows from 2 weeks prepartum to 6 weeks postpartum when compared to cyclic cows. Blood CHO levels have also been shown to be higher in cows exhibiting their first postpartum estrus within 9 weeks compared to those that came into estrus after 9 weeks postpartum (a

significant negative correlation between the day of first postpartum estrus and blood CHO concentration (Veena et al., 2015).

Greater concentrations of plasma CHO are positively associated with the likelihood of conception, estrus expression at first ovulation, and a shorter interval from calving to conception (Westwood et al., 2002). The mechanisms by which CHO may affect the resumption of ovarian activity and fertility of dairy cattle are unclear, but Westwood et al. (2002) hypothesized that improved fertility for cows with greater concentrations of blood CHO may be related to a more positive energy balance. In fact, Lean et al. (1992) found that plasma CHO concentrations were positively correlated with energy balance for dairy cows in early lactation. It is also believed that increased LH pulsatile secretion and resumption of ovarian activity occurs earlier in cows that move toward positive EB earlier (Canfield and Butler, 1990). In addition, in vitro studies (Gwynne and Strauss, 1982) showed a regulatory role for blood CHO concentrations in steroid production by ovarian tissue. In the present study, the EO cows had a greater blood CHO concentration, greater BCS, and potentially greater DMI. These factors combined may have positively affected the hypothalamic-pituitary-ovarian axis leading to earlier postpartum ovulation in EO.

FCHO analysis showed no difference between EO and LO groups during weeks 0 through 4 of the experimental period. As the data continued towards week 4 the lines between the two groups tended to diverge from one another, but not enough to be statistically different from each other (Figure 1.7). The original design of this experiment was focused on investigating potential early predictors of late/early ovulation during the first few weeks after calving. If the measurements for FCHO continued later into the sampling period it is possible that a difference between groups could have been detected.

Additionally, knowing that FCHO is a portion of total CHO we could then hypothesize that if there is a difference between total CHO in these groups there may be a difference in FCHO or even in CHO esters (which can be calculated from these two values; total CHO-FCHO=CHO esters (Kessler et al., 2014).

Total CHO was different in cows when grouped by H and S where H cows had greater blood total CHO when compared to S cows beginning in week 2 until the end of the sampling period (Figure 1.11). Our findings support a previous study (Sepúlveda-Varas et al., 2015) which showed that low CHO concentrations postpartum are closely associated with postpartum health disorders (metritis), and low levels are exacerbated in the face of multiple clinical disease events. Paiano and associates (2020) showed lower CHO levels are indicative of metabolic ketosis, hypocalcemia, and even lameness (Paiano et al., 2019) in the early postpartum period. The overall concentrations and pattern of postpartum CHO concentrations agree with many previous studies that reported a sharp decrease in levels immediately postpartum and a rebound between 3-5 weeks later (Kessler et al., 2014; Gross et al., 2015). We can speculate that cows experiencing reproductive, metabolic, and other diseases involving inflammation demonstrate lower levels of blood CHO concentrations because in the immediate postpartum period there is a shift in nutrient stores and lipid metabolism to support high milk yield (Bauman et al., 1988; Drackley et al., 1999; Lucy et al., 1992; Lucy et al., 2013; Rowlands et al., 1980). Additionally, blood CHO levels are closely associated with the DMI in lactating cows, and several studies have shown a decrease in DMI when disease states are present (Huzzey et al., 2006; Goldhawk et al., 2009; González et al., 2008). It has also been demonstrated that CHO has an important role in inflammatory disease states (Tall and Yvan-Charvet, 2015). CHO is found in high levels

within cells involved in tissue remodeling and wound healing. It is hypothesized that CHO may enhance cell proliferation (Tall and Yvan-Charvet, 2015). Thus, a combination between lactation utilization, disease states, the possibility of lower DMI and CHO utilization during inflammation may provide an explanation for lower blood CHO levels in sick cows.

There was no significant difference in FCHO levels between H and S groups, but the testing for this variable only occurred in week 0 through week 4 (Figure 1.13). There was some separation between the groups in week 4 and again, the differences between groups regarding total CHO concentrations were not detectably different until later in the sampling period.

This study showed no difference in GLU concentrations between EO and LO which is not different from a few other studies. It is well established that GLU levels are the lowest in the early postpartum period. During this window, multiple studies reported that cows with greater concentrations of blood GLU had fewer days open (Reist et al., 2003), a greater chance of conception at first AI following parturition (Garverick et al., 2013), and decreased time between calving and first postpartum ovulation (Jonsson et al., 1997) or estrus (Veena et al., 2015). A study by Moallem et al. (1997) also showed that days to first ovulation, first estrus, and the number of days open were negatively correlated with the level of plasma GLU. Plasma GLU concentrations in this study follow the same general pattern as seen in these previous studies, i.e., greater concentrations before calving, a sharp decrease at parturition, and a slow gradual increase in the weeks following parturition. However, there was no difference seen between either EO/LO or H/S groups in this experiment. The results of studies on the relationship between GLU concentrations in the early postpartum period

and reproductive success are not consistent. For example, Veena et al. (2015) showed there was no difference in blood GLU levels between cows that conceived before or after 3 months postpartum and also showed a positive correlation between the day of conception and GLU levels. Additionally, there was no relationship between greater GLU concentrations and conception rate (Rowlands et al., 2009), days to first ovulation, or days to first behavioral estrus (Abe et al, 1994), or days to conception (Kappel et al., 1984) in dairy cows.

It has also been hypothesized that the effects of greater plasma GLU concentrations on other systems result in earlier ovarian activity postpartum. Greater plasma GLU concentration promotes greater insulin and IGF-I production, which positively affects the hypothalamus, GnRH pulsatility, and subsequent LH release (Le Roy et al., 2008). Thus, cows with greater postpartum blood GLU concentrations may exhibit a shorter time to resumption of cyclicity by having the capacity for ovarian cells to respond to gonadotropin. It has also been shown that circulating IGF-I concentrations may be a hormonal mediator of nutritional regulation which affects reproductive performance in cattle. There have been positive correlations between high IGF-I levels, more positive energy status, and reproductive functionality **(**Zulu et al., 2002). In contrast, Velazquez et al. (2008) reported there were both positive and inverse correlations between circulating IGF-1 concentrations and days to first ovulation across 6 studies with multiparous cows. Velazquez et al. (2008) speculated that many factors may lead to such variation in reported data i.e., milk yield, breed, and genotype. Additionally, the positive correlation between serum and follicular IGF-I is absent in the postpartum period and could explain contradictory reports. It was also noted that studies that included primiparous cows would likely need to be considered

separate from multiparous cows because of IGF-I involvement in body growth in primiparous cows (Velazquez et al., 2008).

No difference was seen in blood GLU concentrations between H and S cows. Some studies suggest that GLU and glutamine are the major fuels used by immune cells and the utilization of GLU is necessary to create an optimal immune response (Ingvartsen and Moyes, 2013). However, Stevenson et al. (2020) reported there was no significant difference in plasma GLU concentrations in cows based on their health status. In contrast, Bicalho and associates (2017) reported that cows with greater levels of plasma GLU at 3 DIM had 3.5 times higher odds of developing clinical endometritis and 6.6 times higher odds of being diagnosed with metritis.

The lack of difference in GLU concentrations in the current study could be for a variety of reasons, including parity where older cows and cows with higher milk yields demonstrated lower GLU levels (Blum et al., 1983). Other factors influencing GLU concentration include dietary intake (Djokovic et al., 2017), genotype (Bossaert et al., 2009; Jaakson et al., 2013), milk yields (Danfær et al., 1994; Lean et al. (1992); Lomax and Baird, 1983), dietary composition (Kennedy et al., 2008; Herbein et al., 1985), and seasons (Herbein et al., 1985) among other factors (Lee et al., 1978).

The overall mean basal levels of SAA were slightly greater (EO= 97 ug/ml; LO= 103 ug/ml) than observed in other studies for healthy cows (Approximately 22-75 ug/ml) (Alsemgesst et al., 1994; Karreman et al., 2000). Serum AA concentrations can be elevated post parturition (Alsemgeest et al., 1993) and can flux because of a large variety of inflammatory diseases (both clinical and subclinical) which prevail in the transition period (Alsemgesst et al., 1994; Biswal et al., 2014; Chan et al., 2010; Dervishi et al., 2016; Gozho

et al., 2005; Gozho et al., 2007; Guzelbektes et al., 2010; Nazifi et al., 2010). Additionally, because of the confinement housing and dietary conditions of the experimental animals, cows may be susceptible to greater basal levels of circulating SAA (Karreman et al., 2000). Due to the housing and dietary considerations as well as the time of sampling; a slightly higher basal level of SAA would be expected.

For the measurements of SAA which were only determined for weeks 1 and 2 of the sampling period, no difference was detected between EO and LO groups. Early ovulators had a very small slope, nearly the same values from week 1 to week 2 whereas the LO group showed a large decrease in SAA concentrations (Figure 1.8). There was a lot of individual cow variation in the data. The original design of the experiment only included using healthy animals, but because of limited cow availability and low numbers of available research animals, both EO and LO groups included sick animals. It has been extensively reported that elevated SAA exists in cows experiencing inflammatory disease and could very well have contributed to the large SAA variation. A similar study including more healthy animals and more frequent measurements, over a longer period, would be beneficial to shed more light on the link between SAA, similar acute phase response markers, and fertility.

When cows were grouped by health status, based on clinical signs of disease, the average overall SAA concentrations were significantly different (S= 138 ± 13.4; H= 76.1 ± 11.1 ug/mL; $P < 0.01$). Serum AA concentrations of S cows were nearly two-fold greater than H cows during week 1 but the concentrations decreased by week 2 where they were 27 ug/ml greater than H cows (Figure 1.14). These findings support many other studies that also found sick cows, for a variety of ailments, had increased SAA concentrations. Specifically, elevated SAA levels have been reported in sole hoof ulcers (Ksenija et al.,

2019), acute laminitis (Ksenija et al., 2019), ketosis (Abuajamieh et al., 2016), in both left and right displaced abomasum cases (Guzelbektes et al., 2010), metritis (Chan et al., 2010: Nazifi et al., 2010; Dervishi 2016), and with cows having either follicular or luteal cysts (Brodzki et al., 2019). The sick population of cows used in this study did not have a continual problem with one disease or health concern, cows determined sick experienced an unconcentrated variety of health issues. Our data suggest that SAA is a detectable marker for diseases with an inflammatory nature; however, it does not appear to be a suitable marker for the prediction of postpartum ovulation. It should be noted that the sampling period in the current study was limited. Nevertheless, because of the somewhat cumbersome laboratory procedures and cost currently associated with SAA testing, it is not a feasible marker to use for general producers.

CONCLUSION

These results provide evidence that BCS and blood CHO concentrations during the early postpartum period may be associated with the timing of the first postpartum ovulation in multiparous cows. Blood CHO and SAA concentrations may be associated with the presence of a disease state in early postpartum cows. Therefore, these markers may be used as a tool to identify cows at risk of being anovulatory due to diseases or other physiological problems.

This data also shows a significant relationship between BCS measurement at 21 DIM and the likelihood of ovulation by 28 DIM (Figure 1.15). As BCS on 21 DIM increased, the probability of ovulation also increased. Additionally, a tendency toward a relationship exists between CHO concentrations at 21 DIM and the likelihood of ovulation by 28 DIM. Similar to BCS, as blood CHO concentrations increased the probability of ovulation by 28 DIM

increased (Figure 1.16). As indicated, utilizing a BCS monitoring system or testing blood CHO in the early postpartum period could help producers identify cows with a higher likely hood of delayed postpartum ovulation, hence providing opportunities for producers to manage these cows.

Additional research to explore reliable, less invasive, and easy-to-use techniques for monitoring these markers would be useful for producers in identifying problem cows. Further research is needed to determine how applicable the implementation of BCS and/or CHO monitoring practices are in dairy management programs.

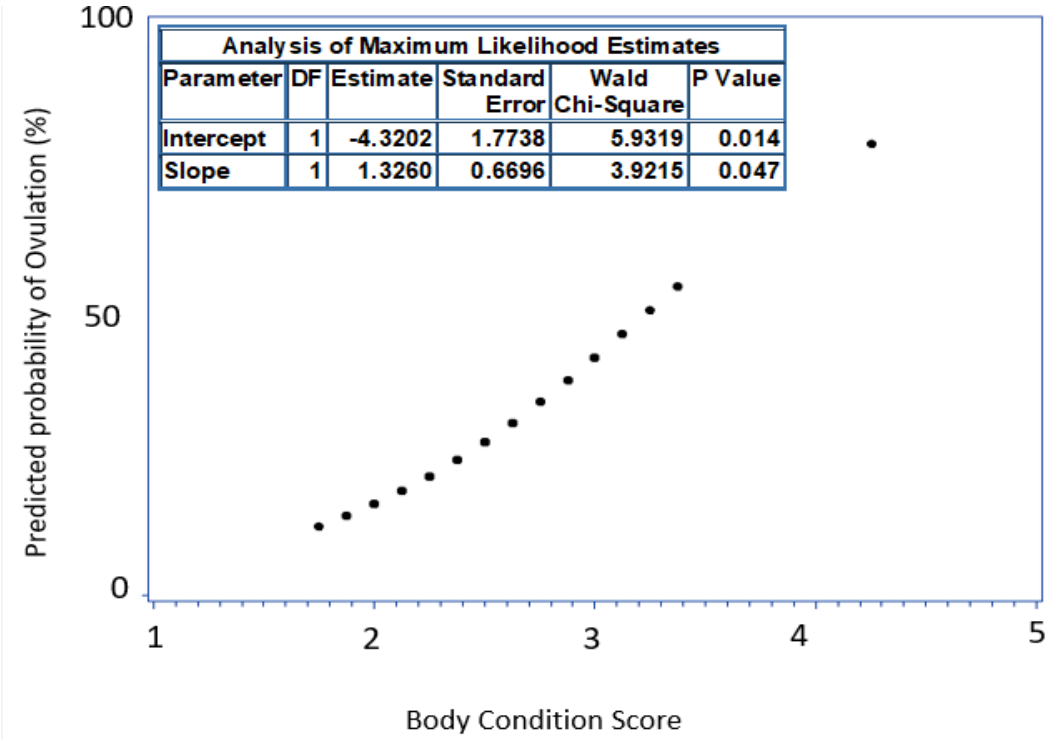

Figure 1.15 Probability of ovulation by 28 DIM based on BCS values on day 21 DIM. The logistic regression equation for the prediction model: ln [P / 1 - P] = b0 + b1x.

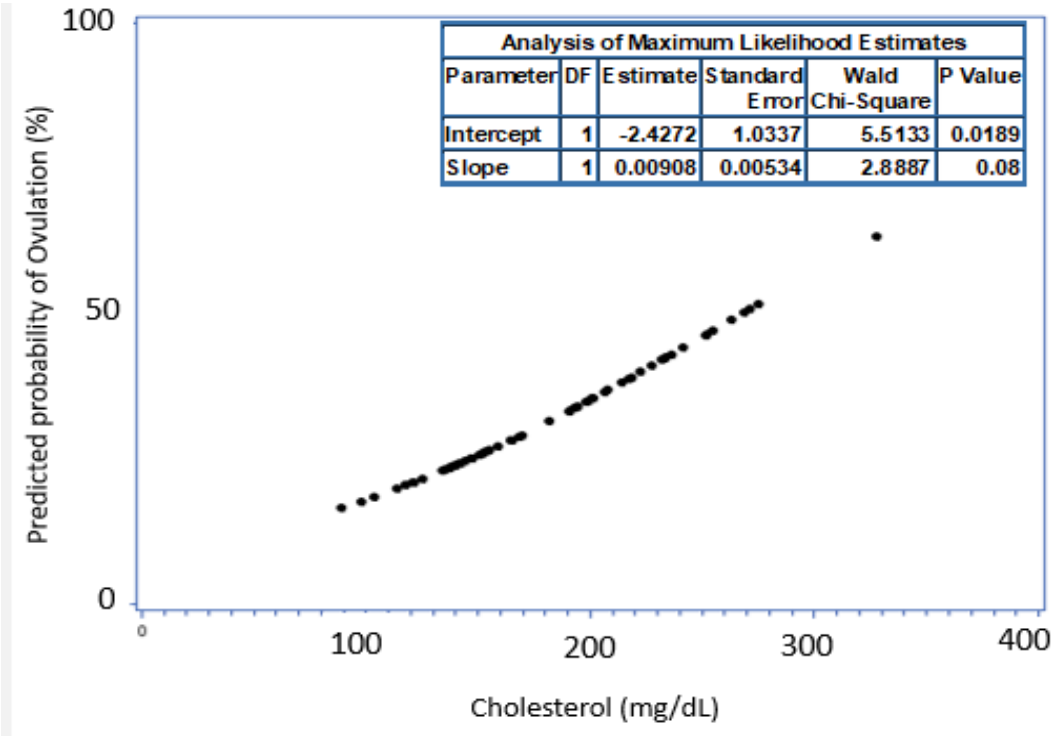

Figure 1.16 Probability of ovulation by 28 DIM based on total blood CHO values on day 21 DIM. The logistic regression equation for the prediction model: ln [P / 1 - P] = b0 + b1x.

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