THESIS

NOVEL STRATEGIES FOR PERIPARTAL HEALTH IMPROVEMENT IN TRANSITION DAIRY COWS

Submitted by

Karol Gilberto Solano-Suárez

Department of Animal Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2019

Master's Committee:

Advisor: Pablo J. Pinedo

Jason Bruemmer
Noa Roman-Muniz
Andrea Bohn

ABSTRACT

NOVEL STRATEGIES FOR PERIPARTAL HEALTH IMPROVEMENT IN TRANSITION DAIRY COWS

The transition period is acknowledged as a time of increased stress and risk to develop infectious, non-infectious, and metabolic diseases due to fetal growth and the onset of lactogenesis. In the past decades, antimicrobial resistance in animals and humans has become an environmental and public health concern, and the restrictions on the use of conventional drugs in organic certified dairy farms encourage the research on novel approaches for the prevention and treatment of infectious diseases in dairy systems. Natural alternatives and biomolecular technologies have been studied to diminish the impact of diseases during the transition period for conventional and organic certified dairy, and a couple of them will be discussed in this thesis.

Chapter 1 comprehend a literature review on transition period elucidating the complex and multifactorial chain of events that lead to make the cow susceptible to develop metabolic and infectious diseases. Metabolic disorders such as negative energy balance and hypocalcemia are connected with the excessive inflammation and cellular immunosuppression occurring around calving. Then, a brief description of other related diseases and new strategies for prevention and treatment of them is discussed.

Chapter 2 describes an experiment using an immunomodulator based on Mycobacterium cell wall fraction (MCWF). We hypothesized that the subcutaneous administration of MCWF within the two weeks before calving and within 24 hours after calving could generate a non-specific cellular immune response capable of reducing the risk of peripartal infectious diseases in dairy cows. Therefore, our objective was to evaluate the effect of a commercial immunomodulator based on MCWF (Amplimune®, NovaVive Inc., Belleville, Canada) on presentation of peripartal diseases and reproductive performance of Holstein cows, assessing cellular immune response and metabolic status. The presentation of clinical metritis, clinical mastitis, and pyometra in MCWF cows were significantly lower compared to CON cows, although the presentation of respiratory disease before 28 days in milk was significantly higher compared to CON cows. The overall reproductive performance was significantly improved in MCWF cows compared to CON cows. Future research at different physiological stages and using different doses and routes of administration is encouraged.

Chapter 3 describes an experiment using a pulsed alternating wavelength system (PAWS). We hypothesized that PAWS could elicit a positive hormonal and metabolic response that might reduce presentation of dystocia, as well as the imbalances and stress around calving, improving peripartal health and subsequent performance in transition dairy cows. Hence, our objective was to evaluate the effect of PAWS on dystocia presentation, peripartal health, activity, and serum levels of melatonin (MEL), serotonin (5-HT), prolactin (PRL), somatotropin (BST), calcium, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) of organic certified Holstein cows. Cows exposed to PAWS reduced the presentation of dystocia and modify NEFA serum levels within 3 days after calving. However, results of melatonin were not available by the time of this writing to clarify the effect of PAWS on these outcomes.

ACKNOWLEDGEMENTS

I would like to thank to my advisor Dr. Pablo Pinedo, and to my lab partners Ana, Diego, Sushil, Luciana, and Catey. Additionally, to the owners and staff of the dairies that let us enter in their facilities and their assistance and help during the projects. Finally, to USDA for the research assistantship that let me come to do this work and create this thesis.

DEDICATION

I would like to dedicate my effort to my mother, Virginia, my aunt, Josefina, my sisters, Yaneth, Eliana, and Derly, and especially to my adored nieces and nephews, Daniela, Camilo, María, Sebastián, Santiago, and Nicolas.

TABLE OF CONTENTS

ABSTRACT ii

ACKNOWLEDGEMENTS iv

DEDICATION v

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER 1: LITERATURE REVIEW ON TRANSITION PERIOD 1
- Metabolic challenge and subsequent performance 2
  - Endocrine implications 2
  - Negative energy balance 4
  - Calcium and mineral disorders 5
- Impairment of the immune function and disease presentation 7
  - Inflammation and immunosuppression 7
  - Relevant health disorders during the transition period 9
- New alternatives to conventional approaches 13
- Tables and figures 17

REFERENCES 22

CHAPTER 2: EFFECT OF MYCOBACTERIUM CELL WALL FRACTION IMMUNOMODULATOR ON PERIPARTAL HEALTH AND REPRODUCTIVE PERFORMANCE IN TRANSITION DAIRY COWS 31
- Summary 31
- Introduction 32
- Materials and methods 34
  - Study population 34
  - Experimental design and treatment allocation 34
  - Case definitions 35
  - Immune and metabolic assessment 37
  - Data management and statistical analyses 38
- Results 39
  - Descriptive statistics 39
  - Disease presentation and fertility 40
  - Cellular immune response and metabolic status 42
- Discussion 42
- Conclusions 47
- Tables and figures 49

REFERENCES 57

CHAPTER 3: EFFECT OF PULSED ALTERNATING WAVELENGTHS SYSTEM (PAWS) ON PERIPARTAL HEALTH AND HORMONAL PROFILING IN TRANSITION DAIRY COWS 62

- Summary 62
- Introduction 63
- Materials and methods 66
  - Study population 66
  - Experimental design and treatment allocation 66
  - Case definitions 67
  - Hormonal and metabolic assessment 69
  - Data management and statistical analyses 69
- Results 70
  - Descriptive statistics 70
  - Peripartal conditions, metabolic and hormonal status 71
- Discussion 71
- Conclusions 74
- Tables and figures 75

REFERENCES 81

LIST OF TABLES

TABLE 2.1- Frequencies of disease presentation comparing *Mycobacterium* cell wall fraction immunomodulator (MCWF) vs. control (CON) group..35

TABLE 2.2- Frequencies of reproductive events comparing *Mycobacterium* cell wall fraction immunomodulator (MCWF) vs. control (CON) group..36

TABLE 2.3- Repeated measures and confidence limits (CL) of back-transformed values for non-esterified fatty acids (NEFA), β-hydroxi butyrate (BHB), and calcium between *Mycobacterium* cell wall fraction (MCWF) and control (CON) group 36

TABLE 3.1- Frequencies of disease presentation for cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows..71

TABLE 3.2- Repeated measures and confidence limits (CL) of back-transformed values for non-esterified fatty acids (NEFA), β-hydroxi butyrate (BHB), and calcium between cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows 71

TABLE 3.3- Repeated measures and confidence limits (CL) of back-transformed values for prolactin (PRL) and bovine somatotropin (bST) between cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows..72

TABLE 3.4- Repeated measures and standard error (SE) of activity 12 hours before expelling of the calf between cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows. 72

LIST OF FIGURES

FIGURE 1.1- Feed intake and negative energy/protein balance due to lactogenesis (Modified from Drackley, 1999)........40

FIGURE 1.2- Relationship between immune competence, metabolic imbalances and inflammation around the transition period (Modified from Trevisi and Minuti, 2018).........40

FIGURE 1.3- Synthesis of NEFA for subsequent secretion of fat in the milk and production of Acetil-CoA in the liver (Modified from Drackley, 1999)........40

FIGURE 1.4- Effect of metabolic alkalosis and hypomagnesemia on the binding of parathyroid hormone (PTH) on its receptors (Modified from Goff, 2008).........40

FIGURE 1.5- Negative effect of inflammation on cellular immune response (Modified from Trevisi and Minuti, 2018). 40

FIGURE 2.1- Survival curves for time to pregnancy comparing *Mycobacterium* cell wall fraction (MCWF) vs. control (CON) group. 41

FIGURE 2.2- Repeated measures of back-transformed leukocytes counts comparing *Mycobacterium* cell wall fraction (MCWF) vs. control (CON) group 42

FIGURE 3.1- Distribution of CON (left, green) and PAWS (right, red) groups within the maternity complex........74

FIGURE 3.2- PAWS lamps (arrow) affixed to wires on the roof for the pen of cows exposed to PAWS (up), and the pen of unexposed CON group (down)........75

CHAPTER 1: LITERATURE REVIEW ON TRANSITION PERIOD

The transition period (TP) is defined as the time from 21 days prior to calving until 21 days after calving. Compared to other stages in the life of a dairy cow, the TP is consistently recognized as a time of increased risk to develop diseases (Drackley, 1999). Lactogenesis is the principal factor inducing and enhancing metabolic imbalances, including negative energy balance (NEB), calcium disorders, and immunologic disruptions due to the abrupt increase of nutrient requirements (**Figure 1.1;** Drackley, 1999; Lacasse *et al.*, 2018). These imbalances make the cows susceptible to develop peripartal diseases like retention of fetal membranes (RMF), uterine infectious diseases (metritis, endometritis, and pyometra), subclinical and clinical hypocalcemia, fatty liver, subclinical and clinical ketosis, mastitis, displaced abomasum, acute or subacute ruminal acidosis (SARA), and lameness (Drackley, 1999; Esposito *et al.*, 2014, Contreras and Sordillo, 2011).

Metabolic imbalances usually precede immunosuppression, although acute phase proteins (APP) and cytokines from placenta (IL1β, IL-8) and liver (serum amyloid A1 – SAA1 –, TNFα) may have an effect on the satiety center of the brain reducing dry matter intake (DMI), promoting hypoinsulinemia, hypoglycemia and lipid mobilization (Wankhade *et al.*, 2017, Maret *et al.*, 2019; Shimizu *et al.*, 2018). Metabolic and immune status can result in a positive or negative feedback depending on the ability of the cow to adapt, and TP naturally determines the upcoming life quality of the animal and profitability for the producer. Therefore, metabolites like non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) and immunologic markers can be use as trustworthy predictors for future performance (Wankhade *et al.*, 2017).

Deleterious effects on the TP are worse if the previous lactation is extended for more than 450 DIM (Maret *et al.*, 2019); hence, the metabolism of glucose and lipids in the TP is highly correlated with the health performance of the cow during mild and late lactation (Maret *et al.*, 2019; Lange *et al.*, 2018). Successful strategies to improve the adaptation of cows to TP, predominantly around nutritional parameters, includes the implementation of a good therapy scheme in the dry-off period and proper environmental management to reduce stressors (Singh, 2019).

Doubling the energy and protein requirements to guarantee a proper body condition around calving, ensuring adequate vitamin y antioxidants supply (vitamin A, B and E, selenium, β-carotene), and supporting mineral demands (calcium and vitamin D, magnesium, phosphorus, dietary cation anion diets – DCAD –) will warrant a better health performance (Reddy *et al.*, 2016; Gilbert, 2016). These programs count on a herd-group level monitoring, based on clinical records (incidences, prevalence, risk factors, protocols to ensure accuracy and reliability of diagnostic tests, prevention and treatment procedures), nutrition (e.g. feed consumption, composition of diets, and BCS), milk yield, and environmental and social management (LeBlanc, 2010; Chapinal *et al.*, 2012; Dänicke *et al.*, 2018). On an individual level, the programs must focus on early diagnosis and treatment of cows at risk of diseases related with metabolic syndromes.

Metabolic challenge and subsequent performance

Endocrine implications

One of the main sources of metabolic impairment and immunosuppression is the robust change around calving on circulating levels of progesterone (P4) and estrogens (E2) with a successive increase in cortisol levels (Gross *et al.*, 2015). This stress response will have a repercussion on subsequent health, welfare, fertility, and production (LeBlanc, 2010). The thyroid gland plays a key role on homeostasis during the TP, especially in heifers experiencing their first pregnancy and lactation. The influence of the thyroid hormones (thyroid-stimulating hormone – TSH –, thyroxin – T4 –, triiodothyronine – T3 –) is greater the week before parturition, and their concentration is higher as the pregnancy advances and the mammary gland adjusts to tolerate and permit lactogenesis. The thyroid hormones regulate metabolic pathways to control energy expenses by the interactions between the brain, adipose tissue, skeletal muscle, liver and pancreas (Steinhoff *et al.*, 2019; Fiore *et al.*, 2015), and thyroid hormones continue to elevate some days after calving while the cow reaches again the state of homeostasis (Fiore *et al.*, 2015).

Somatotropic hormones upregulates the expression of genes that increase the release and action of growth hormones like bovine somatotropin (bST) and insulin-like growth factor 1(IGF-1), stimulating gluconeogenesis and metabolism and transportation of lipids, and downregulating the expression of genes that promotes oxidative stress and inflammation (Silva *et al.*, 2017; Trevisi and Minuti, 2018). If the body delays recovery from the TP, there will be delayed estrous cycle resumption, the endometrium and embryo production on a cellular level could be affected, embryos may be unable to divide and implant properly, and the uterus may fail to recognize the pregnancy (Roche *et al.*, 2018). Recombinant bST was used in the United States during the weeks around TP to enhance somatotropic hormones effects, and during lactation (especially early lactation) to improve general health, reproductive performance and milk yield (Silva *et al.*, 2017; Roche *et al.*, 2018). A deeper description on the details of the metabolic imbalances and related diseases is presented in this chapter.

Negative energy balance

The reduction on DMI due to the physical, metabolic, immune and endocrine changes related to pregnancy and lactogenesis, leads to the NEB **(Figure 1.2)**; in addition, most of the nutrients are partitioned for fetal growth and mammary gland development in a process call homeorhesis (Contreras and Sordillo, 2011; LeBlanc, 2010). Cows with high production of milk are predisposed to mobilize more lipids; therefore, a substantial insulin resistance (i.e. incapacity of the tissues to use glucose effectively), hypoglycemia, and hypoinsulinemia are expected. For example, elevated NEFA concentration can alter inflammatory response leading to an exacerbated immune response harmful for the cow (i.e. excessive reactive oxygen species – ROS – response), and a subsequent impairment of leukocytes activity leading to immunosuppression (Contreras and Sordillo, 2011). Cows with greater NEB in the TP have delayed resumption of ovarian cyclicity as high NEFA concentrations are associated with an impairment on GnRH and LH production (Miqueo *et al.*, 2019). Moreover, cows at greater risk of anestrous are the ones with more probability to present dystocia, displaced abomasum (DA), ketosis, retention of fetal membranes and clinical endometritis (Walsh *et al.*, 2007; Vieira-Neto *et al.*, 2014).

It is possible to create energy from mobilized NEFA by the oxidation of these fatty acids in the Krebs cycle, but if the liver reaches its metabolic capacity to perform oxidation, ketone bodies (acetone, acetoacetic acid, and especially BHB) are produced and accumulated into circulation (ketosis) as an accessible form of energy on behalf of the strong demand of glucose, as shows in **Figure 1.3** (Drackley, 1999; LeBlanc, 2010; Horst *et al.*, 2018). Dextrose-50% and propylene glycol are the principal gluconeogenic therapeutic choice in cases of ketosis (Bashir *et al.*, 2016. Ketosis, often undiagnosed, include clinical signs like a sudden reduction of DMI, rapid loss of BCS, hypoglycemia, a significant drop of milk production, and in extreme cases nervous signs are presented (e.g. aggressiveness) (Bashir *et al.*, 2016). Subclinical ketosis can increase the risk of ruminal microbial disorders, evolving to other diseases like lameness (Bashir *et al.*, 2016; Horst *et al.*, 2018). Odds ratios (OR) for values of NEFA above 0.4 mmol/L and/or BHB above 1.2-1.4 mmol/L within the first 2 weeks after calving (0.7 mmol/L the two weeks before) show significant association with DA (OR = 2-8), clinical ketosis (OR = 4-6), metritis (OR = 2-5), reduced milk yield, retention of fetal membranes (OR = 2-3), endometritis, anestrous (OR= 2-3), and increased severity of mastitis and risk of culling before 60 DIM (Dubuc and Denis-Robichaud, 2017; Duffield *et al.*, 2009; LeBlanc, 2010; Roberts *et al.*, 2012; Chapinal *et al.*, 2011; Pinedo *et al.*, 2017). In general terms for dairy cattle in the United States, an optimal threshold to determine an elevation for NEFA is 0.27 mmol/L within the 2 weeks prepartum, and 0.57 mmol/L within the 2 weeks postpartum; the optimal threshold to determine an elevation for BHB within the 2 weeks postpartum is 10 mg/dL (or 0.96 mmol/L) (Ospina *et al.*, 2010).

Calcium and mineral disorders

Usually, cows with hypocalcemia also have elevated NEFA and BHB levels, providing evidence of the multifactorial and interrelated nature of energy and mineral imbalances in the TP (**Figure 1.2;** Martinez *et al.*, 2012; Chapinal *et al.*, 2011, Trevisi and Minuti, 2018). Calcium (Ca) is involved in processes such as muscular contraction, nerve conduction, cellular membranes, organelles stability, and many other physiological functions. It is hypothesized that as the cow get closer to calving, endocrine changes stimulated by lactogenesis occur in order to control Ca homeostasis. Thus, parathyroid hormone (PTH) is increased in response to hypocalcemia, and PTH related protein (PTHrP) is induced in the mammary gland by the action of serotonin; both will induce a greater intestinal Ca absorption, bone Ca resorption, and lesser Ca renal excretion (Neves *et al.*, 2018; Hernandez *et al.*, 2012; Laporta *et al.*, 2014). **Figure 1.4** shows how increased pH as result of high-cations/low-anions diets (H+ ions must be removed from the blood to reach electroneutrality, leading to metabolic alkalosis), and hypomagnesemia (Mg is an essential co-factor to stimulate PTH receptors), interfere with the ability of PTH to bind its receptors on bone, kidney and intestines (Goff, 2008). If the demand for Ca to make colostrum and milk is excessive, there is inability of the cow to maintain Ca homeostasis, and around 50% of multiparous cows and 25% of primiparous cows will develop SCHC (Goff, 2008, Martinez *et al.*, 2012). Rodriguez et al (2017) reported OR for DA, clinical ketosis, RFM and metritis of 3.7, 5.5, 3.4, and 4.3, respectively, for cows with SCHC after calving, although diagnosis of hypocalcemia within the first DIM may be associated with subsequent increased milk yield in primiparous cows (Martinez *et al.*, 2012, Rodriguez *et al.*, 2017, Neves *et al.*, 2018). Moreover, Martinez *et* al (2012) reported that the total absence of SCHC may represent around 65 and 90 % reduction in the incidence of clinical and toxic metritis, respectively.

The cutoffs to define subclinical hypocalcemia (SCHC) will vary from farm to farm depending on parity distribution and the day relative to calving selected to measure blood Ca, therefore, many cutoffs will be attributable independently to different risk fractions of future diseases (Rodriguez *et al.*, 2017). To summarize, thresholds are indicative of SCHC when Ca levels are below 8.5 mg/dl (2.12 mmol/L) within the first 3 DIM, or below 8.8 mg/dL (2.20 mmol/L) within the first week (Martinez *et al.*, 2012; Chapinal *et al.*, 2011). If the levels of Ca are below 6 mg/dL (1.50 mmol/L), cows are at high risk to develop clinical hypocalcemia (also known as “milk fever”), defined as downer-cows in head-down recumbency with paresis of the four limbs within 24 hours after calving, responding to intravenous Ca administration (Goff, 2008; Mahjoubi et al., 2018).

Preventive programs must focus on providing an adequate supply of magnesium in the diet, as well as on reduction of diet cations (e.g. K) and increment on diet anions (e.g. chloride) to maintain an optimal pH in order to fight the metabolic challenge (i.e. dietary cation-anion difference – DCAD – based diets). The suggested values for DCAD diets is −100 to −150mEq/kg, although it have been associated with decreased DMI and metabolic alkalosis; DCAD between 0 and −30mEq/kg seems to support the cow adequately to prevent hypocalcemia onset, although in general, the DCAD must be carefully adjusted in the farm depending on their results (Bani Hassan *et al.*, 2018; Goff, 2008). Another strategy is the stimulation of PTH at the onset of the TP by reducing moderately the Ca on diet, although this strategy seems to be controversial among producers and scientists, being the efforts on the DCAD approach most imperative to focus (Goff, 2008; Bani Hassan *et al.*, 2018).

Impairment of the immune function and disease presentation

Inflammation and immunosuppression

Neutrophils perform phagocytosis, respiratory burst, and release chemical signals to enhance and amplify the immune response in cases of infectious diseases. They are involved in wound healing and separation and expulsion of placenta, and they are even involved in events like folliculogenesis, corpus luteum formation and luteolysis (angiogenic effect guarantee nutrients release to the ovarian structures) (Alhussien and Dang, 2019).

In the TP there is dysregulation of leukocyte recruitment and antibody response (especially in high milk yield cows) due to high cortisol concentrations by activation of the hypothalamic-pituitary-adrenal axis for the calving process. This reduces expression of cytokines, chemoattractants, platelets activating factors, complement, antimicrobial peptides, important markers for cell adhesion and migration to tissues (e.g. Il-4, IL-17, INFγ, TNFα, L-selectins – CD62L+) and reduce the availability of trace elements in response to colostrum and lactogenesis (e.g. vitamins A and E, Zn, Ca and P) (Meglia *et al.*, 2018; Paibomesai et al, 2018; Alhussien and Dang, 2019; Martin *et al.*, 2016; Stoop *et al.*, 2016; Bassel and Caswell, 2018). Nevertheless, the onset of a resilient acute response may develop systemic inflammation against the host that reduces the cellular immune function (**Figure 1.5**), making difficult for the cow to deal with infectious ailments like mastitis or metritis (Trevisi and Minuti, 2018). As described by Contreras and Sordillo (2011), leukocytes use fatty acids as an energy substrate. The main NEFAs used are palmitate, stearate and oleic acid. Palmitate modifies the structure of leukocytes to boost their function stimulating secretion of ROS by neutrophils, monocytes and endothelial cells, modulating leukocytes gene expression to produce more proinflammatory cytokines in a process defined as palmitoylation (Contreras and Sordillo, 2011; Agrawall *et al.*, 2017). In addition, lipid mediators can stimulate the araquidonic acid pathway of inflammation directly in the cell membrane, and the excess of ROS finish affecting the membrane cells integrity and tissues of the cow. In the presence of infections (especially by gram negative bacteria), leukocytes switch their metabolism from a beneficial oxidative phosphorylation to a potentially harmful anaerobic glycolysis (Horst *et al.*, 2018). Furthermore, a prolonged excess of NEFAs reach a counterproductive effect by lipotoxicity on tissues (necrosis) and leukocytes, affecting oxidative stress, antigen presentation, chemotaxis, diapedesis, reduction of the DNA synthesis, and reduction of cytokines and antibodies production, giving now the pass to immunosuppression (Contreras and Sordillo, 2011; LeBlanc, 2010; Ingvartsen and Moyes, 2013; 2015). Overall, the NEFA concentration may enhance or suppress the immune response depending on time of excess and amount of NEFA released into circulation, and of course, the individual capacity of the cow to deal with this excess.

Relevant health disorders during the transition period

The prevalence of certain diseases in periparturient cows has increased (e.g. clinical mastitis and lameness) or decreased (hypocalcemia, respiratory and digestive problems, RFM and DA) during the last years, although still 30 to 50% of the cows present metabolic or infectious diseases during the TP (LeBlanc, 2010: Bacigalupo, 2017). The liver is one of the main players maintaining homeostasis, it amplifies expression of fat adipocyte binding proteins (FABP) and increases synthesis of proteins (e.g. albumin) to expand oxidant capacity and metabolize fatty acids (Angeli *et al.*, 2019; Contreras and Sordillo, 2011). The liver can process a limited amount of NEFA mobilized from adipose tissue during NEB, releasing ketone bodies and very low-density lipoproteins (VLDL) as sub products **(Figure 1.3)**. If the amount of NEFA exceeds the limit of liver oxidation capacity, hepatic steatosis (i.e. fatty liver) is developed as the hepatocyte is unable to transform triacylglycerol (TG) in VLDL (Drackley, 1999; Horst *et al.*, 2018). Hepatic steatosis develops from the excessive and sustained incorporation of lipids in the form of triacylglycerol molecules (TG) within the endoplasmic reticulum, Golgi apparatus and cytoplasm of hepatocytes (Contreras and Sordillo, 2011). Steatosis cause physical damage to liver cells and induce apoptosis, albumin production is decreased and the integrity and function albumin itself (transportation protein, antioxidant capacity) are decreased by the excess of NEFA, creating a vicious loop on imbalance (Contreras and Sordillo, 2011). Beside the metabolic challenge, there are diseases that have an impact due to opportunistic infections during the TP, like mastitis.

Clinical mastitis (MAST) is a common and recurrent disease that can increase the risk of culling and mortality due to loss of milk and has the potential to become a systemic disease (toxic mastitis), which is a life-threatening illness (Jamali *et al.*, 2018). It is defined as a quarter with signs of inflammation (heat, pain, redness, or swelling) and/or changes in the appearance in the milk (e.g. flakes, clots, pus, etc.) (International Dairy Federation, 1971). MAST is more frequent in multiparous cows with high milk yield, and the recurrence and severity is strongly related with the type of pathogen involved and the management practices of the farm. The lactation stage where more cows experienced MAST is during the first 60 DIM, and is commonly associated with the performance of the cow to adapt to the challenges of the TP and specially, due to the infectious nature of this disease, the risk relies mostly on immune competence of the mammary gland (Jamali *et al.*, 2018; Hussein *et al.*, 2018; Ibrahim *et al.*, 2016). Hence, risk factors among cows presenting MAST are mostly similar to the risk factors that hint at other infectious diseases (i.e. contamination of the tissue), like uterine infectious diseases (Hossein-Zadeh and Ardalan, 2011; Perez-baez *et al.*, 2019).

The risk for uterine infectious diseases (RFM, metritis, endometritis) may be predicted within the 2 weeks around the estimated calving date by measurements in the neutrophil counts, phagocytic activity, oxidative capacity, cortisol levels and expression of TNFα, Il-1, IL-6, IL-8, IL-10 (Kim *et al.*, 2005; Shimizu *et al.*, 2018), as well as the measurements showing NEB (proper uterine involution is strongly associated with NEB), reduced DMI, or low selenium and Vitamin E diet/blood concentrations (LeBlanc, 2010; Gilbert, 2016; Braga Paiano *et al.*, 2019). Neutrophil counts in routine hemograms performed at calving also serve as a predictor to identify animals developing RFM (Moretti *et al.*, 2016). The definition of RFM is the failure of the expulsion of the placenta by 12-24 hours after the expulsion of the offspring, due to proteolytic impairment and malfunction of neutrophils and macrophages, and lack of proinflammatory mediators in the placentomes, with the subsequent lack of detachment of placenta from the maternal caruncles (Patel and Parmar, 2019; Nelli *et al.*, 2019; Gilbert, 2016). Induced parturition, short gestation, abortion, twins, dystocia, fetotomy or cesarean sections, plus metabolic disorders and immunosuppression, are risk factors for RFM (Patel and Parmar, 2019). The subsequent contamination of the uterus with potentially pathogenic bacteria (e.g. *Escherichia coli*, *Arcanobacterium pyogenes*, *Trueperalla pyogenes*) around calving and the previous presentation of RFM are major issues that increase the risk of metritis and endometritis (Wagener *et al.*, 2017; LeBlanc, 2010; Sheldon *et al.*, 2008).

Inflammation of the uterus is part of the physical involution after calving (Genis *et al.*, 2018; Cui *et al.*, 2019), but if the process steps out on control, it will become pathologic and will impair fertility (Magata *et al.*, 2016). Sheldon et al (2006, 2008) define metritis as an infection affecting all the layers of the uterus (endometrium, myometrium and perimetrium) prior to 21 DIM and characterized by an abnormally enlarged uterus; clinical endometritis is defined as an infection involving just the lining layer (endometrium) after 21 DIM. In the case of metritis, if the the discharge is watery, red-brown and fetid, and is associated with signs of systemic illness and fever, is defined as toxic or puerperal metritis; if the discharge is purulent and cows are not systemically ill, is defined as clinical metritis (Sheldon *et al.*, 2006, Sheldon *et al.*, 2008). In the case of clinical endometritis, the discharge is purulent (> 50% pus) or mucopurulent (50% pus, 50% mucus) after the 3rd and 4th weeks after calving, respectively. In the case of subclinical endometritis there is no evident identifiable vaginal discharge, but uterine cytology shows more than 18% or more than 5-10% of neutrophils at 21-33 and 34-62 DIM, respectively (Wagener *et al.*, 2017; Sheldon *et al.*, 2006). Pyometra is defined as pus in the uterus in the presence of a persistent corpus luteum and closed cervix; the definition may be complemented with history of anestrous (Sheldon *et al.*, 2006, Sheldon, 2008).

Uterine infections have a direct impact on increasing the odds of pregnancy loss, but the inflammation developed during the TP (and inflammatory diseases developed in the middle and late lactation) is associated with reduced fertilization of oocytes, development of follicles, impaired embryo implantation and growth of conceptus, regardless of where in the body the inflammatory diseases occurred (Ribeiro, 2017; Ribeiro *et al.*, 2016; 2013; Velazquez *et al.*, 2019). The detrimental effects happen on a systemic level, also affecting the outcomes from synchronization protocols (Ribeiro *et al.*, 2016). Some diseases involve anatomic and physiological issues more than the immune competence, like the ones related with the health of the rumen.

Ruminal microorganisms live in symbiosis (mutualism) with cattle: they receive nutrients from the host food and in exchange, they degrade cellulose to provide short-chain fatty acids (SCFA) as the main source of energy, proteins and vitamins (especially B complex) to be absorbed in the ruminal wall for the cow (Zebeli *et al.*, 2015). Ruminal microorganisms need a gradually introduction (weeks) to high-energy diets. If microorganisms fail to adapt to the sudden increment of rapid degradation starch (e.g. grain, silage, molasses and other sources of readily fermentable carbohydrates), will favor the growth of the amylolytic species and the reduction of the fibrolytic ones (i.e. dysbiosis), promoting the growth of lactic archaea and bacteria, developing ruminal acidosis (pH < 5.8) (Zebeli *et al.*, 2015; Snyder and Credille, 2017). In a complex progress of events, either acute ruminal acidosis or SARA advance to laminitis, rumen ulcerations, liver abscessation, and thromboembolic respiratory disease (Snyder and Credille, 2017). Overall, specially SARA will affect deeply the metabolism, general health, milk yield, welfare and fertility of the cow. Producers must guarantee a gradual energy increase in the TP while providing sufficient physically effective fiber, feed additives to reduce the risk of SARA (e.g. bicarbonate and ionophores), proper mixture of rations in order to reduce food sorting behavior of cows, and monitoring of rumination activity to warrant the alkalinizing effect of saliva (Zebeli *et al.*, 2015; Oetzel G R, 2017).

Another digestive issue related with the TP is DA. The rumen (and part of the gravid uterus) occupies the left side of the abdomen avoiding an unusual movement of the abomasum to that side, but immediately after calving, the uterus dramatically decreases in size and the DMI is reduced, conferring the space of the rumen for the abomasum to displace (Caixeta *et al.*, 2018). The mechanisms behind DA are complex; hypocalcemia (for muscular contractibility) and the reduction in the DMI will predispose to a considerable insulin resistance and reduction glucose levels, affecting abomasum motility and permitting gas accumulation. These, plus the availability of empty space, can results in DA (Duffield *et al.*, 2009; LeBlanc, 2010).

New alternatives to conventional approaches

The complex interactions between metabolism and immune function have resulted in de development of biological markers used as early as in the dry-off period to predict problems and hopefully, prevent disease in the TP (Wisnieski *et al.*, 2019). Antibiotics are still used in diseases like mastitis (Ruegg, 2018), although the inappropriate use and increasing antimicrobial resistance in animals and humans have become a topic of environmental and public health (Lhermie *et al.*, 2019; 2017). In addition, the restrictions of conventional drugs (e.g. antibiotics, NSAIDs and hormones) in organic certified dairy farms stimulate the initiative to develop new strategies to alleviate the impact of the TP (Pinedo and Velez, 2019; Pinedo *et al.*, 2015). With metritis, for example, studies have shown that cows treated with natural infusions based on plants extracts can properly recover and improve overall reproductive performance in organic certified farms (Pinedo *et al.*, 2017; Pinedo *et al.*, 2015). Thus, preventive therapy options such as immunomodulators (Tom *et al.*, 2019; Bradford, 2011; Nickerson *et al.*, 2019; Van Schyndel *et al.*, 2018; Fabris *et al.*, 2017) or the evaluation of breeding traits to boost the genetic inheritance of the immune function for next generations (Stoop *et al.*, 2016), are growing and gaining space on the market as valuable tools.

Many strategies have been utilized to reduce the impact of TP, from nonsteroidal anti-inflammatory drugs (NSAIDs) (Montgomery *et al.*, 2019) to molecular and environmental strategies. It is important to consider that the interventions cannot work alone, and that the TP is always complex and multifactorial at environmental, cow, nutritional, and molecular levels (Sundrum, 2015). Interventions will depend on the farm and owner options and preferences. Real-time monitoring of health, feeding (rumination and eating times), drinking, number of bouts (times that the animal stands up or lies down) and standing/lying activity can be done using automated livestock behavior monitoring sensors (e.g. accelerometers). For example, cows usually drink and eat less and the number of boats is greater (the cow becomes restless and anxious) close to the calving (Van Dixhoorn *et al.*, 2018; Huzzey *et al.*, 2005). These devices are also useful to detect heat or even predict the onset of certain diseases like ketosis (Itle *et al.*, 2015). Serotonin plays a role on DMI, glucose and Ca metabolism; hence, peripartal concentration of serotonin may serve as a reliable predictor of a complete set of postpartum metabolic related syndromes (Laporta *et al.*, 2013). Prepartum intravenous infusions of serotonin (1 mg/kg of BW) for at least 4 days before the estimated calving date have shown a positive effect on the prevention of hypocalcemia as serotonin induces PTHrP in the mammary gland independently of PTH concentration and stimulation (Hernández-Castellano *et al.*, 2017). Oral Ca supplementation within the first week after calving in cows has shown divided results based on parity and the outcome evaluated; for example, it may have a detrimental effect on milk yield in primiparous cows, may improve fertility in multiparous cows, but depending on the time relative to the diagnosis of metritis, it may or may not reduce the incidence of uterine infectious diseases in multiparous cows (Martinez *et al.*, 2016; Pinedo *et al.*, 2017). Genomic selection procedures intended to identify cows resistant, tolerant or resilient to environmental and/or health treats have received more attention over the last years (König and May, 2018). Robust statistical models have been developed to predict the breeding value of future generations for adaptability traits related to metabolic and environmental challenges inherent to the TP. Currently, selection decisions focus less on production traits, and more on the physiological, anatomical, immune, and reproductive expressions (König and May, 2018; Weller *et al.*, 2017).

New studies assessing novel strategies to diminish the impact of diseases during the TP on organic certified dairy cows are in process of development, and a couple of them will be discussed in this thesis. Chapter 2 describes an experiment using an immunomodulator based on *Mycobacterium* cell wall fraction (MCWF). We hypothesized that the subcutaneous administration of MCWF within the two weeks before calving and within 24 hours after calving could generate a non-specific cellular immune response capable of reducing the risk of peripartal infectious diseases in dairy cows. Therefore, our objective was to evaluate the effect of a commercial immunomodulator based on MCWF (Amplimune®, NovaVive Inc., Belleville, Canada) on presentation of peripartal diseases and reproductive performance of Holstein cows, assessing cellular immune response and metabolic status. Chapter 3 describes an experiment using a pulsed alternating wavelength system (PAWS). We hypothesized that PAWS could elicit a positive hormonal and metabolic response that might reduce presentation of dystocia, as well as the imbalances and stress around calving, improving peripartal health and subsequent performance in transition dairy cows. Hence, our objective was to evaluate the effect of PAWS on dystocia presentation, peripartal health, activity, and serum levels of melatonin (MEL), serotonin (5-HT), prolactin (PRL), somatotropin (BST), calcium, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) of organic certified Holstein cows.

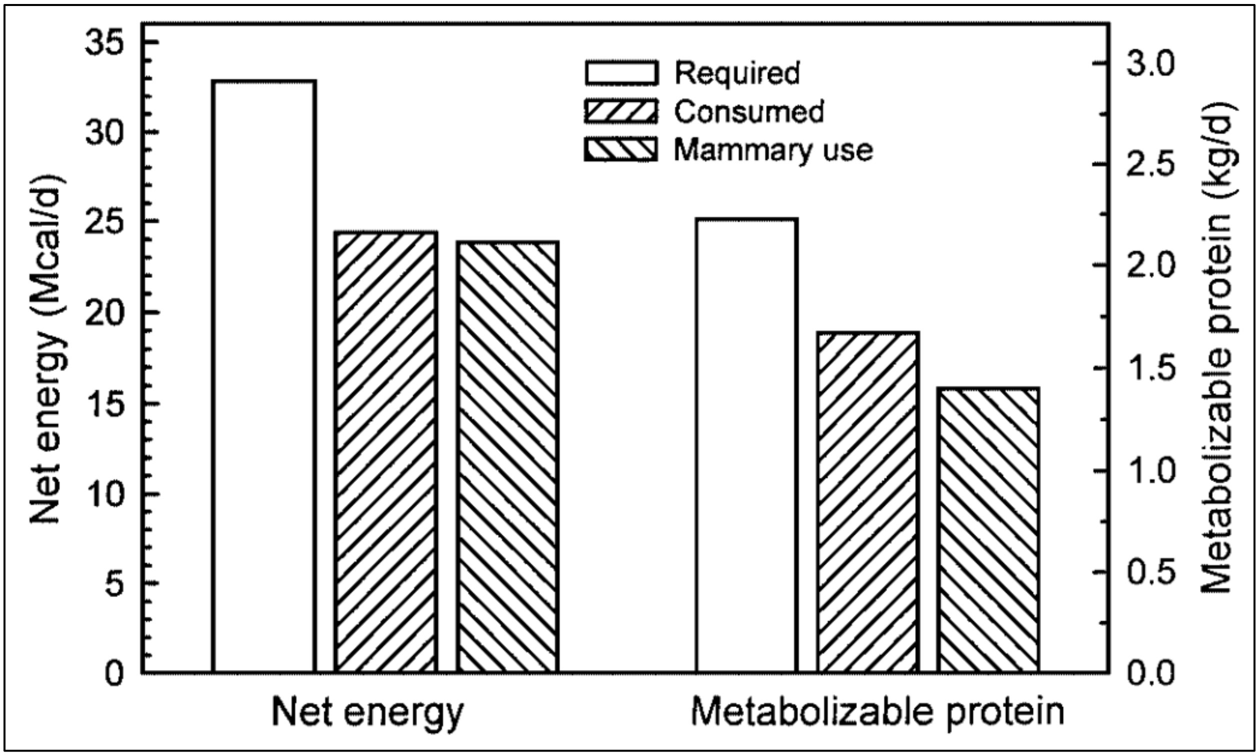

FIGURE 1.1. Feed intake and negative energy/protein balance due to lactogenesis. The energy and proteins levels required by the cow are not properly consumed, and most of the nutrients are directed to the mammary gland to support milk production (Modified from Drackley, 1999).

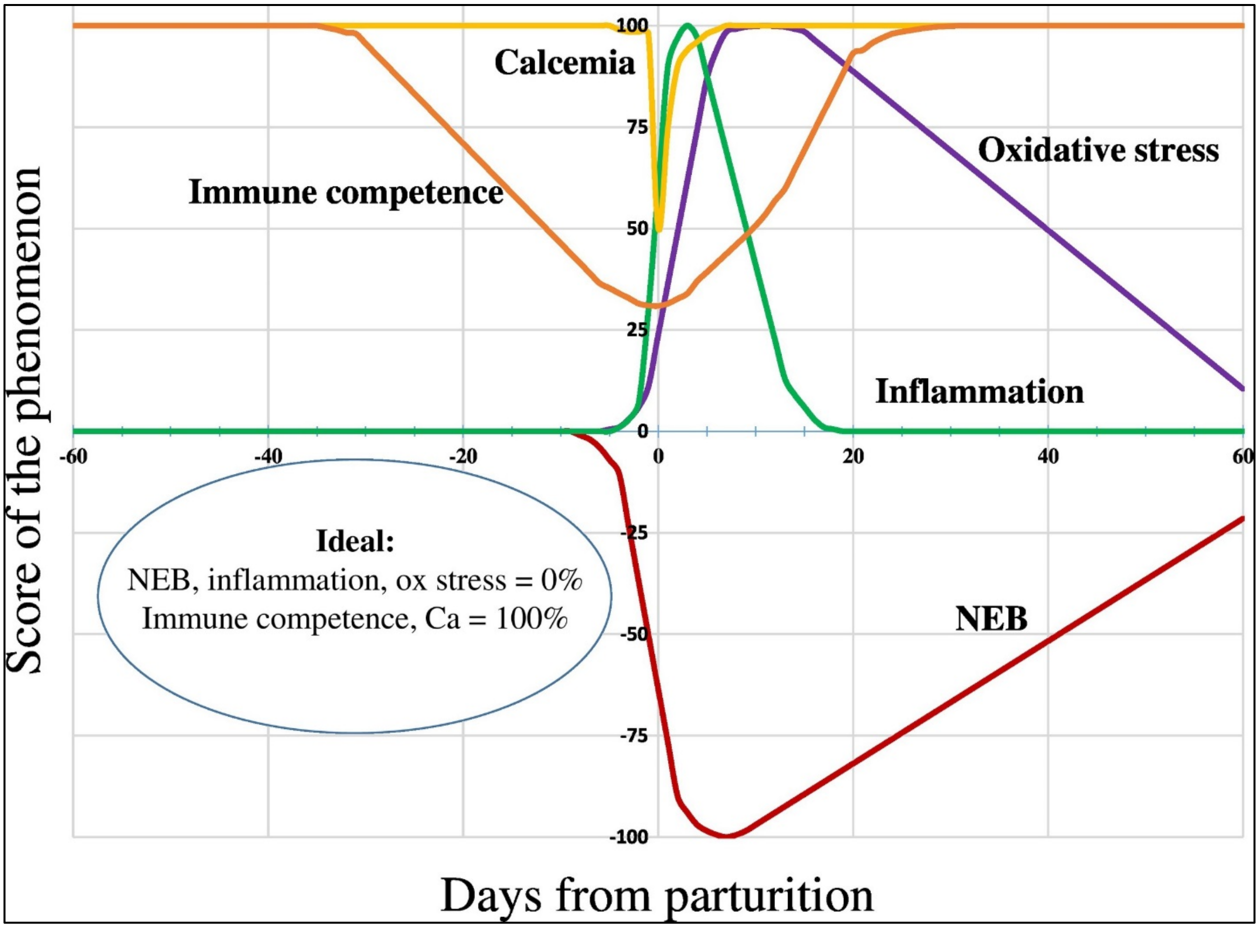

FIGURE 1.2. Relationship between immune competence, metabolic imbalances and inflammation around the transition period. As parturition get closer, the risk of immunosuppression, hypocalcemia, negative energy balance (NEB), inflammation, and oxidative stress, increase. In addition, total recovery of homeostasis after calving may take weeks (Modified from Trevisi and Minuti, 2018).

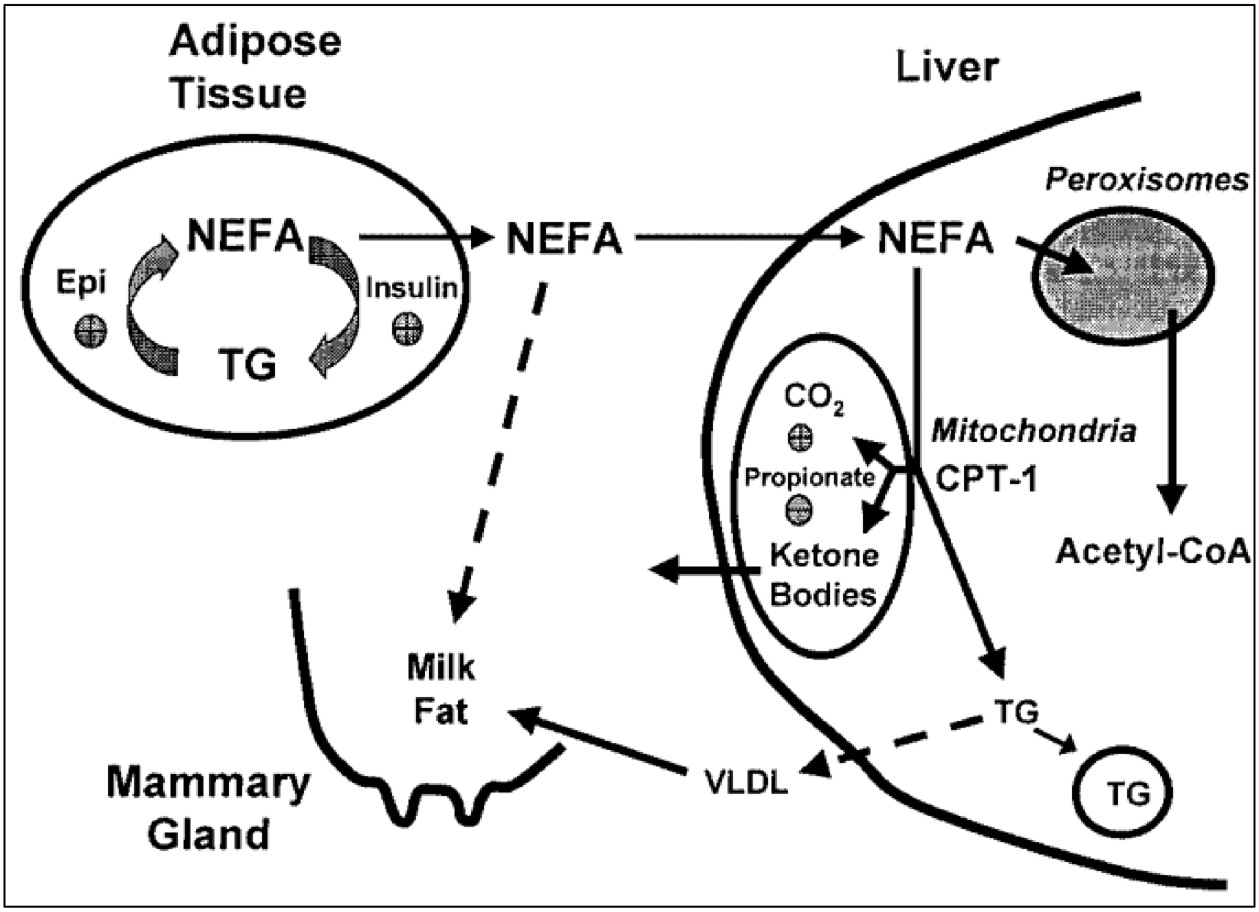

FIGURE 1.3. Synthesis of NEFA for subsequent secretion of fat in the milk and production of Acetil-CoA in the liver. Disproportionate formation of ketone bodies and accumulation of triacylglycerol (TG) in the hepatocyte appear when the amounts of NEFA exceeds the limit of fat oxidation in the liver, leading to ketosis and fatty liver presentation, respectively (Modified from Drackley, 1999).

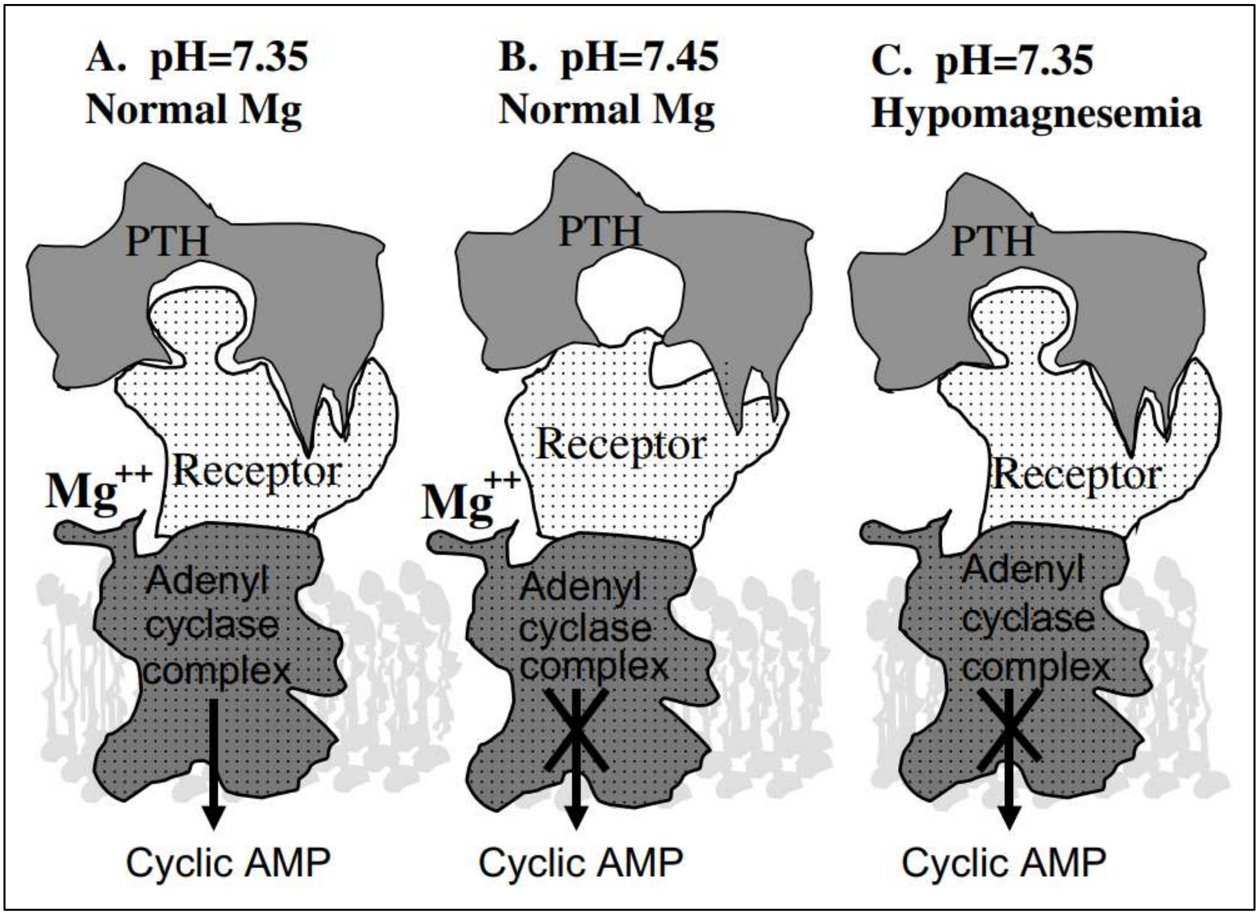

FIGURE 1.4. Effect of metabolic alkalosis and hypomagnesemia on the binding of parathyroid hormone (PTH) on its receptors. Alkalosis damage the physicochemical integrity of the receptor, and magnesium (Mg) is an important cofactor to activate the second messenger system within the target cells (Modified from Goff, 2008).

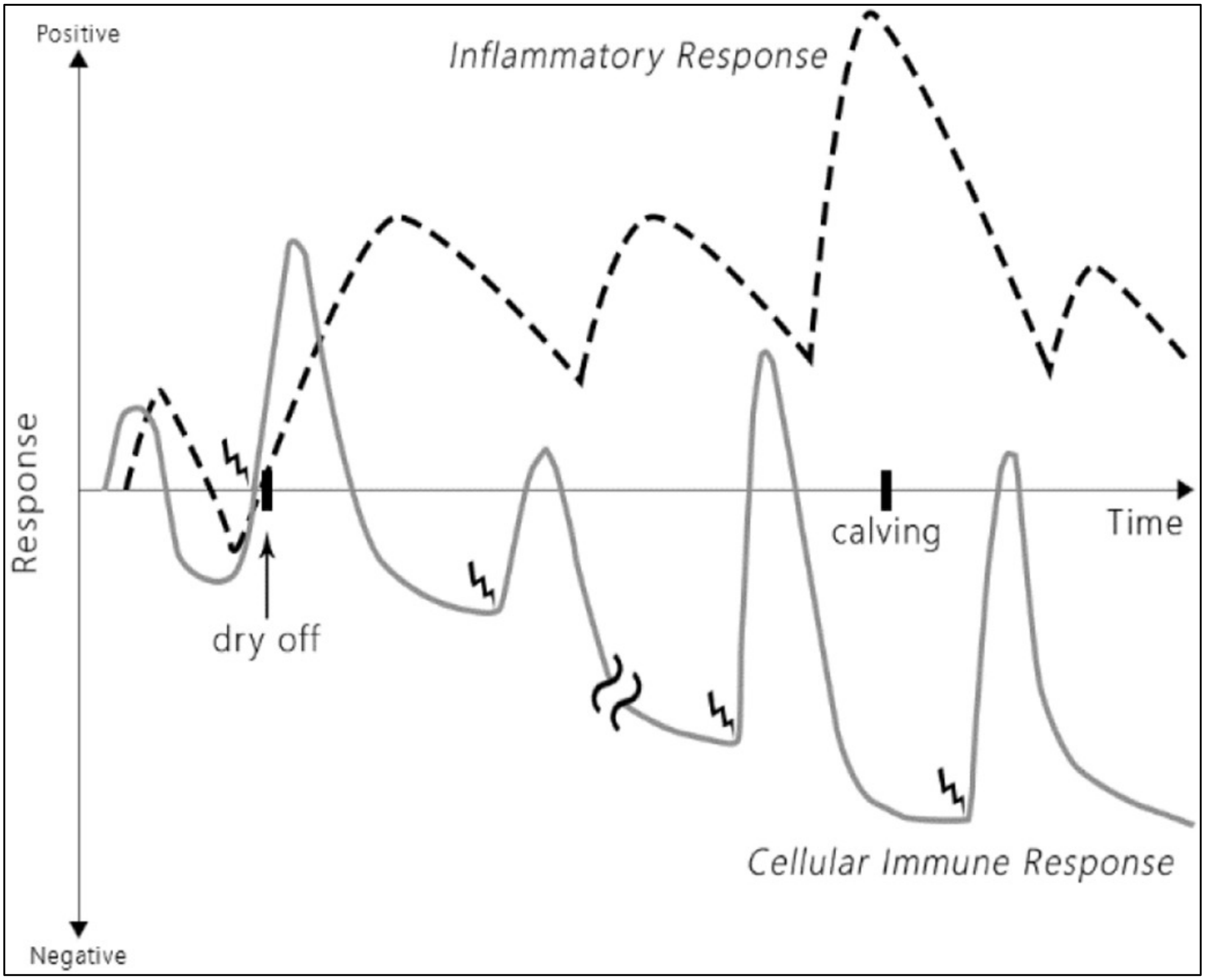

FIGURE 1.5. Negative effect of inflammation on cellular immune response. As parturition get closer, the inflammatory response increase with the production of reactive oxygen species (ROS), leading to cellular immunosuppression (Modified from Trevisi and Minuti, 2018).

REFERENCES

Agrawal, A., Khan, M. J., Graugnard, D. E., Vailati-Riboni, M., Rodriguez-Zas, S. L., Osorio, J. S., & Loor, J. J. (2017). Prepartal energy intake alters blood polymorphonuclear leukocyte transcriptome during the peripartal period in holstein cows. Bioinformatics and biology insights, 11, 1177932217704667.

Alhussien, M. N., & Dang, A. K. (2019). Potential roles of neutrophils in maintaining the health and productivity of dairy cows during various physiological and physiopathological conditions: a review. Immunologic research, 67(1), 21-38.

Angeli, E., Trionfini, V., Gareis, N. C., Matiller, V., Huber, E., Rey, F., ... & Hein, G. J. (2019). Protein and gene expression of relevant enzymes and nuclear receptor of hepatic lipid metabolism in grazing dairy cattle during the transition period. Research in veterinary science, 123, 223-231.

Bacigalupo S. P. 2017. Time lost to disease in dairy cattle: associa5tions between two consecutive lactations. Master thesis, Colorado State University, Fort Collins.

Bani Hassan, E., Nouri, M., Vogrin, S., & Pyman, M. (2018). Can neutral dietary cation–anion difference (DCAD) decrease occurrence of clinical periparturient hypocalcaemia in dairy cattle?. Australian veterinary journal, 96(7), 269-273.

Bashir, S., Ashraf, I., Burkhari, S., & Dar, A. A. (2016). Clinical ketosis in lactating dairy cows and its therapeutic management. J. Livest. Sci., 7, 72-74.

Bassel, L. L., & Caswell, J. L. (2018). Bovine neutrophils in health and disease. Cell and tissue research, 371(3), 617-637.

Bradford, B. J. (2017). IMMUNITY, INFLAMMATION, AND THE TRANSITION COW. AABP Proc 44, 48–52.

Braga Paiano, R., Becker Birgel, D., & Harry Birgel Junior, E. (2019). Uterine Involution and Reproductive Performance in Dairy Cows with Metabolic Diseases. Animals, 9(3), 93.

Caixeta, L. S., Herman, J. A., Johnson, G. W., & McArt, J. A. (2018). Herd-level monitoring and prevention of displaced abomasum in dairy cattle. Veterinary Clinics: Food Animal Practice, 34(1), 83-99.

Chapinal, N., Carson, M., Duffield, T. F., Capel, M., Godden, S., Overton, M., ... & LeBlanc, S. J. (2011). The association of serum metabolites with clinical disease during the transition period. Journal of dairy science, 94(10), 4897-4903.

Chapinal, N., LeBlanc, S. J., Carson, M. E., Leslie, K. E., Godden, S., Capel, M., ... & Duffield, T. F. (2012). Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. Journal of dairy science, 95(10), 5676-5682.

Contreras, G. A., & Sordillo, L. M. (2011). Lipid mobilization and inflammatory responses during the transition period of dairy cows. Comparative Immunology, Microbiology and Infectious Diseases, 34(3), 281-289.

Cui, L., Wang, H., Ding, Y., Li, J., & Li, J. (2019). Changes in the blood routine, biochemical indexes and the pro-inflammatory cytokine expressions of peripheral leukocytes in postpartum dairy cows with metritis. BMC veterinary research, 15(1), 157.

Dänicke, S., Meyer, U., Kersten, S., & Frahm, J. (2018). Animal models to study the impact of nutrition on the immune system of the transition cow. Research in veterinary science, 116, 15-27.

Drackley, J. K. (1999). Biology of dairy cows during the transition period: The final frontier?. Journal of dairy science, 82(11), 2259-2273.

Dubuc, J., & Denis-Robichaud, J. (2017). A dairy herd-level study of postpartum diseases and their association with reproductive performance and culling. Journal of dairy science, 100(4), 3068-3078.

Duffield, T. F., Lissemore, K. D., McBride, B. W., & Leslie, K. E. (2009). Impact of hyperketonemia in early lactation dairy cows on health and production. Journal of dairy science, 92(2), 571-580.

Esposito, G., Irons, P. C., Webb, E. C., & Chapwanya, A. (2014). Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Animal reproduction science, 144(3-4), 60-71.

Fabris, T. F., Laporta, J., Corra, F. N., Torres, Y. M., Kirk, D. J., McLean, D. J., ... & Dahl, G. E. (2017). Effect of nutritional immunomodulation and heat stress during the dry period on subsequent performance of cows. Journal of dairy science, 100(8), 6733-6742.

Fiore, E., Piccione, G., Gianesella, M., Praticò, V., Vazzana, I., Dara, S., & Morgante, M. (2015). Serum thyroid hormone evaluation during transition periods in dairy cows. Archives Animal Breeding, 58(2), 403-406.

Genís, S., Arís, A., Kaur, M., & Cerri, R. L. (2018). Effect of metritis on endometrium tissue transcriptome during puerperium in Holstein lactating cows. Theriogenology, 122, 116-123.

Gilbert, R. O. (2016). Management of reproductive disease in dairy cows. Veterinary Clinics: Food Animal Practice, 32(2), 387-410.

Goff, J. P. (2008). The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. The veterinary journal, 176(1), 50-57.

Gross, J. J., Wellnitz, O., & Bruckmaier, R. M. (2015). Cortisol secretion in response to metabolic and inflammatory challenges in dairy cows. Journal of animal science, 93(7), 3395-3401.

Hernandez, L. L., Gregerson, K. A., & Horseman, N. D. (2012). Mammary gland serotonin regulates parathyroid hormone-related protein and other bone-related signals. American Journal of Physiology-Endocrinology and Metabolism, 302(8), E1009-E1015.

Hernández-Castellano, L. E., Hernandez, L. L., Sauerwein, H., & Bruckmaier, R. M. (2017). Endocrine and metabolic changes in transition dairy cows are affected by prepartum infusions of a serotonin precursor. Journal of dairy science, 100(6), 5050-5057.

Horst, E. A., Kvidera, S. K., Al-Qaisi, M., Abeyta, M. A., Rhoads, R. P., & Baumgard, L. H. (2018). Energy cost of inflammation in dairy cows. In 27th Annual Tri-State Dairy Nutrition Conference, 16-18 April 2018, Fort Wayne, Indiana, USA (pp. 65-79). Ohio State University.

Hossein-Zadeh, N. G., & Ardalan, M. (2011). Cow-specific risk factors for retained placenta, metritis and clinical mastitis in Holstein cows. Veterinary research communications, 35(6), 345-354.

Hussein, H. A., El, K. A. E. H. A., Razik, A. M. G., Elbayoumy, M. K., Abdelrahman, K. A., & Hosein, H. I. (2018). Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle. Veterinary world, 11(1), 34.

Huzzey, J. M., Von Keyserlingk, M. A. G., & Weary, D. M. (2005). Changes in feeding, drinking, and standing behavior of dairy cows during the transition period. Journal of Dairy Science, 88(7), 2454-2461.

Ibrahim, H. M. M., El-seedy, Y. Y., & Gomaa, N. A. (2016). Cytokine response and oxidative stress status in dairy cows with acute clinical mastitis. J Dairy Vet Anim Res, 3(1), 00064.

Ingvartsen, K. L., & Moyes, K. M. (2015). Factors contributing to immunosuppression in the dairy cow during the periparturient period. Japanese Journal of Veterinary Research, 63(Supplement 1), S15-S24.

Ingvartsen, K. L., & Moyes, K. (2013). Nutrition, immune function and health of dairy cattle. Animal, 7(s1), 112-122.

International Dairy Federation 1971. A monograph of bovine mastitis. Annual bulletin – International Dairy federation, 1971, pt 2. Secrétariat general, Bruxelles, Belgium.

Itle, A. J., Huzzey, J. M., Weary, D. M., & Von Keyserlingk, M. A. G. (2015). Clinical ketosis and standing behavior in transition cows. Journal of dairy science, 98(1), 128-134.

Jamali, H., Barkema, H. W., Jacques, M., Lavallée-Bourget, E. M., Malouin, F., Saini, V., ... & Dufour, S. (2018). Invited review: Incidence, risk factors, and effects of clinical mastitis recurrence in dairy cows. Journal of dairy science, 101(6), 4729-4746.

KIM, I. H., NA, K. J., & YANG, M. P. (2005). Immune responses during the peripartum period in dairy cows with postpartum endometritis. Journal of Reproduction and Development, 0510050026-0510050026.

König, S., & May, K. (2019). Invited review: Phenotyping strategies and quantitative-genetic background of resistance, tolerance and resilience associated traits in dairy cattle. Animal, 13(5), 897-908.

Lacasse, P., Vanacker, N., Ollier, S., & Ster, C. (2018). Innovative dairy cow management to improve resistance to metabolic and infectious diseases during the transition period. Research in veterinary science, 116, 40-46.

Lange, J., Ganesh, S., Meier, S., Kay, J. K., Crookenden, M. A., Walker, C. G. ... & Heiser, A. (2018). Far-off and close-up feeding levels affect immunological performance in grazing dairy cows during the transition period. Journal of animal science, 97(1), 192-207.

Laporta, J., Moore, S. A. E., Peters, M. W., Peters, T. L., & Hernandez, L. L. (2013). Circulating serotonin (5-HT) concentrations on day 1 of lactation as a potential predictor of transition-related disorders. Journal of dairy science, 96(8), 5146-5150.

LeBlanc, S. (2010). Monitoring metabolic health of dairy cattle in the transition period. Journal of reproduction and Development, 56(S), S29-S35.

Lhermie, G., Wernlii, D., Jørgensen, P. S., Kenkel, D., Tauer, L. W., & Gröhn, Y. T. (2019). Global resistance to antimicrobials and their sustainable use in agriculture. The Lancet Planetary Health, 3(3), e109-e110.

Lhermie, G., Gröhn, Y. T., & Raboisson, D. (2017). Addressing antimicrobial resistance: an overview of priority actions to prevent suboptimal antimicrobial use in food-animal production. Frontiers in microbiology, 7, 2114.

Magata, F., Kitaoka, R., Morino, I., Teramura, M., Kawashima, C., Haneda, S., & Shimizu, T. (2016). Long-term impact of puerperal metritis on the profiles of peripheral blood leukocytes in peripartum dairy cows. Animal Science Journal, 87(1), 151-155.

Mahjoubi, E., Zahmatkesh, D., Hossein-Yazdi, M., Khabbazan, M. H., & Samadian, M. R. (2018). Test of the relationship between prepartum milk leakage and blood calcium concentration in Holstein cows—An observational study. Journal of dairy science, 101(4), 3579-3582.

Marett, L. C., Auldist, M. J., Wales, W. J., Macmillan, K. L., Dunshea, F. R., & Leury, B. J. (2019). Responses to metabolic challenges in dairy cows with high or low milk yield during an extended lactation. Journal of dairy science, 102(5), 4590-4605.

Martin, C. E., Paibomesai, M. A., Emam, S. M., Gallienne, J., Hine, B. C., Thompson-Crispi, K. A., & Mallard, B. A. (2016). Cytokine profiles from blood mononuclear cells of dairy cows classified with divergent immune response phenotypes. Journal of dairy science, 99(3), 2364-2371.

Martinez, N., Risco, C. A., Lima, F. S., Bisinotto, R. S., Greco, L. F., Ribeiro, E. S., ... & Santos, J. E. P. (2012). Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. Journal of dairy science, 95(12), 7158-7172.

Martinez, N., Sinedino, L. D. P., Bisinotto, R. S., Daetz, R., Risco, C. A., Galvão, K. N., ... & Santos, J. E. P. (2016). Effects of oral calcium supplementation on productive and reproductive performance in Holstein cows. Journal of dairy science, 99(10), 8417-8430.

Meglia, G. E., Johannisson, A., Petersson, L., & Waller, K. P. (2018). Investigation in blood Leukocytes and Neutrophils in Periparturient Dairy Cow. Scientific Journal of Animal and Veterinary Science, 1(1), 6.

Miqueo, E., Chiarle, A., Giuliodori, M. J., & Relling, A. E. (2019). Association between prepartum metabolic status and resumption of postpartum ovulation in dairy cows. Domestic Animal Endocrinology.

Montgomery, S. R., Mamedova, L. K., Zachut, M., Kra, G., Häussler, S., Vaughn, M. ... & Bradford, B. J. (2019). Effects of sodium salicylate on glucose kinetics and insulin signaling in postpartum dairy cows. Journal of dairy science, 102(2), 1617-1629.

Moretti, P., Probo, M., Cantoni, A., Paltrinieri, S., & Giordano, A. (2016). Fluctuation of neutrophil counts around parturition in Holstein dairy cows with and without retained placenta. Research in veterinary science, 107, 207-212.

Nelli, R. K., De Koster, J., Roberts, J. N., de Souza, J., Lock, A. L., Raphael, W., ... & Contreras, G. A. (2019). Impact of uterine macrophage phenotype on placental retention in dairy cows. Theriogenology, 127, 145-152.

Neves, R. C., Leno, B. M., Bach, K. D., & McArt, J. A. A. (2018). Epidemiology of subclinical hypocalcemia in early-lactation Holstein dairy cows: the temporal associations of plasma calcium concentration in the first 4 days in milk with disease and milk production. Journal of dairy science, 101(10), 9321-9331.

Nickerson, S. C., Kautz, F. M., Ely, L. O., Rowson, A. D., Hurley, D. J., Chapman, J. D., & McLean, D. J. (2019). Effects of an immunomodulatory feed additive on intramammary infection prevalence and somatic cell counts in a dairy herd experiencing major health issues. Research in veterinary science, 124, 186-190.

Oetzel, G. R. (2017). Diagnosis and management of subacute ruminal acidosis in dairy herds. Veterinary Clinics: Food Animal Practice, 33(3), 463-480.

Ospina, P. A., Nydam, D. V., Stokol, T., & Overton, T. R. (2010). Association between the proportion of sampled transition cows with increased nonesterified fatty acids and β-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. Journal of Dairy Science, 93(8), 3595-3601.

Ospina, P. A., Nydam, D. V., Stokol, T., & Overton, T. R. (2010). Associations of elevated nonesterified fatty acids and β-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. Journal of dairy science, 93(4), 1596-1603.

Ospina, P. A., Nydam, D. V., Stokol, T., & Overton, T. R. (2010). Evaluation of nonesterified fatty acids and β-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. Journal of dairy science, 93(2), 546-554.

Paibomesai, M. A., Sharif, S., Karrow, N., & Mallard, B. A. (2018). Type I and type II cytokine production of CD4+ T-cells in immune response biased dairy cattle around calving. Veterinary immunology and immunopathology, 199, 70-76.

Patel, R. V., & Parmar, S. C. (2016). Retention of fetal membranes and its clinical perspective in bovines. Sch J Agric Vet Sci, 3(2), 111-116.

Pérez-Báez, J., Risco, C. A., Chebel, R. C., Gomes, G. C., Greco, L. F., Tao, S., ... & Staples, C. R. (2019). Association of dry matter intake and energy balance prepartum and postpartum with health disorders postpartum: Part I. Calving disorders and metritis. Journal of dairy science, 102(10), 9138-9150.

Pérez-Báez, J., Risco, C. A., Chebel, R. C., Gomes, G. C., Greco, L. F., Tao, S., ... & Staples, C. R. (2019). Association of dry matter intake and energy balance prepartum and postpartum with health disorders postpartum: Part II. Ketosis and clinical mastitis. Journal of dairy science, 102(10), 9151-9164.

Pinedo, P. J., Velez, J. S., Bothe, H., Merchan, D., Piñeiro, J. M., & Risco, C. A. (2015). Effect of intrauterine infusion of an organic-certified product on uterine health, survival, and fertility of dairy cows with toxic puerperal metritis. Journal of dairy science, 98(5), 3120-3132.

Pinedo, P., Velez, J., Solano, G., Rodriguez, N., Naves, J., Schuenemann, G. M., & Risco, C. (2017). Effect of oral calcium administration on the cure and reproductive performance of Holstein cows diagnosed with puerperal metritis. Journal of dairy science, 100(4), 2917-2927.

Pinedo, P. J., & Velez, J. (2019). Invited Review: Unique reproductive challenges for certified organic dairy herds. Applied Animal Science, 35(4), 416-425.

Reddy, P. R. K., Raju, J. K., Reddy, A. N., Reddy, P. P. R., & Hyder, I. (2016). Transition period and its successful management in dairy cows. Indian J. Nat. Sci, 7(38), 11691-11699.

Ribeiro, E. S., Lima, F. S., Greco, L. F., Bisinotto, R. S., Monteiro, A. P. A., Favoreto, M., ... & Santos, J. E. P. (2013). Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. Journal of Dairy Science, 96(9), 5682-5697.

Ribeiro, E. S., Gomes, G., Greco, L. F., Cerri, R. L. A., Vieira-Neto, A., Monteiro Jr, P. L. J., ... & Santos, J. E. P. (2016). Carryover effect of postpartum inflammatory diseases on developmental biology and fertility in lactating dairy cows. Journal of dairy science, 99(3), 2201-2220.

Ribeiro E. S. (2017). Factors Affecting Preimplantation Embryonic Development in Dairy Cows. WCDS Advances in Dairy Technology (29), 185-197
.

Roberts, T., Chapinal, N., LeBlanc, S. J., Kelton, D. F., Dubuc, J., & Duffield, T. F. (2012). Metabolic parameters in transition cows as indicators for early-lactation culling risk. Journal of dairy science, 95(6), 3057-3063.

Roche, J. R., Burke, C. R., Crookenden, M. A., Heiser, A., Loor, J. L., Meier, S., ... & Turner, S. A. (2018). Fertility and the transition dairy cow. Reproduction, Fertility and Development, 30(1), 85-100.

Rodríguez, E. M., Arís, A., & Bach, A. (2017). Associations between subclinical hypocalcemia and postparturient diseases in dairy cows. Journal of dairy science, 100(9), 7427-7434.

Ruegg, P. L. (2018). Making antibiotic treatment decisions for clinical mastitis. Veterinary Clinics: Food Animal Practice, 34(3), 413-425.

Sheldon, I. M., Lewis, G. S., LeBlanc, S., & Gilbert, R. O. (2006). Defining postpartum uterine disease in cattle. Theriogenology, 65(8), 1516-1530.

Sheldon, I. M., Williams, E. J., Miller, A. N., Nash, D. M., & Herath, S. (2008). Uterine diseases in cattle after parturition. The Veterinary Journal, 176(1), 115-121.

Shimizu, T., Morino, I., Kitaoka, R., Miyamoto, A., Kawashima, C., Haneda, S., & Magata, F. (2018). Changes of leukocyte counts and expression of pro-and anti-inflammatory cytokines in peripheral leukocytes in periparturient dairy cows with retained fetal membranes. Animal Science Journal, 89(9), 1371-1378.

Silva, P. R. B., Soares, H. F., Braz, W. D., Bombardelli, G. D., Clapper, J. A., Keisler, D. H., & Chebel, R. C. (2017). Effects of treatment of periparturient dairy cows with recombinant bovine somatotropin on health and productive and reproductive parameters. Journal of dairy science, 100(4), 3126-3142.

Silva, P. R. B., Weber, W. J., Crooker, B. A., Collier, R. J., Thatcher, W. W., & Chebel, R. C. (2017). Hepatic mRNA expression for genes related to somatotropic axis, glucose and lipid metabolisms, and inflammatory response of periparturient dairy cows treated with recombinant bovine somatotropin. Journal of dairy science, 100(5), 3983-3999.

Singh, D. N. (2019). Managemental Strategies of Farm Animals During Transition Period. Call for Editorial Board Members, 5(1), 31.

Snyder, E., & Credille, B. (2017). Diagnosis and treatment of clinical rumen acidosis. Veterinary Clinics: Food Animal Practice, 33(3), 451-461.

Steinhoff, L., Jung, K., Meyerholz, M. M., Heidekorn-Dettmer, J., Hoedemaker, M., & Schmicke, M. (2019). Thyroid hormone profiles and TSH evaluation during early pregnancy and the transition period in dairy cows. Theriogenology, 129, 23-28.

Stoop, C. L., Thompson-Crispi, K. A., Cartwright, S. L., & Mallard, B. A. (2016). Variation in production parameters among Canadian Holstein cows classified as high, average, and low immune responders. Journal of dairy science, 99(6), 4870-4874.

Sundrum, A. (2015). Metabolic disorders in the transition period indicate that the dairy cows' ability to adapt is overstressed. Animals, 5(4), 978-1020.

Tom, J. K., Albin, T. J., Manna, S., Moser, B. A., Steinhardt, R. C., & Esser-Kahn, A. P. (2018). Applications of immunomodulatory immune synergies to adjuvant discovery and vaccine development. Trends in biotechnology.

Trevisi E., and Minuti A. "Assessment of the innate immune response in the periparturient cow." Research in veterinary science 116 (2018): 47-54.

Van Schyndel, S. J., Carrier, J., Pascottini, O. B., & LeBlanc, S. J. (2018). The effect of pegbovigrastim on circulating neutrophil count in dairy cattle: A randomized controlled trial. PloS one, 13(6), e0198701.

van Dixhoorn, I. D. E., de Mol, R. M., van der Werf, J. T. N., van Mourik, S., & van Reenen, C. G. (2018). Indicators of resilience during the transition period in dairy cows: A case study. Journal of dairy science, 101(11), 10271-10282.

Vieira-Neto, A., Gilbert, R. O., Butler, W. R., Santos, J. E. P., Ribeiro, E. S., Vercouteren, M. M., ... & Galvão, K. N. (2014). Individual and combined effects of anovulation and cytological endometritis on the reproductive performance of dairy cows. Journal of dairy science, 97(9), 5415-5425.

Velázquez, M. M. L., Peralta, M. B., Angeli, E., Stassi, A. F., Gareis, N. C., Durante, L., ... & Ortega, H. H. (2019). Immune status during postpartum, peri-implantation and early pregnancy in cattle: An updated view. Animal reproduction science.

Wagener, K., Gabler, C., & Drillich, M. (2017). A review of the ongoing discussion about definition, diagnosis and pathomechanism of subclinical endometritis in dairy cows. Theriogenology, 94, 21-30.

Walsh, R. B., Kelton, D. F., Duffield, T. F., Leslie, K. E., Walton, J. S., & LeBlanc, S. J. (2007). Prevalence and risk factors for postpartum anovulatory condition in dairy cows. Journal of dairy science, 90(1), 315-324.

Wankhade, P. R., Manimaran, A., Kumaresan, A., Jeyakumar, S., Ramesha, K. P., Sejian, V., ... & Varghese, M. R. (2017). Metabolic and immunological changes in transition dairy cows: A review. Veterinary world, 10(11), 1367.

Weller, J. I., Ezra, E., & Ron, M. (2017). Invited review: a perspective on the future of genomic selection in dairy cattle. Journal of dairy science, 100(11), 8633-8644.

Wisnieski, L., Norby, B., Pierce, S. J., Becker, T., Gandy, J. C., & Sordillo, L. M. (2019). Predictive models for early lactation diseases in transition dairy cattle at dry-off. Preventive veterinary medicine, 163, 68-78.

Zebeli, Q., Ghareeb, K., Humer, E., Metzler-Zebeli, B. U., & Besenfelder, U. (2015). Nutrition, rumen health and inflammation in the transition period and their role on overall health and fertility in dairy cows. Research in veterinary science, 103, 126-136.

CHAPTER 2: EFFECT OF MYCOBACTERIUM CELL WALL FRACTION IMMUNOMODULATOR ON PERIPARTAL HEALTH AND REPRODUCTIVE PERFORMANCE IN TRANSITION DAIRY COWS

Summary

The transition period is a challenging time for dairy cows, characterized by the occurrence of metabolic imbalances and infectious diseases due to immune suppression. Our objective was to evaluate the effect of a commercial immunomodulator based on *Mycobacterium* cell wall fraction (MCWF; Amplimune®, NovaVive Inc., Belleville, Canada) on disease presentation and reproductive performance of Holstein cows. Cellular immune response, metabolic status, general health, and production were assessed up to 150 days in milk. A total of 136 Holstein cows were grouped by parity and randomly assigned to a treatment group (MCWF; n = 65) receiving 5 mL of Amplimune subcutaneously at enrollment (2 to 12 days before calving) and at calving (1 ± 1 days in milk), or a control group (CON; n = 71) receiving 5 mL of saline solution subcutaneously at the same time points. Blood samples for the immune and metabolic assessment were collected at enrollment, at 2 days post enrollment, at calving, and at d 3, 7, and 14 after calving. The frequency (%) of clinical metritis in MCWF cows was 24 (36.9) vs. 36 (50.7) in CON cows for all cows (P = 0.038), and 17 (34) vs. 28 (52.8) for multiparous cows (P = 0.025). The frequency (%) of clinical mastitis before 28 DIM in MCWF cows was 4 (6.3) vs. 14 (19.7) in CON cows (P = 0.014) for all cows, and for clinical mastitis before 60 DIM was 0 (0) vs. 5 (27.8) for primiparous cows (P = 0.043). The frequency (%) of pyometra in MCWF cows was 0 (0) vs. 5 (9.4) in CON cows for multiparous cows (P = 0.035). The frequency (%) of respiratory disease in MCWF cows was 5 (7.7) vs. 0 (0) in CON cows for all cows (P = 0.023). The frequency (%) of cows inseminated and cows pregnant at first insemination in MCWF cows was 44 (97.8) vs. 46 (86.8) in CON cows and 16 (35.6) vs. 10 (18.9) for multiparous cows (P = 0.044 and 0.033, respectively). The frequency (%) of cows pregnant before 100 DIM and by 150 DIM in MCWF cows was 30 (50.9) and 40 (67.8) vs. 23 (32.4) and 36 (50.7) in CON cows for all cows (P = 0.015 and 0.021, respectively); these effects were especially significant in multiparous cows (P = 0.043 and 0.008, respectively). The median number of days (CI) to pregnancy in MCWF cows was 96 (83-139) vs. 150 (110-150) in CON cows for all cows (P = 0.025); this effect was especially significant in multiparous cows (P = 0.005). There was not significant (P > 0.05) effect of MCWF on the presentation of other diseases, white blood cell counts or metabolic status.

In conclusion, the presentation of clinical metritis, clinical mastitis, and pyometra in MCWF cows were significantly lower compared to CON cows, although the presentation of respiratory disease before 28 days in milk was significantly higher compared to CON cows. The overall reproductive performance was significantly improved in MCWF cows compared to CON cows. Future research at different physiological stages and using different doses and routes of administration is encouraged.

Introduction

The transition period represents a challenging time in the life cycle of dairy cows. Reduced dry matter intake resulting in negative energy balance (NEB), inflammation, and immunosuppression are typical conditions of this stage that favor the presentation of metabolic and infectious disorders (Esposito *et al*, 2014). Consequently, diseases such as mastitis and metritis are some of the most common clinical conditions reported by producers in this period and infertility, strongly related to metabolic status and uterine health, represents the most frequent cause of culling and economic losses (NAHMS, USDA, 2014; Braga Paiano *et al*, 2019).

Most cows affected by infectious diseases receive antimicrobial therapy, and cephalosporins are the main antimicrobial type selected (NAHMS, USDA, 2014; Haimerl *et al*, 2016). The constant use of antibiotics increases the risk to antimicrobial resistance in animals and humans (Lhermie *et al*, 2019; 2016), becoming an environmental and public health concern (Spielmeyer *et al*, 2018). On the other hand, restrictions on the use of these treatment options in organic certified dairy farms encourage the exploration of new strategies for the treatment and prevention of infectious diseases in dairy systems (NOP, 2015). Natural alternatives to antibiotics (Pinedo *et al*, 2017; 2015), feed additives (Nickerson *et al*, 2019), and biomolecular technologies (Van Schyndel *et al*, 2018) have been emerging over the last few decades, and immunomodulators based on *Mycobacterium* cell wall fraction (MCWF) are part of these new strategies.

Mycobacterium species can incite strong humoral and cellular immune responses in the host. They have been reported to have anticancer effect in humans and animals (Subramanian *et al*, 2016; Filion *et al*, 2017), and research in dairy calves shows a reduction in the incidence and severity of diarrhea and pneumonia (Romanowski *et al*, 2017; Nosky *et al*, 2017). In addition, repeated doses of MCWF in lactating dairy cows are associated with the reduction of clinical signs related to persistent *Mycoplasma bovis* infection (Masic *et al*, 2017).

We hypothesize that the subcutaneous administration of MCWF within the two weeks before calving and within 24 hours after calving could generate a non-specific immune response capable of reducing the risk of peripartal infectious diseases in dairy cows. Therefore, our objective was to evaluate the effect of a commercial immunomodulator based on MCWF (Amplimune®, NovaVive Inc., Belleville, Canada) on presentation of peripartal diseases, reproductive performance, and milk yield of Holstein cows, assessing cellular immune response, metabolic status, general health, and milk yield.

Materials and methods

All the procedures conducted on this research were approved on 06/06/2018 by the Institutional Animal Care and Use Committee (IACUC) at Colorado State University (Fort Collins, CO) in the protocol 18-7874A.

Study population

This study was conducted in a dairy under certified organic management located in northern Colorado, USA. Holstein cows were enrolled between August 2018 and December 2018 at the maternity facility. Cows within 21 d of the expected due date were housed in a pen with wood shavings-bedding and free access to an adjacent dry lot. Fresh cows were moved to a milking facility and housed in sand-bedding free-stalls and free access to an adjacent a dry lot. Milking cows received a total mixed ration (TMR) twice a day according to the National Research Council recommendations for transition and lactating cows (NRC, 2001) and had *ad libitum* access to clean water and shade. After calving, all cows were milked twice a day in a 60-stalls rotary parlor with an average milk yield of 30 kg/cow/day. Colored chalk was used for estrous detection and no hormones for synchronization were administered, following the organic certification prohibitions. In the absence of chalk, heat was confirmed by the presence of clear mucus from the vagina and artificial insemination (AI) was performed. Pregnancy diagnosis was performed by transrectal ultrasound examination at 40 days after AI. Non-pregnant cows with more than 5 AI or more than 180 DIM were moved to a pen with a clean-up bull.

Experimental design and treatment allocation

A total of 136 Holstein cows at an organic certified dairy farm in northern Colorado were grouped by parity with a goal of approximately 35% primiparous and 65% multiparous cows, based on the farm population distribution. Between August and December of 2018, cows were randomly assigned into 1 of 2 treatment groups: (1) *Mycobacterium* cell wall fraction (MCWF, n = 65), 1.25 mg (5 ml) subcutaneous (Amplimune®; NovaVive Inc., Belleville, Canada) at enrollment (7 ± 5 d before calving) and at calving (1 ± 1 DIM); or (2) control group (CON, n = 71), receiving 5 mL of 0.9% sterile saline solution subcutaneous at the same time points.

Blood samples were collected from the coccygeal vessels of the tail at enrollment, at 2 d post enrollment, at calving, and at d 2, 7, and 14 after calving for immune and metabolic assessment. Trained personnel and farm veterinarians performed daily general health inspections, including clinical mastitis diagnosis at any time within 28 DIM in the milking parlor. In addition, a milk sample was collected at 14 ± 3 DIM for somatic cell count (SCC). One of the authors (GSS) performed the metritis diagnosis at 7 and 14 DIM (and at 21 in cows diagnosed with metritis at 14 DIM). Clinical endometritis was assessed at 28 DIM and cows were body condition scored (Ferguson *et al*, 1994) at calving, and at d 7, d 14 and d 28 after calving. After 28 DIM, the cows stopped sharing the same pen are were sent to different pens along the farm.

Overall health disorders and reproductive performance were assessed up to 150 DIM. Farm workers, veterinarians, and the author performing metritis and clinical endometritis assessment were blinded to the treatment assignment of the study cows.

Case definitions

A stillbirth was defined as a calf that was either born dead or died within the first 24 hours after birth, and a case of retention of fetal membranes was a cow unable to expel the placenta within 24 hours after the expulsion of the offspring. Farm-trained workers diagnosed clinical ketosis (KET) by putting a drop of blood on acetoacetic acid reagent strips (Ketostix; Bayer Corporation, Elkhart, IN); cows with ketosis were treated with 500 ml i.v. of dextrose 5% during 3 to 5 days according with the farm health protocol. Cows with clinical hypocalcemia (CHC) were identified as a downer-cow (in head-down recumbency with paresis of the four limbs) within 24 hours after parturition responding to intravenous calcium administration (Mahjoubi *et al*, 2018).

Diagnosis of uterine infectious disease (UID) was done by transrectal massage of the uterus or using a Metricheck device (Metricheck, SimcroTech, Hamilton, New Zealand). Puerperal or toxic metritis (PMET) was defined as an abnormally enlarged uterus and a fetid, watery, reddish/brownish uterine discharge, associated with signs of systemic illness (e.g. reduced milk yield and appetite, dullness) and fever (≥ 39.5˚C) within 21 days after calving. Clinical metritis (CMET) was defined as purulent uterine discharge within 21 days after parturition, in the absence of systemic illness and fever. Clinical endometritis (ENDO) at 28 DIM was defined as vaginal discharge with a content of pus greater than the content of mucus (more than 50% pus) noticeable in the vagina (Sheldon *et al*, 2006). According to the farm health protocol, all cows diagnosed with PMET received an intrauterine (i.u.) infusion of Optimum UterFlush (Van Beek Natural, Science, Orange City, IA), administered every other day for 3 times, plus intravenous (i.v.) hypertonic saline solution, i.v. dextrose 5%, and oral aspirin, and cows diagnosed with ENDO received 500 ml i.u. of dextrose 5% (Pinedo *et al*, 2017; 2015). Cows with pyometra were diagnosed by the farm veterinarian by transrectal uterine ultrasonography, and the disorder was defined as mixed echo density fluid (pus) in the presence of a persistent corpus luteum and history of anestrous, usually after around 40 DIM (Sheldon *et al*, 2006). Due to restrictions in the use of hormones in the organic certified systems, no therapeutic actions were taken in pyometra cases.

Clinical mastitis (MAST) was defined as a mammary quarter with signs of inflammation (heat, pain, redness, or swelling) and/or changes in the appearance in the milk (e.g. flakes, clots, pus, etc.) (International Dairy Federation. 1971). According to the farm health protocol, deep stripping (hand milking) until the disappearance of the symptoms was the treatment approach for cows with MAST. If the milk appearance was watery, transparent/yellowish, and occasionally fetid smell, in the presence of inflammation of the quarter and clinical signs of systemic toxemia (e.g. fever, depression, inappetence), the mastitis was defined as toxic mastitis.

Veterinarians of the farm diagnosed displacement of abomasum by auscultation of a metallic ‘ping’ sound in the upper left flank side of the abdomen, parallel with history of reduced consumption, reduced milk yield and lack of rumen motility (Caixeta et al., 2018). Trained farm workers diagnosed ruminal acidosis mainly by the presence of profuse watery diarrhea and parallel history of reduced consumption, reduced milk yield, and poor body condition score (Oetzel, 2017). Trained workers and veterinarians diagnosed respiratory disease by the auscultation of lungs sounds and the presence of cough, polypnea and nasal discharge.

Immune and metabolic assessment

A subsample of 34 cows (MCWF = 16, CON = 18) was randomly selected for WBC counts analyses, and a subsample of 42 cows (MCWF = 21, CON = 21) was randomly selected to assess the peripartal metabolic status (serum calcium, non-esterified fatty acids (NEFA), and β-hydroxybutyrate (BHB). Blood samples were collected in the morning (0600 to 0800 h) at enrollment (7 ± 5 days before calving), 2 days after enrollment, calving (1 ± 1 DIM), 2 days after calving, and at 7 and 14 DIM using 10 ml EDTA BD and serum Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood for hemograms was homogenized and stored in a transportation cooler filled with ice packs, and processed within 12 hours of collection for WBC in the Clinical Pathology Lab of the veterinary Teaching Hospital at Colorado State University (Fort Collins, CO). Samples for metabolic status were stored in a transportation cooler filled with ice packs, and the serum was separated within 12 hours of collection (centrifuged at

3,500 rpm for 15 minutes at room temperature) and stored frozen at -20°C from 1 to 7 months until the moment of metabolites determination.

Cut-off values for serum NEFA concentrations to determine negative energy balance were 0.27 mmol/L at calving, or 0.57 mmol/L at 7 DIM. Cows with serum BHB > 0.96 mmol/L at calving or at 7 DIM were considered as hyperketonemic (Ospina *et al*, 2010). Cut-off values for subclinical hypocalcemia were Ca < 2.12 mmol/L at calving (Martinez *et al*, 2012; Goff, 2008) and < 2.20 mmol/L at 7 DIM (Chapinal *et al*, 2011).

Data management and statistical analyses

The sample size was calculated to detect a difference of 20 percentage points in the proportion of pregnant cows by 150 DIM in favor of MCWF group. Based on the farm data, the anticipated proportion of pregnant cows in the CON group was 25%. Considering power = 80% and confidence = 95%, the number of cows required to show a significant difference between the 2 treatment groups was estimated in 70 cows per group (PROC POWER, SAS 9.4).

Randomization of cows was performed using Microsoft® Office Excel (Excel RAND function) based on a list from the farm with the cows expected to calve within 2 weeks at the time of enrollment. Due to lack of normality, white blood cell counts (10^3 cells/µL) reported by the lab were Log10-transformed and a repeated measures anova was performed. Then, the Log-10 LSM and confidence intervals were back transformed in an Excel sheet (=POWER(10,value)) to obtain again the cell counts (10^3 cells/µL) of leukocytes (**13.** Van Schyndel, 2017). A similar process was performed with the values for serum NEFA, BHB, calcium, and SCC in milk, as none of these parameters had a normal distribution. Binary variables, such as the presentation of peripartal diseases, were analyzed using Chi square and Fisher's exact test (PROC FREQ, SAS 9.4) and logistic regression was performed in variables with frequencies ≥ 1 (PROC GLIMMIX, SAS 9.4).

Variables with repeated measures, such as the WBC counts or metabolic status assessment, were analyzed using repeated measures anova analysis (PROC MIXED, SAS 9.4) and one-way ANOVA followed by a Tukey test (PROC GLM, SAS 9.4) and least square mean analyses (LSMEANS statement, SAS).

Fertility parameters (e.g. time to first AI or time to pregnancy), culling, and survival assessment were evaluated with survival curves analyses (PROC LIFETEST, SAS 9.4), and a Cox proportional regression model was used to evaluate the effect of multiple variables on the risk of these variables (PROC PHREG, SAS 9.4).

Results

Descriptive statistics

Overall, 136 Holstein cows were enrolled: 103 multiparous (MCWF = 50, CON = 53) and 33 primiparous (MCWF = 15, CON = 18) for a total of 65 MCWF cows and 71 CON cows. A total of 18 cows left the herd during the follow up period (3 died, 15 culled) with 3 and 4 cows not reaching the assessment at 14 DIM and at 28 DIM, respectively. The main reasons for culling and death were puerperal metritis and digestive disorders complicated with respiratory disease (n = 9). Other causes for culling were toxic mastitis (n = 4), lameness (n = 3), low production (n = 1) and reproductive related issues (n = 1).

The average ± SD lactation number were 2.66 ± 1.34 and 2.48 ± 1.32 for MCWF and CON, respectively. The average ± SD number of days from enrollment to calving were 7.02 ± 4.37 and 7.80 ± 5.56 for MCWF and CON, respectively. The average ± SD BCS at 1, 7, 14 and 28 DIM were 2.85 ± 0.21, 2.70 ± 0.19, 2.62 ± 0.17, 2.49 ± 0.20 and 2.85 ± 0.22, 2.71 ± 0.18, 2.59 ± 0.17, 2.48 ± 0.20 for MCWF and CON, respectively. None of these descriptive variables was significantly different between the groups.

Disease presentation and fertility

Table 2.1. shows the frequencies of disease for all the cows and by parity. The frequency (%) of clinical metritis in MCWF cows was 24 (36.9) vs. 36 (50.7) in CON cows for all cows (P = 0.038), and 17 (34) vs. 28 (52.8) for multiparous cows (P = 0.025). The frequency (%) of pyometra in MCWF cows was 0 (0) vs. 5 (9.4) in CON cows for multiparous cows (P = 0.035). There was not significant effect of MCWF on the presentation of other uterine diseases.

The frequency (%) of clinical mastitis before 28 DIM in MCWF cows was 4 (6.3) vs. 14 (19.7) in CON cows (P = 0.014) for all cows, and for clinical mastitis before 60 DIM was 0 (0) vs. 5 (27.8) for primiparous cows (P = 0.043). For all cows, the odds (95% confidence interval) of presenting with clinical mastitis before 30 days in milk for cows receiving MCWF were 0.27 (0.08 – 0.87) times the odds of CON cows (P = 0.029). There was not significant effect of MCWF on the presentation clinical mastitis from 28 to 60 DIM, 60 to 100 DIM, and 100 to 150 DIM, or toxic mastitis at any point from calving to 150 DIM.. The SCC (log10-transformed values) measured at 14 ± 3 DIM showed no significant differences between treatment groups (LSM (SE) MCWF: 221×10^3 cells (1.30); CON: 195×10^3cells (1.25); P = 0.708). Similarly, the proportions of cows above the 200,000 cell/µL was not different between treatment groups (MCWF = 21 cows; CON = 26 cows; OR (95% CI) = 1.08 (0.49 – 2.35); P = 0.853). However, the SCC assessment was performed in the pen of the fresh cows, which was inhabited just with clinically healthy cows. As the cows diagnosed with clinical mastitis were moved immediately to the hospital, no SCC assessment was performed on mastitic cows.

The frequency (%) of respiratory disease in MCWF cows was 5 (7.7) vs. 0 (0) in CON cows for all cows (P = 0.023), although there was not significant effect of MCWF on the presentation of respiratory disease from 28 to 150 DIM. There was statistical tendencies for

MCWF to increase the presentation of stillbirth calves (P = 0.107), and to increase or reduce the presentation of lameness depending on the time of diagnosis and parity (Table 2.1). There was not significant effect of MCWF on the presentation of other diseases (Table 2.1).

Table 2.2. shows the frequencies of cows inseminated and cows diagnosed pregnant, for all the cows and by parity. The frequency (%) of multiparous cows inseminated and cows pregnant at first artificial insemination (AI) in MCWF cows was 44 (97.8) vs. 46 (86.8) in CON cows and 16 (35.6) vs. 10 (18.9) (P = 0.044 and 0.033, respectively). The frequency (%) of cows pregnant before 100 DIM and by 150 DIM in MCWF cows was 30 (50.9) and 40 (67.8) vs. 23 (32.4) and 36 (50.7) in CON cows for all cows (P = 0.015 and 0.021, respectively). The frequency (%) of cows pregnant before 100 DIM and by 150 DIM in MCWF cows was 23 (51.1) and 30 (66.7) vs. 13 (24.5) and 22 (41.5) in CON cows for multiparous cows (P = 0.043 and 0.008, respectively). The odds of conception before 100 DIM for all cows and for multiparous cows were 2.17 (1.06 – 4.35) and 3.23 (1.37 – 7.69) times greater for MCWF cows than for CON cows, respectively (P = 0.034 and 0.008). The odds of pregnancy by 150 DIM in all cows and in multiparous cows were 2.04 (1.00 – 4.17) and 2.78 (1.23 – 6.25) times greater for MCWF cows than for CON cows, respectively (P = 0.050 and 0.014). Figure 2.1 shows the survival curves for time to pregnancy, for all cows and by parity. The median number of days from calving to pregnancy (open days) were 96 (83 - 139) for MCWF cows and 150 (110 - 150) for CON cows, for all cows (P = 0.025). This effect was especially significant in multiparous cows (Figure 2.1.). The hazard ratio for a cow to get pregnant was 1.605 (P = 0.040) for MCWF vs. CON. There was not difference in the mean (SE) number of AI per pregnancy (2.15 (0.21) and 1.78 (0.14) for MCWF and CON, respectively (P = 0.148)).

Regarding survival, MCWF cows had a tendency to leave the farm (culled/dead) earlier than CON cows (P = 0.078). Any group reached the median for the number of days to culling/death, but the LSM (SE) were 68.9 (14.4) for MCWF cows and 102.5 (20.4) for CON cows (P = 0.198).

Cellular immune response and metabolic status

Treatment did not have a significant effect on any of the WBC counts at enrollment, 2 days after enrollment, calving, 2 days after calving, and at 7 and 14 DIM (Figure 2.2). Treatment did not have a significant effect on any of the metabolites analyzed for metabolic status assessment at calving or at 7 DIM (Tables 2.3). Analyzing the metabolic status using the cut-off values, no cows presented elevated BHB at calving, and no significant differences were found when comparing the proportions of cows above the thresholds for BHB at 7 DIM (MCWF: 2 (4.76%), CON: 2 (4.76%); P = 0.999). There was not significant effect of MCWF for NEFA at calving (MCWF: 18 (42.9%), CON: 20 (47.6%); P = 0.316) or at 7 DIM (MCWF: 11 (26.2%), CON: 11 (26.2%); P = 0.999), and there was not significant difference for subclinical hypocalcemia at calving (MCWF: 7 (16.67%), CON: 8 (19.05%); P = 0.748) or at 7 DIM (MCWF: 4 (9.52%), CON: 3 (7.14%); P = 0.680). Proportion of cows with elevated fatty acids at both times (calving and 7 DIM), or hypocalcemia at both times were not different between groups (P = 0.757 and 0.636, respectively).

Discussion

The objective of the present study was to evaluate the effect of a commercial immunomodulator using MCWF (Amplimune®, NovaVive Inc., Belleville, Canada) on cellular immune response, presentation of peripartal diseases, metabolic status, reproductive performance and milk yield of Holstein cows. To our knowledge, this is the first research conducted with MCWF in transition dairy cows. All the study cows shared the same pen during the first 28 DIM, and then they were moved to other pens in the farm, changing the environmental and management conditions. This factor set the decision of analyzing disease presentation before and after 28 DIM.

Similar studies in neonatal calves showed that a single dose of MCWF (250 µg i.v.) significantly reduced the severity of clinical signs and mortality associated to enterotoxigenic *Escherichia coli* (ETEC, strain K99+) induced diarrhea (Romanowski *et al*, 2017). Beside the reduction of general morbidity, MCWF also improved the average daily gain on feedlot calves, displaying a positive impact on production parameters (Nosky *et al*, 2017). In adult cows, a case-report stated that MCWF reduced clinical signs associated with persistent *Mycoplasma bovis* infection (i.e. pneumonia, otitis, arthritis and mastitis); despite the absence of a control, the author supported the effect of MCWF on the reduction of these signs after observing the evolution of these cows during four years with repeated doses approximately every month, (Masic *et al*, 2017). These investigations highlights the effect to immunomodulatory compounds in the *Mycobacterium* species cell wall, such as muramyl dipeptide (MDP) and trehalose 6,6'-dimycolate (TDM). Recent studies with mice immunized with MDP have shown evidence of IgG, IgA, and cytokines increase in serum (IL-2, IL-4, IL-6 and IFN-γ), a significant increase in serum subpopulations of lymphocytes T (CD3+, CD4+ and CD8+) and dendritic cells (Zhou *et al*, 2018; Christiana *et al*, 2016). The activation of macrophages by TDM (as happens in granulomas formation), is well known and has been demonstrated *in vitro* and *in vivo*. The component is one of the most abundant mycolates (lipoglycans) on the surface of *Mycobacteria*, and it is responsible of the host vasculature remodeling in the lungs and excessive inflammation as in the case of tuberculosis (Walton *et al*, 2018; Liu *et al*, 2018). Cases of respiratory disease appeared in MCWF cows that previously were diagnosed with toxic metritis (n = 2), clinical ketosis (n = 1), acidosis (n = 1), and/or displacement of abomasum (n = 1); hence, it is probable that these comorbidities made the cows more susceptible to develop respiratory signs due to an excessive reaction to the TDM of the MCWF. There was a tendency for MCWF toward an increased number (%) of stillbirth calves compared to CON (3 (4.6) vs. 0 (0); P = 0.107), and toward an increase/reduction in the presentation of lameness (depending on the time of diagnosis and parity). However, more research is necessary to elucidate the effect of MCWF on these conditions, as there is not an apparent direct effect of MCWF on the death of a calf or the incidence of lameness, and it is possible that the effect on these tendencies was due to random effects.

The isolation of *Mycobacterium* fractions, such as MDP and TDM has been suggested for therapeutic utilization in products based on MCWF (Liu *et al*, 2018; Huber *et al*, 2016). It is plausible to speculate that MCWF may improve the leukocytes cell adhesion to pathogens, antigen presentation, phagocytic capacity, and antimicrobial activity, stimulating the humoral and cellular branches of the immune system before the appearance of an infectious disease. These individual or combined effects may explain the reduction in presentation of clinical mastitis before 28 DIM in cows receiving MCWF (OR = 0.27 (0.08 – 0.87) relative to CON cows; P = 0.029).

In our study, MCWF had also a significant impact on general fertility, particularly in multiparous cows by improving the proportion of pregnancy before 100 DIM and by 150 DIM, and by reducing the number of days from calving to conception (open days). There was a significant effect for MCWF to reduce the risk of clinical metritis and pyometra, especially in multiparous cows, which could have contributed with a better reproductive performance. Studies with MCWF in mares with post-breeding endometritis showed a reduction of cytotoxic factors that could threaten the uterus, favoring a better cleansing of uterine fluids and providing a uterine environment similar to that of normal mares resistant to endometritis (Woodward *et al*, 2013; Christoffersen *et al*, 2012, Fumuso *et al*, 2007, 2003; Rogan *et al*, 2017).

According to the studies performed in calves and lactating cows (Romanowski *et al*, 2017; Nosky *et al*, 2017, Masic *et al*, 2017), the main effect of MCWF is to stimulate the immune response; however, anti-inflammatory interleukins (e.g. IL-10) have been show to increase after MCWF administration in mares (Fumuso *et al*, 2007). In the case of the cows in our experiment, it is inferable that MWCF was able to improve the uterine environment, refining the endometrium for future pregnancy development and better reproductive performance. However, it is difficult and problematic to infer conclusions from studies performed in horses; hence, uterine cytological analyses are encouraged for future studies to assess in more detail this hypothesis of MCWF in dairy cows.

Leukocytes, either PMN or mononuclear cells, are produced and completely matured in the bone marrow, although lymphocytes require an extra development and proliferation in lymphoid tissues. Eosinophils and basophils are principally released in the presence of allergens (Jones and Allison, 2007). Lymphocytes produce antibodies (B type), regulate the immune system, are cytotoxic (T type), and to secrete different types of cytokines (IFNγ, IL-4, IL-17A) around calving (Jones and Allison, 2007, Paibomesai *et al*, 2018). Monocytes migrate to tissues to become macrophages and phagocyte microorganisms, particles or cell debris, and are an important cell line after calving alongside with neutrophils (Jones and Allison, 2007; Meglia *et al*, 2018). Neutrophils are the first line that migrate to tissues affected by foreign material or an infection; within 2 hours after the onset of the stimulus, they are ready on site undertaking chemotaxis, phagocytosis, and cellular adhesion, presenting antigens, and secreting cytokines (IL-1β, IL-4, IL-8 and INFγ) (Jones and Allison, 2018; Meglia *et al*, 2018; Alhussien *et al*, 2019). It has been described the possible role of neutrophils on ovarian function in dairy cows (folliculogenesis, corpus luteum formation and regression), fertilization and embryo implantation (Alhussien *et al*, 2019), and in transition cows, due to the metabolic, physiological and immunological challenge, neutrophils defends the cow against opportunistic pathogens, play a major role separating fetal membranes after calving, and maintain the host immune tolerance against uterine and mammary infections (Alhussien *et al*, 2019).

Neutropenia around calving appears to be a strong indicator of future ailments in dairy cows, thus, neutrophils recruitment by the uterus and subsequent activation around calving is desirable (Moretti *et al*, 2016; Shimizu *et al*, 2018). Research with a granulocyte-colony stimulating factor evaluated the circulating neutrophil count in dairy cows 24 hours after the administration of the product at enrollment and at calving, showing a marked and sustained rise of neutrophil counts for more than 2 weeks (Van Schyndel *et al*, 2018). However, this study did not report health performance. Another study with the same immunostimulant found a detrimental effect on clinical health of periparturient cows despite the WBC stimulation; the explanation provided by the authors relies on the low incidence of disease and the reduction of power analysis due to low subsample size (Zinicola *et al*, 2018). Nightingale *et al* (2016) mentioned that cows in the early postpartum with an exceeded acute phase response (associated with excess of haptoglobin) may have cellular immunosuppression, observed by the reduction of neutrophils count, lymphocytes reduced function and cytokines secretion. These deleterious effects were also associated with a subsequent poor reproductive performance (Nightingale *et al*, 2015). Therefore, cellular immune suppression around calving may be one of the reason of why MCWF could not have a significant effect on white blood cell (WBC) counts. Another possible explanation of why MCWF did not showed a significant effect on WBC might be that the effect happened previously of after the time of measurement (Christoffersen *et al*, 2012). In addition, the low presentation and the dispersion in the number of cases for the diseases between 28 and 150 DIM forced to count them all in a single period, affecting the power to analyze the effect of MCWF on disease presentation.

The studies on granulocyte-colony stimulating factor provided ideas on how to address our research regarding the blood sampling times for WBC counts examination (Van Schyndel *et al*, 2018); however, the effect of MCWF seems to be supported by potential cytokine interactions as suggested in studies with mares. It is encouraged for future studies to evaluate the humoral response along with the cellular assessment, cytokines such as IL-1β, IL-2, Il-4, IL-6, IL-8, Il-10, IL-12, TNFα, and IFNγ, as well as measurement of serum amyloid (SAA) and haptoglobin (HP) (Christoffersen *et al*, 2012; Fumuso *et al*, 2007, 2003; Rogan *et al*, 2017; Jones and Allison, 2007; Meglia *et al*, 2018; Alhussien *et al*, 2019; Nightingale *et al*, 2015).

The innate immunity goes through many adaptations especially around calving, leading to impairments in the general immune function. However, the metabolic status and the nutritional strategy have a big influence on relieving the cow from these alterations and the subsequent inflammatory responses, improving also lately the milk production (Esposito *et al*, 2013; Mezzetti *et al*, 2017). Immunomodulatory feed additives (mainly vitamins and micronutrients) given since the dry period to the first month of lactation improves the general metabolic status of the cow and stimulates PMN and interleukins mRNA expression, hence, improving the general health and logically the milk yield (Nickerson *et al*, 2019; Mezzetti *et al*, 2017). However, there are no implications to suggest a direct effect of MCWF on metabolic status or milk yield, its effect is presumably on the humoral immune response without any implicit nutritional interaction.

Conclusions

The administration of MCWF at the doses and times considered in this study may reduce presentation of clinical metritis, clinical mastitis within the two months after calving, and pyometra, and may improve the reproductive performance by 100 and 150 DIM. However, MCWF may potentially increase the presentation of respiratory diseases within the first month after calving. Future researches with the addition of uterine cytology, and cytokines and acute phase proteins assessment, are encouraged.

TABLE 2.1: Frequencies of disease presentation comparing *Mycobacterium* cell wall fraction immunomodulator (MCWF) vs. control (CON) group. Data for all cows and blocked by parity (primiparous = first lactation; multiparous ≥ 2 lactations) is shown.

Disease	Parity	Treatment		Fisher's Exact Test
		MCWF, n (%)	CON, n (%)	
Stillbirth	All cows	3/65 (4.6)	0 /71 (0)	0.107
	Multiparous	2/50 (4.0)	0/53 (0)	0.233
	Primiparous	1/15 (6.7)	0/18 (0)	0.455
Retention of fetal membranes	All cows	4/65 (6.2)	2/71 (2.8)	0.214
	Multiparous	4/50 (8.0)	1/53 (1.9)	0.139
	Primiparous	0/15 (0)	1/18 (5.6)	0.546
Clinical hypocalcemia	All cows	0/65 (0)	1/71 (1.4)	0.522
	Multiparous	0/50 (0)	1/53 (1.8)	0.515
	Primiparous	0/15 (0)	0/18 (0)	.
Clinical ketosis	All cows	7/65 (10.8)	7/71 (9.9)	0.218
	Multiparous	6/50 (12.0)	6/53 (11.3)	0.239
	Primiparous	1/15 (6.7)	1/18 (5.6)	0.511
Puerperal (toxic) Metritis	All cows	7/65 (10.8)	8/71 (11.3)	0.214
	Multiparous	6/50 (12.0)	3/53 (5.7)	0.148
	Primiparous	1/15 (6.7)	5/18 (27.8)	0.116
Clinical metritis	All cows	24/65 (36.9)	36/71 (50.7)	0.038
	Multiparous	17/50 (34.0)	28/53 (52.8)	0.025
	Primiparous	7/15 (46.7)	8/18 (44.4)	0.272
Clinical endometritis	All cows	18/61 (29.5)	23/71 (32.4)	0.141
	Multiparous	10/47 (21.3)	14/53 (26.4)	0.156
	Primiparous	8/14 (57.1)	9/18 (50.0)	0.258
Clinical mastitis, 0 to 28 DIM	All cows	4/64 (6.3)	14/71 (19.7)	0.014
	Multiparous	4/49 (8.2)	10/53 (18.9)	0.070
	Primiparous	0/15 (0)	4/18 (22.2)	0.075
Clinical mastitis, 0 to 60 DIM	All cows	7/59 (11.9)	16/71 (22.5)	0.054
	Multiparous	7/45 (15.6)	11/53 (20.8)	0.168
	Primiparous	0/14 (0)	5/18 (27.8)	0.043
Clinical mastitis, 28 to 60 DIM	All cows	3/59 (5.1)	5/71 (7.0)	0.261
	Multiparous	3/45 (6.7)	4/53 (7.6)	0.300
	Primiparous	0/14 (0)	1/18 (5.6)	0.563
Clinical mastitis, 60 to 100 DIM	All cows	7/59 (11.9)	6/71 (8.5)	0.187
	Multiparous	5/45 (11.1)	5/53 (9.4)	0.250
	Primiparous	2/14 (14.3)	1/18 (5.6)	0.330
Clinical mastitis, 100 to 150 DIM	All cows	7/59 (11.9)	5/70 (7.1)	0.158
	Multiparous	4/45 (8.9)	3/52 (5.8)	0.256
	Primiparous	3/14 (21.4)	2/18 (11.1)	0.277

TABLE 2.1: Continuation.

Disease	Parity (P)	Treatment (T)		Fisher's Exact Test
		MCWF, n (%)	CON, n (%)	
Pyometra	All cows	2/64 (3.1)	6/71 (8.5)	0.134
	Multiparous	0/49 (0)	5/53 (9.4)	0.035
	Primiparous	2/15 (13.3)	1/18 (5.6)	0.346
Toxic mastitis	All cows	1/65 (1.5)	3/71 (4.2)	0.273
	Multiparous	1/50 (2.00)	3/53 (5.7)	0.265
	Primiparous	0/15 (0)	0/18 (0)	.
General digestive disorders	All cows	6/65 (9.2)	5/71 (7.0)	0.221
	Multiparous	5/50 (10.0)	5/53 (9.4)	0.258
	Primiparous	1/15 (6.7)	0/18 (0)	0.455
Displacement of abomasum	All cows	2/65 (3.1)	1/71 (1.4)	0.360
	Multiparous	2/50 (4.0)	1/53 (1.9)	0.367
	Primiparous	0/15 (0)	0/18 (0)	.
Ruminal acidosis	All cows	2/65 (3.1)	1/71 (1.4)	0.360
	Multiparous	1/50 (2.0)	1/53 (1.9)	0.505
	Primiparous	1/15 (6.7)	0/18 (0)	0.455
Respiratory disease	All cows	9/65 (13.85)	4/71 (5.6)	0.064
	Multiparous	7/50 (14.0)	3/53 (5.7)	0.099
	Primiparous	2/15 (13.3)	1/18 (5.6)	0.346
Respiratory before 28 DIM	All cows	5/65 (7.7)	0/71 (0)	0.023
	Multiparous	4/50 (8.0)	0/53 (0)	0.052
	Primiparous	1/15 (6.7)	0/18 (0)	0.455
Respiratory after 28 DIM	All cows	4/61 (6.6)	4/71 (5.6)	0.275
	Multiparous	3/47 (6.4)	3/53 (5.7)	0.319
	Primiparous	1/14 (7.1)	1/18 (5.6)	0.508
Lameness	All cows	7/65 (10.8)	12/71 (16.9)	0.118
	Multiparous	5/50 (5.0)	11/53 (27.6)	0.072
	Primiparous	2/15 (13.3)	1/18 (5.6)	0.346
Lameness before 28 DIM	All cows	3/65 (4.6)	0/71 (0)	0.107
	Multiparous	3/50 (6.0)	0/53 (0)	0.111
	Primiparous	0/15 (0)	0/18 (0)	.
Lameness after 28 DIM	All cows	7/65 (10.8)	12/71 (16.9)	0.118
	Multiparous	5/50 (10.0)	11/53 (20.8)	0.072
	Primiparous	2/15 (13.3)	1/18 (5.7)	0.346

TABLE 2.2: Frequencies of reproductive events comparing *Mycobacterium* cell wall fraction immunomodulator (MCWF) vs. control (CON) group. Data for all cows and blocked by parity (primiparous: first lactation; multiparous ≥ 2 lactations) is shown.

Condition	Parity	Treatment		Fisher's Exact Test
		MCWF, n (%)	CON, n (%)	
Cows inseminated	All cows	55/59 (93.2)	63/71 (88.7)	0.168
	Multiparous	44/45 (97.8)	46/53 (86.8)	0.044
	Primiparous	11/14 (78.6)	17/18 (94.4)	0.182
Pregnancy at first AI	All cows	19/59 (32.2)	16/71 (22.5)	0.074
	Multiparous	16/45 (35.6)	10/53 (18.9)	0.033
	Primiparous	3/14 (21.4)	6/18 (33.3)	0.241
Pregnancy before 100 DIM	All cows	30/59 (50.9)	23/71 (32.4)	0.015
	Multiparous	23/45 (51.1)	13/53 (24.5)	0.043
	Primiparous	7/14 (50.0)	10/18 (55.6)	0.266
Pregnancy by 150 DIM	All cows	40/59 (67.8)	36/71 (50.7)	0.021
	Multiparous	30/45 (66.7)	22/53 (41.5)	0.008
	Primiparous	10/14 (71.4)	14/18 (77.8)	0.291

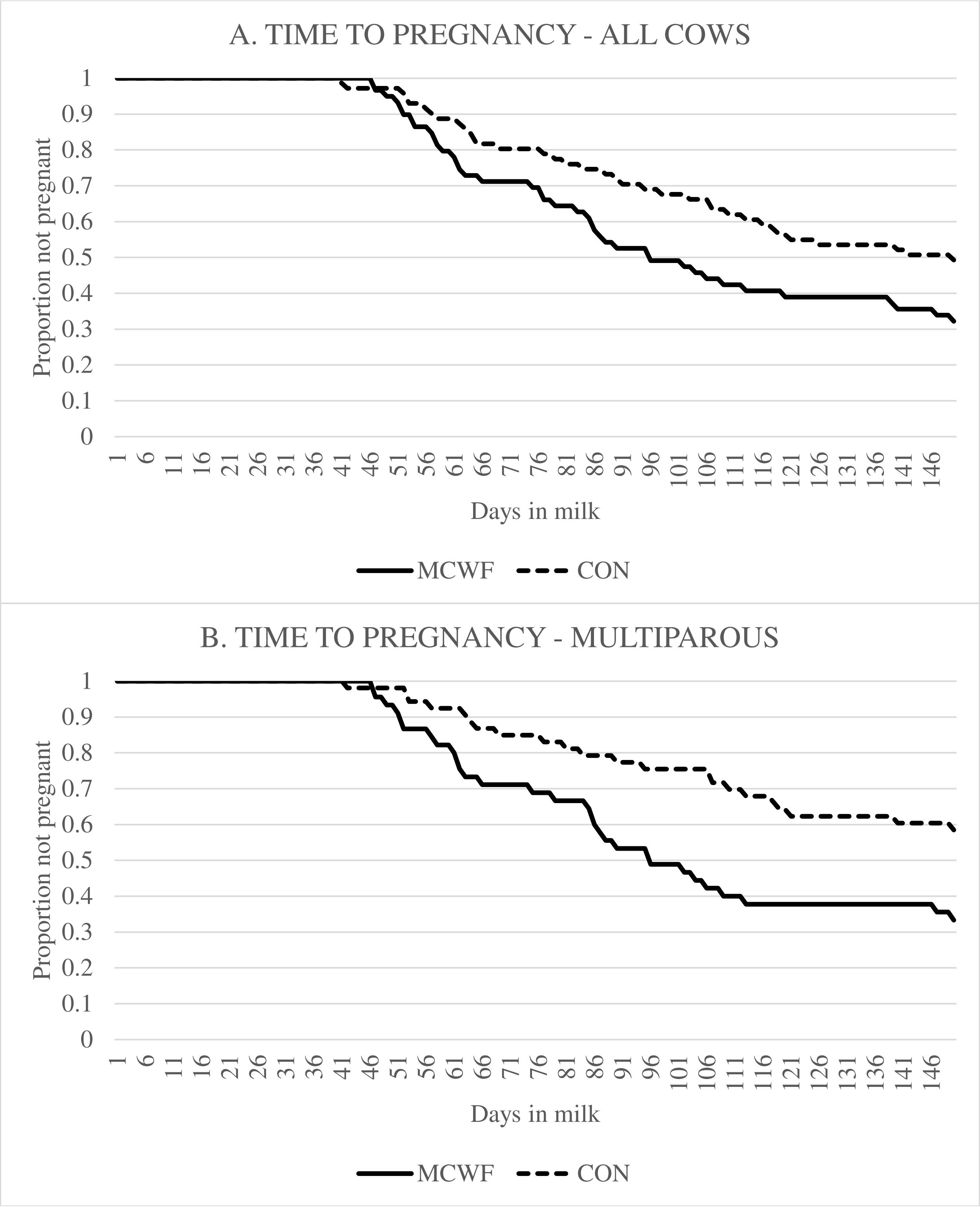

FIGURE 2.1: Survival curves for time to pregnancy comparing *Mycobacterium* cell wall fraction (MCWF) vs. control (CON) group. Curves for all cows and blocked by parity (primiparous: first lactation; multiparous ≥ 2 lactations) is shown.

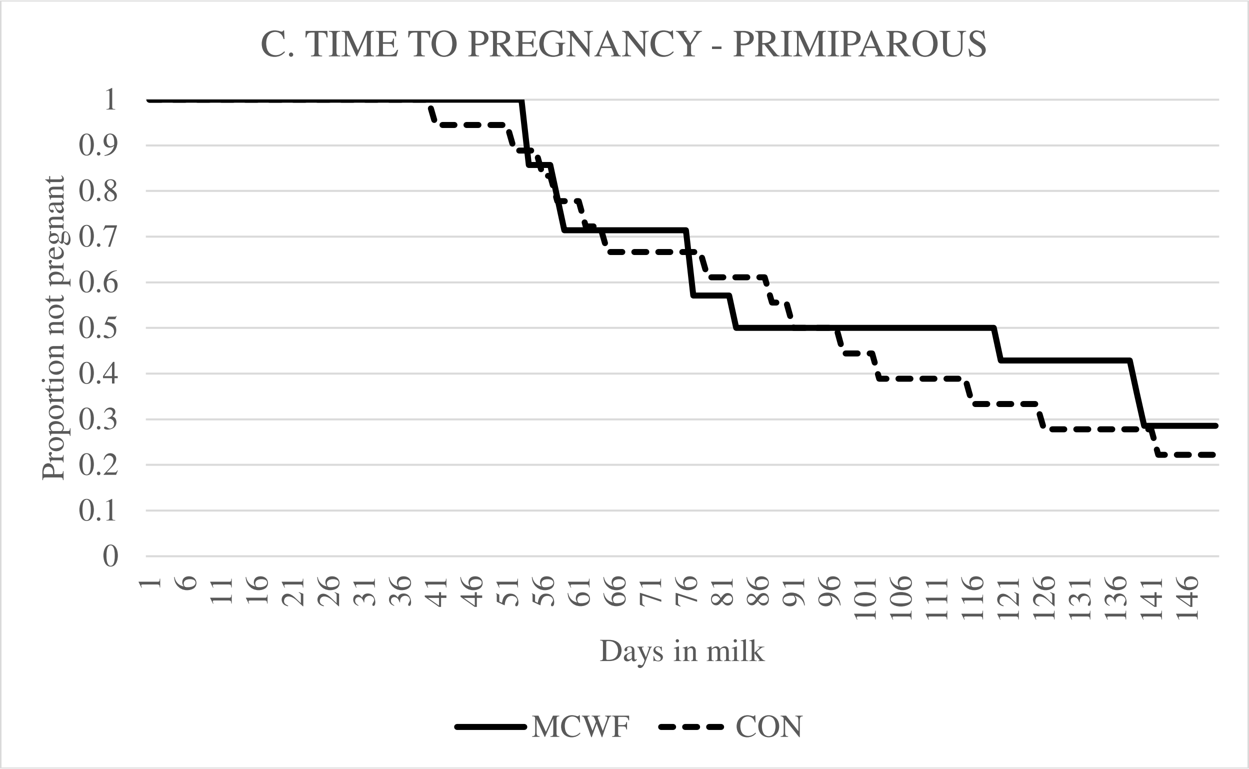

FIGURE 2.1: Continuation.

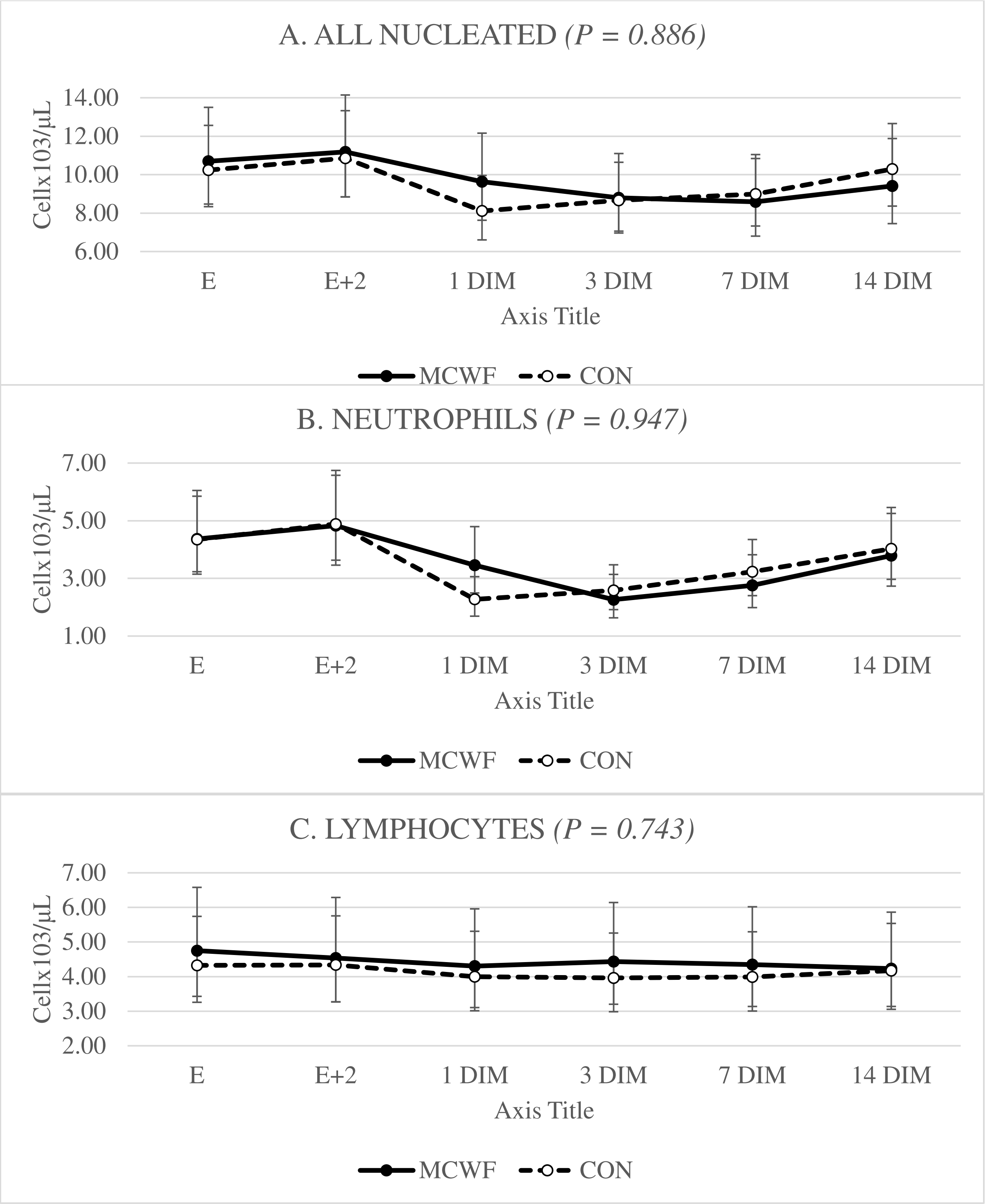

FIGURE 2.2: Repeated measures of back-transformed leukocytes counts comparing *Mycobacterium* cell wall fraction (MCWF) vs. control (CON) group. The model include interaction with parity (P-value for treatment effect). The bars represent confidence limits for the estimates.

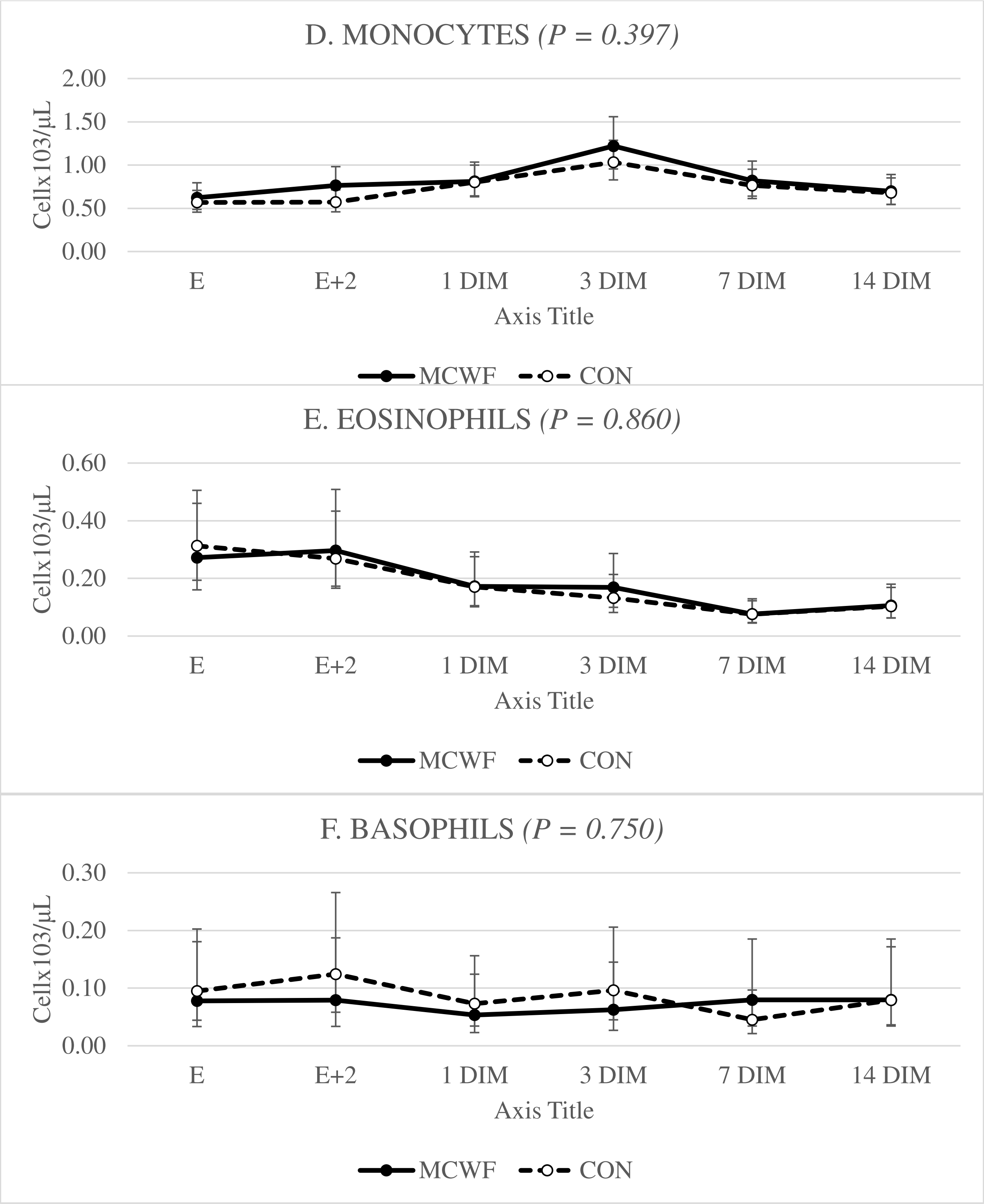

FIGURE 2.2: Continuation.

TABLE 2.3: Repeated measures and confidence limits (CL) of back-transformed values for non-esterified fatty acids (NEFA), β-hydroxi butyrate (BHB), and calcium between *Mycobacterium* cell wall fraction (MCWF) and control (CON) group. The model include interaction with parity.

Metabolite	Treatment (T)	LSM (CL)		Interaction P-values				
		1 DIM	7 DIM	T	P	t	T x P	T x t
NEFA (mmol/L)	MCWF	0.63 (0.48-0.83)	0.58 (0.44-0.77)	0.775	0.831	0.143	0.075	0.477
	CON	0.71 (0.55-0.91)	0.56(0.44-0.72)					
BHB (mmol/L)	MCWF	0.37 (0.30-0.47)	0.49 (0.39-0.61)	0.742	0.523	0.002	0.519	0.620
	CON	0.37 (0.31-0.45)	0.45 (0.37-0.55)					
Calcium (mmol/L)	MCWF	2.05 (1.96-2.14)	2.14 (2.05-2.23)	0.865	0.046	<0.001	0.967	0.369
	CON	2.03 (1.95-2.11)	2.17 (2.09-2.26)					

REFERENCES

Alhussien, M.N. & Dang, A.K. 2019. Potential roles of neutrophils in maintaining the health and productivity of dairy cows during various physiological and physiopathological conditions: a review. Immunol Res 67: 21. DOI: https://doi.org/10.1007/s12026-019-9064-5

Braga Paiano, R., Becker Birgel, D., & Harry Birgel Junior, E. 2019. Uterine Involution and Reproductive Performance in Dairy Cows with Metabolic Diseases. Animals, 9(3), 93. Doi:10.3390/ani9030093.

Caixeta, L. S., Herman, J. A., Johnson, G. W., & McArt, J. A. (2018). Herd-level monitoring and prevention of displaced abomasum in dairy cattle. Veterinary Clinics: Food Animal Practice, 34(1), 83-99.

Chapinal, N., Carson, M., Duffield, T.F., Capel, M., Godden, S., Overton, M., Santos, J.E.P., LeBlanc, S.J. 2011. The association of serum metabolites with clinical disease during the transition period. J. Dairy Sci. 2011; 94: 4897–4903. DOI: https://doi.org/10.3168/jds.2010-4075

Christiana I, Aparnaa R., Rohit R., Nageswara Rao D., Kaliraj P. 2016. Effect of muramyl dipeptide and alum adjuvants on immunization with Filarial multi antigen peptide vaccine in mice model. Helminthologia, 53, 3: 224 – 232, 2016. DOI: 10.1515/helmin-2016-0022.

Christoffersen M., Woodwar E.M., Bojesen A.M., Petersen M.R., Squires E.L., Lehn-Jensen H., Troedsson M.H.T. 2012. Effect of immunomodulatory therapy on the endometrial inflammatory response to induced infectious endometritis in susceptible mares. Theriogenology. 2012; 78(5):991-1004. DOI: https://doi.org/10.1016/j.theriogenology.2012.04.016

Esposito, G., Irons, P. C., Webb, E. C., Chapwanya, A. 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Animal Reproduction Science, 144 (3-4), 60-71. Doi: 10.1016/j.anireprosci.2013.11.007.

Ferguson J. D., Galligan D. T., Thomsen N. 1991. Principal Descriptors of Body Condition Score in Holstein Cows. Journal of Dairy Science, Volume 77 (9), 2695-2703. Doi: 10.3168/jds.S0022-0302(94)77212-X

Filion C. M., Rodrigues L., Johannes C., Masic A. The in vitro and in vivo anti-cancer potential of mycobacterium cell wall fraction (MCWF) against canine transitional cell carcinoma of the urinary bladder. 2017. Acta Veterinaria-Beograd 2017, 67 (4), 477-494. Doi: 10.1515/acve-2017-0039.

Fumuso EA, Aguilar J, Giguire S, Rivulgo M, Wade J, Rogan D. 2007. Immune parameters in mares resistant and susceptible to persistent post-breeding endometritis: effects of immunomodulation. Vet Immunol Immunop 2007;118:30–39. DOI: https://doi.org/10.1016/j.vetimm.2007.04.009.

Fumuso EA, Giguire S, Wade J, Rogan D, Videla-Dorna I, Bowden RA. Endometrial IL-1(beta), IL-6 and TNF-(alpha), mRNA expression in mares resistant or susceptible to post breeding endometritis: effects of estrous cycle, artificial insemination and immunomodulation. 2003. Vet Immunol Immunop 2003; 96:31– 41. DOI: https://doi.org/10.1016/S0165-2427(03)00137-5

Goff J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Vet. J. 176:50–57.

Haimerl, P., Arlt, S., Borchardt, S., & Heuwieser, W. 2017. Antibiotic treatment of metritis in dairy cows – A meta-analysis. Journal of Dairy Science, 100(5), 3783-3795. doi: 10.3168/jds.2016-11834

Huber A, Kallerup R.S., Korsholm K.S. 2016. Trehalose diester glycolipids are superior to the monoesters in binding to Mincle, activation of macrophages in vitro and adjuvant activity in vivo. Innate Immun 22:405–418. DOI: https://doi.org/10.1177/1753425916651132.

International Dairy Federation 1971. A monograph of bovine mastitis. Annual bulletin – International Dairy federation, 1971, pt 2. Secrétariat general, Bruxelles, Belgium.

Jones M. L. and Allison R. W. 2007. Evaluation of the Ruminant Complete Blood Cell Count. Veterinary Clinics of North America: Food Animal Practice, Volume 23, Issue 3, 2007, Pages 377-402, ISSN 0749-0720, DOI: https://doi.org/10.1016/j.cvfa.2007.07.002.

Lhermie, G., Gröhn, Y. T., & Raboisson, D. 2017. Addressing Antimicrobial Resistance: An Overview of Priority Actions to Prevent Suboptimal Antimicrobial Use in Food-Animal Production. Frontiers in microbiology, 7, 2114-2114. doi: 10.3389/fmicb.2016.02114

Lhermie, G., Wernli, D., Jørgensen, P. S., Kenkel, D., Tauer, L. W., & Gröhn, Y. T. 2019. Global resistance to antimicrobials and their sustainable use in agriculture. The Lancet Planetary Health, 3(3), e109-e110. doi: 10.1016/S2542-5196(18)30251-1

Liu, D., B. C. L. Chan, L. Cheng, M. S. M. Tsang, J. Zhu, C. W. Wong, D. Jiao, H. Y. T. Chan, P. C. Leung and C. W. K. Lam. 2018. Sophora flavescens protects against mycobacterial trehalose dimycolate-induced lung granuloma by inhibiting inflammation and infiltration of macrophages. Sci. Rep. 8, 3903. DOI: https://doi.org/10.1038/s41598-018-22286-w.

Mahjoubi, E., Zahmatkesh D, Hossein-Yasdi M, Khabbazan MH, Samadian MR 2018. Short communication: Test of the relationship between prepartum milk leakage and blood calcium concentration in Holstein cows — an observational study." Journal of Dairy Science 101(4): 3579-3582. Doi: 10.3168/jds.2017-13767

Martinez, N., Risco, C. A., Lima, F. S., Bisinotto, R. S., Greco, L. F., Ribeiro, E. S., Maunsell, F. Galvão, K., Santos, J. E. P. 2012. Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. Journal of Dairy Science, 95(12), 7158-7172. doi: https://doi.org/10.3168/jds.2012-5812

Masic, A., Prunic, B., Bugarski, D., Petrovic, T., Apic, J., Barna, T., and Milovanovic, A. 2017. Immunomodulator-Mycobacterium Cell Wall Fraction as an aid in control of persistent Mycoplasma bovis infection in dairy cows. Veterinarski Glasnik, 71(1), 58-68. Doi: 10.2298/VETGL170308003M

Meglia G. E., Johannisson A., Petersson L., Persson Waller K. Investigation in blood Leukocytes and Neutrophils in Periparturient Dairy Cow. 2018. Sci J of Ani and Vet Sci. 2018; 1(1): 001-009. https://www.onomyscience.com/onomy/articles/SJAVS-ID-01.pdf.

Mezzetti, M. New Insight on Metabolism and the Immune Response from Dry-Off to Early Lactation. 2017. Università Cattolica del Sacro Cuore, XXXI ciclo, a.a. 2017/18, Piacenza. http://hdl.handle.net/10280/59476.

Moretti P., Probo M., Cantoni A., Paltrinieri S., Giordano A. 2016. Fluctuation of neutrophil counts around parturition in Holstein dairy cows with and without retained placenta. Research in Veterinary Science, Volume 107, 2016, Pages 207-212, DOI: https://doi.org/10.1016/j.rvsc.2016.06.015.

National Animal Health Monitoring System – NAHMS, USDA – 2014. Health and Management Practices on U.S. Dairy Operations

National Organic Farming Handbook, National Organic Program (NOP). 2015. United States Department of Agriculture

National Research Council 2001. Nutrient requirements of dairy cattle (7th rev. ed. Natl. Acad. Sci., Washington, DC.).

Nightingale C.R., Sellers M.D., Ballou M.A. 2015. Elevated plasma haptoglobin concentrations following parturition are associated with elevated leukocyte responses and decreased subsequent reproductive efficiency in multiparous Holstein dairy cows. Veterinary Immunology and Immunopathology, Volume 164, Issues 1–2, pages 16-23. DOI: https://doi.org/10.1016/j.vetimm.2014.12.016.

Nickerson, S. C., Kautz, F. M., Ely, L. O., Rowson, A. D., Hurley, D. J., Chapman, J. D., & McLean, D. J. 2019. Effects of an immunomodulatory feed additive on intramammary infection prevalence and somatic cell counts in a dairy herd experiencing major health issues. Research in Veterinary Science, 124, 186-190. doi: 10.1016/j.rvsc.2019.03.013

Nosky B, Biwer J, Alkemade S, Prunic B, Milovanovic A, Maletic M, and Masic A. 2017. Effect of a Non-Specific Immune Stimulant (Amplimune™) on the Health and Production of Light Feedlot Calves. J Dairy Vet Anim Res 6(3): 00179. Doi: 10.15406/jdvar.2017.06.00179

Oetzel, G. R. (2017). Diagnosis and management of subacute ruminal acidosis in dairy herds. Veterinary Clinics: Food Animal Practice, 33(3), 463-480.

Ospina, P. A., Nydam, D. V., Stokol, T., & Overton, T. R. 2010. Evaluation of nonesterified fatty acids and β-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. J. Dairy Sci. 93 :546–554. doi: 10.3168/jds.2009-2277

Paibomesai M.A., Sharif S., Karrow N., Mallard B.A. 2018. Type I and type II cytokine production of CD4+ T-cells in immune response biased dairy cattle around calving. Veterinary Immunology and Immunopathology, Volume 199, 2018, Pages 70-76. DOI: https://doi.org/10.1016/j.vetimm.2018.03.001.

Pinedo, P. J., Velez, J. S., Bothe, H., Merchan, D., Piñeiro, J. M., & Risco, C. A. 2015. Effect of intrauterine infusion of an organic-certified product on uterine health, survival, and fertility of dairy cows with toxic puerperal metritis. Journal of Dairy Science, 98(5), 3120-3132. doi: 10.3168/jds.2014-8944

Pinedo, P., Velez, J., Solano, G., Rodriguez, N., Naves, J., Schuenemann, G. M., & Risco, C. 2017. Effect of oral calcium administration on the cure and reproductive performance of Holstein cows diagnosed with puerperal metritis. Journal of Dairy Science, 100(4), 2917-2927. doi: 10.3168/jds.2016-11577

Rogan D., Fumuso E., Rodríguez E., Wade J., Sánchez Bruni S.F. 2007. Use of a Mycobacterial Cell Wall Extract (MCWE) in Susceptible Mares to Clear Experimentally Induced Endometritis With Streptococcus zooepidemicus. Journal of Equine Veterinary Science, Volume 27, Issue 3, 2007, Pages 112-117. DOI: https://doi.org/10.1016/j.jevs.2007.01.010.

Romanowski, R., Culbert, R., Alkemade, S., Medellin-Peña, M. J., Bugarski, D., Milovanovic, A., Nesic, S., & Masic, A. 2017. Mycobacterium cell wall fraction immunostimulant (AMPLIMUNE™) efficacy in the reduction of the severity of ETEC induced diarrhea in neonatal calves, Acta Veterinaria, 67(2), 222-237. doi: 10.1515/acve-2017-0019

Sheldon, I. M., Lewis, G. S., LeBlanc, S., & Gilbert, R. O. 2006. Defining postpartum uterine disease in cattle. Theriogenology, 65(8), 1516-1530. Doi: 10.1016/j.theriogenology.2005.08.021

Shimizu T., Morino I., Kitaoka R., Miyamoto A., Kawashima C., Haneda S., Magata F. 2018. Changes of leukocyte counts and expression of pro- and anti-inflammatory cytokines in peripheral leukocytes in periparturient dairy cows with retained fetal membranes. Animal Science Journal, Volume89, Issue9, Pages 1371-1378. DOI: https://doi.org/10.1111/asj.13065.

Spielmeyer, A. 2018. Occurrence and fate of antibiotics in manure during manure treatments: A short review. Sustainable Chemistry and Pharmacy, 9, 76-86. doi:https://doi.org/10.1016/j.scp.2018.06.004

Subramanian M., In L. L. A., Kumar A., Ahmed N., Nagoor N. H. 2016. Cytotoxic and apoptotic effects of heat killed *Mycobacterium indicus pranii* (MIP) on various human cancer cell lines. Sci. Rep. 6, 19833; doi: 10.1038/srep19833 (2016).

Van Schyndel, S. J., Carrier, J., Bogado Pascottini, O., & LeBlanc, S. J. 2018. The effect of pegbovigrastim on circulating neutrophil count in dairy cattle: A randomized controlled trial. PLOS ONE, 13(6), e0198701. doi: 10.1371/journal.pone.0198701

Walton E. M., Cronan M. R., Cambier C.J., Rossi A., Marass M., Foglia M. D., Brewer W. J., Poss K. D., Stainier D.Y.R., Bertozzi C.R., Tobin D.M. 2018. Cyclopropane Modification of Trehalose Dimycolate Drives Granuloma Angiogenesis and Mycobacterial Growth through Vegf Signaling. Cell Host & Microbe, 24 (4), 514-525.e6. DOI: 10.1016/j.chom.2018.09.004.

Woodward E.M., Christoffersen M., Campos J., Horohov D.W., Scoggin K.E., Squires E., Troedsson M. H. T. 2013. An investigation of uterine nitric oxide production in mares susceptible and resistant to persistent breeding induced endometritis, and the effects of immunomodulation. Reprod Domest Anim, 48 (4) (2013), pp. 554-561. DOI: https://doi.org/10.1111/rda.12124

Zhou C.J., Chen J., Hou J.B., Zheng Y., Yu Y.N., He H., Zhang Y.P., Feng X.L., Zheng Q.S.. 2018. The Immunological Functions of Muramyl Dipeptide Compound Adjuvant on Humoral, Cellular-mediated and Mucosal Immune Responses to PEDV Inactivated Vaccine in Mice. Protein and Peptide Letters 25(10): 908-913. DOI: 10.1097/SHK.0000000000001077.

Zinicola, M., Korzec, H., Teixeira, A. G. V., Ganda, E. K., Bringhenti, L., Tomazi, A. C.C. H., Gilbert R. O., Bicalho, R. C. 2018. Effects of Pegbovigrastim administration on periparturient diseases, milk production, and reproductive performance of Holstein cows. Journal of Dairy Science, 101(12), 11199-11217. DOI: https://doi.org/10.3168/jds.2018-14869.

CHAPTER 3: EFFECT OF PULSED ALTERNATING WAVELENGTHS SYSTEM (PAWS) ON PERIPARTAL HEALTH AND HORMONAL PROFILING IN TRANSITION DAIRY COWS.

Summary

The transition period is a challenging stage for dairy cows, with many sources of stress characterizing the imbalances of this time. Manipulation of the type of light exposure could be a non-invasive technique to improve performance, health, and well-being at this phase. Our objective was to evaluate the effect of a recently developed technology based on a Pulsed Alternating Wavelengths System (PAWS; Xiant Technologies, Inc., Greely CO) on dystocia presentation, peripartal health and serum levels of prolactin (PRL) and somatotropin (bST) in Holstein cows. In addition, activity and serum concentrations of calcium, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) were assessed. A total of 82 Holstein cows were blocked by parity and randomly assigned into a treatment group exposed to PAWS (PAWS; n=40) from 3 to 11 days before calving, or a control group (CON; n=42) non-exposed during the same time period. Blood samples for hormonal and metabolic assessment were collected at -1, -2 and -3 days relative to calving, and at 1 and 3 days in milk (DIM). There was a significant reduction on the presentation of dystocia in favor of PAWS ($P = 0.031$). In addition, NEFA serum levels were higher in PAWS cows at 1 and 3 DIM ($P = 0.013$), and PRL was higher at 1 DIM in CON unexposed cows ($P < 0.001$). No significant effects were found in other peripartal conditions, activity, general metabolic status, or bST. In conclusion, exposure to PAWS may reduce the presentation of dystocia and modify NEFA concentrations around calving. Future evaluation of melatonin and BCS is encouraged to confirm the effect of PAWS on these outcomes.

Introduction

The transition period is the most challenging time for dairy cows, and is characterized essentially by a suboptimal immune response and nutritional, physiological, and metabolic imbalances due to the start of a new lactation and the calving process itself (Esposito et al, 2014; Aleri et al, 2016). The main approach to diminish this stress involves nutrition schemes, although development of new strategies applied to management are important to reduce the susceptibility to peripartal and subsequent diseases (Aleri et al, 2016; Sordillo, 2016). Parallel regulation of the metabolic and immune conditions, as well as the hormonal status is a suitable option.

Examples of this approach include manipulating bovine somatotropin (bST), a growth-promoting protein secreted in the pituitary gland that stimulates cell regeneration and reproduction mediated by insulin-like growth factor-I (IGF-I), as synthetic analogues are used in dairy cattle to increase milk production (WHO, 2014). Other examples include multiples strategies for calcium regulation. Calcium is involved in processes such as muscular contraction, nerve conduction, cellular membranes stability and many other physiological functions. Parathyroid hormone (PTH), calcitriol and calcitonin are the principal hormones (calciotropic hormones) controlling calcium homeostasis by the directive of calcium intestinal absorption, renal calcium reabsorption and bone calcium resorption.

In addition, endocrine pathways including hormones such as serotonin and prolactin (PRL) are also tightly involved in the process of calcium regulation (Hernández-Castellano et al, 2019). Prolactin, also secreted in the pituitary gland, is primarily known for its role in colostrogenesis and lactogenesis, and is capable to act on calcium circulating levels similarly as the calciotropic hormones, acting on intestines, kidneys and bones. Prolactin also stimulates the synthesis of serotonin in the mammary gland and increases its blood concentration. Serotonin (5-

hydroxytryptamine; 5-HT), derived from tryptophan and known for its effect on immunity, behavior and reproduction, stimulates the paracrine secretion of parathyroid hormone-related protein (PTHrP) in the mammary gland during lactation, promoting a biological PTH-like activity acting on bone calcium resorption (Hernández-Castellano et al, 2019; Hernandez, 2018). Glucose is also regulated by 5-HT; therefore, higher levels of 5-HT can reduce the impact of clinical and subclinical metabolic diseases like hypocalcemia and ketosis. These reasons make of 5-HT a post-partum health predictor within the first 24 hours after calving (Laporta et al, 2013). Serotonin (5-HT) and calcium are simultaneously at peak levels around 24 hours before calving; PTHrP start increasing 24 to 48 hours after calving, and follow a similar pattern along with the concentration of 5-HT, in response to the levels of calcium to the circulation from the mammary gland (Weaver et al, 2016).

Another hormone also derived from tryptophan is melatonin, produced from 5-HT in the pineal gland or multiple organs and cells, is the circadian pacemaker, it enhances the cellular immune response (lymphocytes), has a powerful and complete antioxidant effect, and is responsible of seasonal breeding (Hardeland et al, 2006). Through photoperiod and the influence of melatonin, a shorter peripartal light exposure can increase the availability of serum calcium by synthesis of vitamin D stimulation, improves dry matter intake, and boost milk yield by upturning PRL levels (Özçelik et al, 2017). Melatonin may also improve the calving easiness by reducing the levels of environmental stress and improving hormonal synchronization around parturition, permitting a better dilation of the cervix, vulva and vagina (Hardeland et al, 2006, Funnell and Hilton, 2016). Thus, hormonal control of melatonin and its benefits by manipulation of the circadian cycle (manipulation of light exposure) is a promissory strategy, especially in organic dairy farms where the use of conventional drugs is restricted (NOP, 2015).

Irregularities in melatonin rhythm in humans disturb the circadian cycle (chronodisruption) and, therefore, affect the overall health. The disorders depend on the light intensity, the time of exposure and the spectrum of the light (Bonmati-Carrion et al, 2014). Blue light is the most chronodisruptive of the wavelengths; contrary, red light could stimulate visual photoreceptors to increase levels of melatonin around sleep time, potentially adjusting the circadian clock and improving the quality of sleep (Bonmati-Carrion et al, 2014; Ho Mien et al, 2014).

In animals, studies in broiler chickens indicated that artificial light manipulated adequately might have positive biological responses manifested by better production and welfare (Yang et al, 2018), and similar effects happen in dairy cows (Penev et al, 2014). In addition to the general effects on health and the circadian cycle, melatonin has an important effect on reproductive performance at different physiological stages; it supports proper oocyte development, favors embryo implantation, and enforces an adequate fetal growth (Carlomagno et al, 2018).

One of the methods of manipulating light exposure at strategic points is the use of pulsed alternating wavelengths system (PAWS). A clinical pilot trial comparing the hormonal (serotonin and cortisol) and neurotransmitter (serotonin) status of dairy calves exposed to PAWS indicated a significant impact for PAWS on serum melatonin concentrations (Pinedo et al, 2019), leading the base for the current research.

In this study, we hypothesized that exposure to PAWS for at least three days before calving could elicit a positive hormonal and metabolic response that might reduce presentation of dystocia, as well as the imbalances and stress around calving, improving peripartal health and subsequent performance in transition dairy cows. Hence, our objective was to evaluate the effect of PAWS on dystocia presentation, peripartal health, activity, and serum levels of melatonin (MEL), serotonin

(5-HT), prolactin (PRL), somatotropin (BST), calcium, non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) of organic certified Holstein cows.

Materials and methods

The procedures completed on this research were approved on 05/11/2018 by the Institutional Animal Care and Use Committee (IACUC) at Colorado State University (Fort Collins, CO) in the protocol 18-7885A.

Study population

This study was conducted in a dairy farm under certified organic management located in northern Colorado, USA. Holstein cows were enrolled between December 2018 and February 2019 at the maternity facility. Cows that were 14 days from the expected due date were housed in pens with wood shavings-bedded floor with *ad libitum* access to clean water. Cows received a total mixed ration (TMR) twice a day according to the National Research Council recommendations for transition cows (NRC, 2001).

Experimental design and treatment allocation

A total of 82 Holstein cows were grouped by parity with a goal of enrolling approximately 35% primiparous and 65% multiparous cows, based on the farm population distribution. Cows were randomly assigned into 1 of 2 treatment groups: (1) exposed to a Pulsed Alternating Wavelengths System (PAWS, n = 40; Xiant Technologies, Inc., Greely CO) within 7±4 days before calving; or (2) a non-exposed control group during the same period (CON, n = 40; Figure 3.1). Pen in PAWS group had lamps affixed to wires on the roof that were constantly on (Figure 3.2). No study cows had access to the yard, and the groups remained always with 10 cows per study pen in a 48m^2 area. As the cows were calving, the pens were filled continuously to maintain the 10 cows per pen. Blood samples were collected at enrollment, -1, -2 and -3 days related to calving, and 1 and 3 DIM for hormonal and metabolic assessment. Trained personnel and farm veterinarians performed daily general health since enrollment to 28 DIM. After 28 DIM, the cows stopped sharing the same pen and were sent to different pens along the farm.

Case definitions

Farm workers performed supervisory rounds every 30 minutes around the maternity pens to detect cows in labor of calving by detection of the amniotic sac (water bag) or the calf. After this moment, if a cow was unable to expel the calf within two hours, it was assisted and considered as a dystocia case (Funnell and Hilton, 2016). A stillbirth was defined as a calf that was either born dead or died within the first 24 hours after birth, and a case of retention of fetal membranes was a cow unable to expel the placenta within 24 hours after the expulsion of the offspring. Farm-trained workers diagnosed clinical ketosis (KET) by putting a drop of blood on acetoacetic acid reagent strips (Ketostix; Bayer Corporation, Elkhart, IN). Cows with clinical hypocalcemia were identified as downer-cows (in head-down recumbency with paresis of the four limbs) 24 hours after parturition responding to intravenous calcium administration (Mahjoubi et al, 2018). Data from clinical diseases from calving to 28 DIM were obtained from PCDART software used to record keeping in the farm.

Diagnosis of uterine infectious disease (UID) was done by transrectal massage of the uterus, collecting the discharge with a Metricheck device (Metricheck, SimcroTech, Hamilton, New Zealand). Puerperal or toxic metritis (PMET) was defined as an abnormally enlarged uterus and a fetid, watery, reddish/brownish uterine discharge, associated with signs of systemic illness (e.g. reduced milk yield and appetite, dullness) and fever (≥ 39.5˚C) within 21 days after calving. Clinical metritis (CMET) was defined as purulent uterine discharge within 21 days after parturition, in the absence of systemic illness and fever. Clinical endometritis (ENDO) DIM was defined as vaginal discharge with a content of pus greater than the content of mucus (more than 50% pus) noticeable in the vagina between 21 and 28 DIM, for this study (Sheldon et al, 2006). According to the farm health protocol, all cows diagnosed with PMET received an intrauterine (i.u.) infusion of Optimum UterFlush (Van Beek Natural, Science, Orange City, IA), administered every other day for 3 times, plus intravenous (i.v.) hypertonic saline solution, i.v. dextrose 5%, and oral aspirin, and cows diagnosed with ENDO received 500 ml i.u. of dextrose 5% (Pinedo et al, 2017; 2015).

Clinical mastitis (MAST) was defined as a mammary quarter with signs of inflammation (heat, pain, redness, or swelling) and/or changes in the appearance in the milk (e.g. flakes, clots, pus, etc.) (International Dairy Federation. 1971). According to the farm health protocol, deep stripping (hand milking) until the disappearance of the symptoms was the treatment approach for cows with MAST. If the milk was watery, transparent/yellowish, and occasionally fetid smell, in the presence of inflammation of the quarter and clinical signs of systemic toxemia (e.g. fever, depression, inappetence, etc.), the mastitis was defined as toxic mastitis. Veterinarians of the farm diagnosed displacement of abomasum by auscultation of a metallic ‘ping’ sound in the upper left flank side of the abdomen, parallel with history of reduced consumption, reduced milk yield and lack of rumen motility (Caixeta et al., 2018). Trained farm workers diagnosed ruminal acidosis mainly by the presence of profuse watery diarrhea and parallel history of reduced consumption, reduced milk yield, and poor body condition score (Oetzel, 2017). Trained workers and veterinarians diagnosed respiratory disease by the auscultation of lungs sounds and the presence of cough, polypnea and nasal discharge.

Hormonal and metabolic assessment

A subsample of 30 cows (PAWS = 17, CON = 13) was selected to determine serum levels of PRL and bST, and a subsample of 46 cows (PAWS = 26, CON = 20) was selected to measure serum levels of NEFA, BHB and calcium. Blood samples were collected in the morning (0600 to 0700 h) at enrollment (7 ± 4 days before calving) in the maternity, and every morning (0800 to 0900 h) at 1 and 3 DIM after milking, using 10 ml serum Vacutainer® tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Samples were stored in a transportation cooler filled with ice packs, and the serum was separated within 12 hours of collection (centrifuged at 2800 rpm for 12 minutes at room temperature). Subsequently, samples were stored frozen at -80˚C from 1 to 3 months until the moment of hormones and metabolites determination.

Cut-off values for serum NEFA concentrations to determine negative energy balance were 0.27 mmol/L at calving and 0.57 mmol/L at 7 DIM. Cows with serum BHB > 0.96 mmol/L at calving or at 7 DIM were considered as hyperketonemic (Ospina et al, 2010). Cut-off values for subclinical hypocalcemia were Ca < 2.12 mmol/L at calving (Martinez et al, 2012; Goff, 2008) and < 2.20 mmol/L at 7 DIM (Chapinal et al, 2011).

All cows had an IceQube accelerometer (IceRobotics, Edinburgh, Scotland) previously attached in one of the legs for activity assessment (lying and standing time, number of bouts, and activity). Records for behavior analysis were obtained to assess 12 hours before expeling of the calf, which was determined observing video records from on-pen cameras.

Data management and statistical analyses

The sample size was calculated to detect a difference of 14 percentage points in the proportion of dystocia cases in favor of PAWS group. Based on the farm data, the anticipated proportion of cows with dystocia in the CON group was 15%. Considering power = 80% and confidence = 95%, the number of cows required to show a significant difference between the 2 treatment groups was estimated in 46 cows per group (PROC POWER, SAS 9.4).

Randomization of cows was performed using Microsoft® Office Excel (Excel RAND function) based on a list from the farm with the cows expected to calve within 2 weeks at the time of enrollment. Due to lack of normality, hormones and metabolites values provided by the lab were Log10-transformed and a repeated measures anova was performed. Then, the Log-10 LSM and confidence intervals were back transformed in an Excel sheet (=POWER(10,value)) to obtain again the hormones and metabolites values. Binary variables, such as the presentation of peripartal diseases, were analyzed using Chi square and Fisher's exact test (PROC FREQ, SAS 9.4) and logistic regression was performed in variables with frequencies ≥ 1 (PROC GLIMMIX, SAS 9.4). Variables with repeated measures were analyzed using repeated measures anova analysis, followed by a Tukey test (PROC GLM, SAS 9.4), and a least square mean analyses (LSMS statement, SAS 9.4).

Results

Descriptive statistics

Overall, 82 Holstein cows were enrolled: 59 multiparous (MCWF = 29, CON = 30) and 23 primiparous (MCWF = 11, CON = 12) for a total of 40 MCWF cows and 42 CON cows. One cow (primiparous, PAWS) left the herd at 11 DIM due to death by toxic metritis and respiratory disease. The average ± SD lactation number were 2.80 ± 1.83 and 2.52 ± 1.55 for PAWS and CON, respectively. The average ± SD number of days from enrollment to calving were 4.00 ± 2.74 and 5.00 ± 2.75 for PAWS and CON, respectively. None of these descriptive variables were significantly different between treatment groups.

Peripartal conditions, metabolic and hormonal status

Treatment had a significant effect on the presentation of dystocia. The frequency (%) of dystocia in cows exposed to PAWS was 0 (0) vs. 5 (11.9) in CON cows (P = 0.031). There was not significant effect of PAWS on the presentation of other diseases or health conditions from enrollment to 28 DIM (Table 3.1).

The LSM and confidence limits (CL) for NEFA at 1 and 3 DIM for PAWS vs CON (mmol/L) were 0.73 (0.58-0.90) vs 0.46 (0.36-0.59), and 0.64 (0.51-0.79) vs 0.49 (0.39-0.63), respectively (P = 0.013). PAWS did not have significant effect on BHB or calcium (Table 3.2), PRL or bST (Table 3.3).

There was a tendency of PAWS to reduce the number ± SE of bouts (5.5 ± 0.8 vs 8.4 ± 1.3; P = 0.065) during the last 12 hours before expelling of the offspring; however, no significant effect was found regarding other activity parameters. The mean (SE) number of steps, lying time (minutes/12h) and number of bouts (number of times the cows went down to lie on the ground within these 12 hours) are shown in Table 3.4.

Discussion

The objective of the present study was to evaluate the effect of a Pulsed Alternating Wavelengths System (PAWS; Xiant Technologies, Inc., Greely CO) on dystocia presentation, peripartal health, activity, and serum levels of prolactin (PRL), and somatotropin (bST), alongside with the assessment of serum concentrations of non-esterified fatty acids (NEFA), β-hydroxybutyrate (BHB) and calcium in Holstein organic certified cows.

This research is a continuation of a small-scale pilot trial comparing melatonin, cortisol and 5-HT serum concentrations in PAWS exposed dairy calves vs unexposed controls (Pinedo et al, 2019). Calves were housed in individual hutches. The hutches in the PAWS exposed group had interior lamps affixed to the roof of the hutch that were continually on. The pilot trial indicated a significant effect for PAWS on serum melatonin concentrations, but no significant effect was found on 5-HT or cortisol concentrations.

In our study, cows exposed to PAWS had some interference from the normal lights in the facility since in the night those lights were always on, whereas in the pilot trial the calves exposed had a most straight impact from PAWS within the hutch during the night.

PAWS may have reduced stress by improving hormonal synchronization and the subsequent reduction in the number of dystocia (Funnell and Hilton, 2016). However, the cases of dystocia presented in our study were related to other aspects such as calf oversize (n = 3) and abnormal calf position (n = 3), making possible that the reduction on dystocia was not associated to PAWS but with these random effects. More details are needed to get a reliable assumption of PAWS and melatonin secretion effect on this variable (Funnell and Hilton, 2016, Zaborski et al, 2009). The activity, especially the lying time, is a parameter that indicates cow comfort and welfare in lactating cows (Hendricks et al, 2019). The statistical tendency found in our study was related to the number of bouts (number of times that the animal lied down), but this difference did not affect the lying time itself. All the cows, regardless of the treatment group, spent around 75% of the time standing, which is expected, as the time window where the activity was measured was in the last 12 hours before calving, period when restlessness, anxiety and uneasiness increase as the moment of expelling of the offspring gets closer.

The principal regulator of PRL is dopamine, and the main physiological PRL induction is the onset of lactation around calving (Lacasse et al, 2016; 2019). Long-day photoperiod (i.e. less MEL secretion) increases serum PRL circulating levels and reduce the availability of PRL receptors (PRL-R) on tissues like liver, mammary gland and the immune system; on the other hand, short-day photoperiod (i.e. more MEL secretion) decrease PRL circulating levels and increase the availability of PRL-R in the same tissues (Collier et al, 2006; Dahl et al, 2004). In theory, PAWS exposed cows could have more PRL-R available and therefore, more sensitivity to PRL circulating molecules, whereas CON cows may have had less PRL-R available and consequently more PRL on blood circulation after the peak of PRL due to the onset of lactation (Dahl et all, 2004). However, no significant effect was found in PRL serum levels; melatonin concentrations need to be evaluated in order to elicit a better conclusion about PRL.

Peripartal bST concentrations were always higher in CON cows (even at enrollment), compared to PAWS exposed cows. Photoperiod appears not to have a spontaneous effect on bST circulating concentration, but it has an impact on circulating levels of IGF-I (which has a strong correlation with bST); comparable with PRL, circulating levels of IGF-I increases on long-day photoperiods, and vice versa (Collier et all, 2006). A study imitating day light exposure using light-emitting diodes (LED) in lactating cows showed that cows exposed to LED for more than 6 hours/day for 7 months had also a significant increase on IGF-1 (Jin-Ryong et al, 2018). However, unexposed CON cows presented higher levels of bST since the enrollment, compared to PAWS, showing that an external factor beside the exposure to PAWS could have influenced the outcome.

PAWS exposed cows presented elevated NEFA at 1 and 3 DIM compared with CON. As MEL is a hormone that decreases metabolic activity in diurnal animals, like cows, the increase of body fat content during the exposure to PAWS would be expected, allowing a greater release of fatty acids during and after the calving process as happened in PAWS exposed cows (Penev et al, 2014). Penev *et al* (2014) also refers to the effect of MEL on IGF-I modulation and the joint effect of these hormones on dry matter intake and nutritional performance. A body condition score (BCS) assessment is encouraged for future studies in order to clear this supposition.

There was no significant difference between groups on serum calcium concentrations after calving (1 and 3 DIM). However, the main effect of daylight on calcium homeostasis relies on the synthesis of calcitriol (1,25-VitD), an essential component for calcium intestinal absorption and renal reabsorption (Özçelik et al, 2017). In this sense, both groups had relatively the same amount of exposure to natural day light (PAWS exposed – east side – received daylight in the morning, CON unexposed – west side – received daylight in the afternoon; Figure 3.1), allowing the same chance for Vitamin D synthesis.

Conclusions

Cows exposed to PAWS had less cases of dystocia and greater NEFA serum levels within 3 days after calving. No significant effects were found on the other outcomes. Future approaches including MEL and BCS assessment are necessary to support properly an inference about the effect of PAWS on the results obtained.

TABLE 3.1: Frequencies of disease presentation for cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows. Data for all cows and blocked by parity (primiparous = first lactation; multiparous ≥ 2 lactations) is shown.

Variable	Parity (P)	Treatment (T)		Fisher's Exact Test
		PAWS, n (%)	CON, n (%)	
Dystocia	All cows	0/40 (0)	5/42 (11.9)	0.031
	Multiparous	0/29 (0)	4/30 (13.3)	0.060
	Primiparous	0/11 (0)	1/12 (8.3)	0.522
Stillbirth	All cows	3/40 (7.5)	1/42 (2.4)	0.237
	Multiparous	0/29 (0)	1/30 (3.3)	0.509
	Primiparous	3/11 (27.2)	0/12 (0)	0.093
Retention of fetal membranes	All cows	0/40 (0)	1/42 (2.4)	0.512
	Multiparous	0/29 (0)	1/30 (3.3)	0.509
	Primiparous	0/11 (0)	0/12 (0)	.
Clinical hypocalcemia	All cows	2/40 (5)	3/42 (7.1)	0.329
	Multiparous	2/29 (6.9)	3/30 (10.0)	0.329
	Primiparous	0/11 (0)	0/12 (0)	.
Clinical ketosis	All cows	2/40 (5.0)	2/42 (4.8)	0.384
	Multiparous	1/29 (3.5)	2/30 (6.7)	0.388
	Primiparous	1/11 (9.1)	0/12 (0)	0.478
Puerperal (toxic) metritis	All cows	4/40 (10.0)	2/42 (4.8)	0.225
	Multiparous	3/29 (10.3)	1/30 (3.3)	0.241
	Primiparous	1/11 (9.1)	1/12 (8.3)	0.521
Clinical endometritis	All cows	3/40 (7.5)	3/42 (7.1)	0.324
	Multiparous	3/29 (10.3)	3/30 (10.0)	0.329
	Primiparous	0/11 (0)	0/12 (0)	.
Clinical mastitis	All cows	5/40 (12.5)	9/42 (21.4)	0.133
	Multiparous	3/29 (10.3)	4/30 (13.3)	0.294
	Primiparous	2/11 (18.2)	5/12 (41.7)	0.178
Digestive disorders	All cows	0/40 (0)	1/42 (2.4)	0.512
	Multiparous	0/29 (0)	1/30 (3.3)	0.509
	Primiparous	0/11 (0)	0/12 (0)	.
Respiratory disease	All cows	1/40 (2.5)	2/42 (4.8)	0.389
	Multiparous	1/29 (3.5)	1/30 (3.3)	0.509
	Primiparous	0/11 (0)	1/12 (8.3)	0.522
Lameness	All cows	1/40 (2.5)	2/42 (4.8)	0.389
	Multiparous	1/29 (3.5)	1/30 (3.3)	0.509
	Primiparous	0/11 (0)	1/12 (8.3)	0.522

TABLE 3.2: Repeated measures and confidence limits (CL) of back-transformed values for non-esterified fatty acids (NEFA), β-hydroxi butyrate (BHB), and calcium between cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows. Times of collection were 1 day in milk (1 DIM) and 3 days in milk (3 DIM).

Variable	Treatment (T)	LSM (CL)		Interaction P-values				
		1 DIM	3 DIM	T	Parity (P)	t	T x P	T x t
NEFA (mmol/L)	PAWS	0.73 (0.58-0.90)	0.64 (0.51-0.79)	0.013	0.014	0.732	0.002	0.262
	CON	0.46 (0.36-0.59)	0.49 (0.39-0.63)					
BHB (mmol/L)	PAWS	0.66 (0.59-0.74)	0.83 (0.74-0.93)	0.906	0.014	<0.001	0.017	0.217
	CON	0.69 (0.61-0.79)	0.78 (0.68-0.88)					
Calcium (mmol/L)	PAWS	2.14 (2.02-2.26)	2.09 (1.98-2.21)	0.752	0.013	0.774	0.115	0.601
	CON	2.13 (2.00-2.27)	2.14 (2.01-2.28)					

TABLE 3.3: Repeated measures and confidence limits (CL) of back-transformed values for prolactin (PRL) and bovine somatotropin (bST) between cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows. Times of collection were enrollment (E), 1 day before calving (!dbc), and1 day in milk (1 DIM).

Variable	Treatment (T)	LSM (CL)			Interaction P-values				
		E	1 dbc	1 DIM	T	Parity (P)	t	T x P	T x t
PRL (ng/mL)	PAWS	576 (400-830)	1332 (924-1918)	4987 (3463-7183)	0.223	0.011	<0.001	0.720	0.795
	CON	793 (525-1199)	1836 (1215-2775)	5712 (3780-8634)					
bST (ng/mL)	PAWS	9.72 (6.87-13.76)	7.27 (5.13-10.29)	8.55 (6.04-12.11)	0.147	0.410	0.022	0.018	0.580
	CON	12.67 (8.57-18.74)	10.84 (7.33-16.04)	13.09 (8.85-19.36)					

TABLE 3.4: Repeated measures and standard error (SE) of activity 12 hours before expelling of the calf between cows exposed to pulsed alternating wavelengths systems (PAWS) vs. unexposed control (CON) cows.

Variable	Treatment (T)	12 hours before calving		Interaction P-values				
		Mean	SE	T	P	t	T x P	T x t
Number of steps	PAWS	251	51	0.642	0.933	0.125	0.770	0.919
	CON	301	92					
Lying time (h:mm:ss)	PAWS	3:44:01	0:34:58	0.892	0.181	0.222	0.378	0.909
	CON	3:53:46	1:02:33					
Number of bouts	PAWS	5.5	0.8	0.065	0.138	0.140	0.083	0.203
	CON	8.4	1.3					

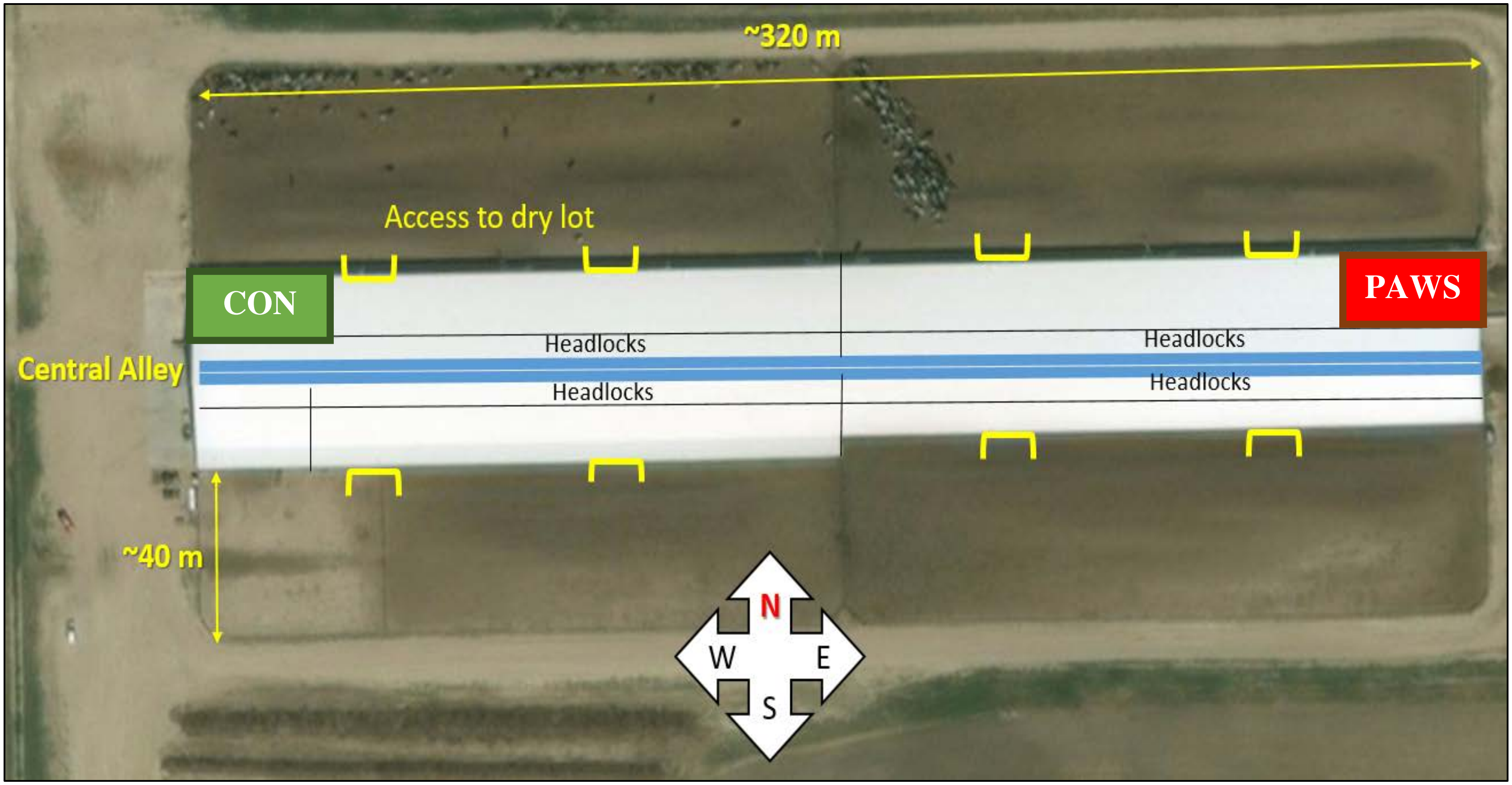

FIGURE 3.1: Distribution of CON (left, green) and PAWS (right, red) groups within the maternity complex.

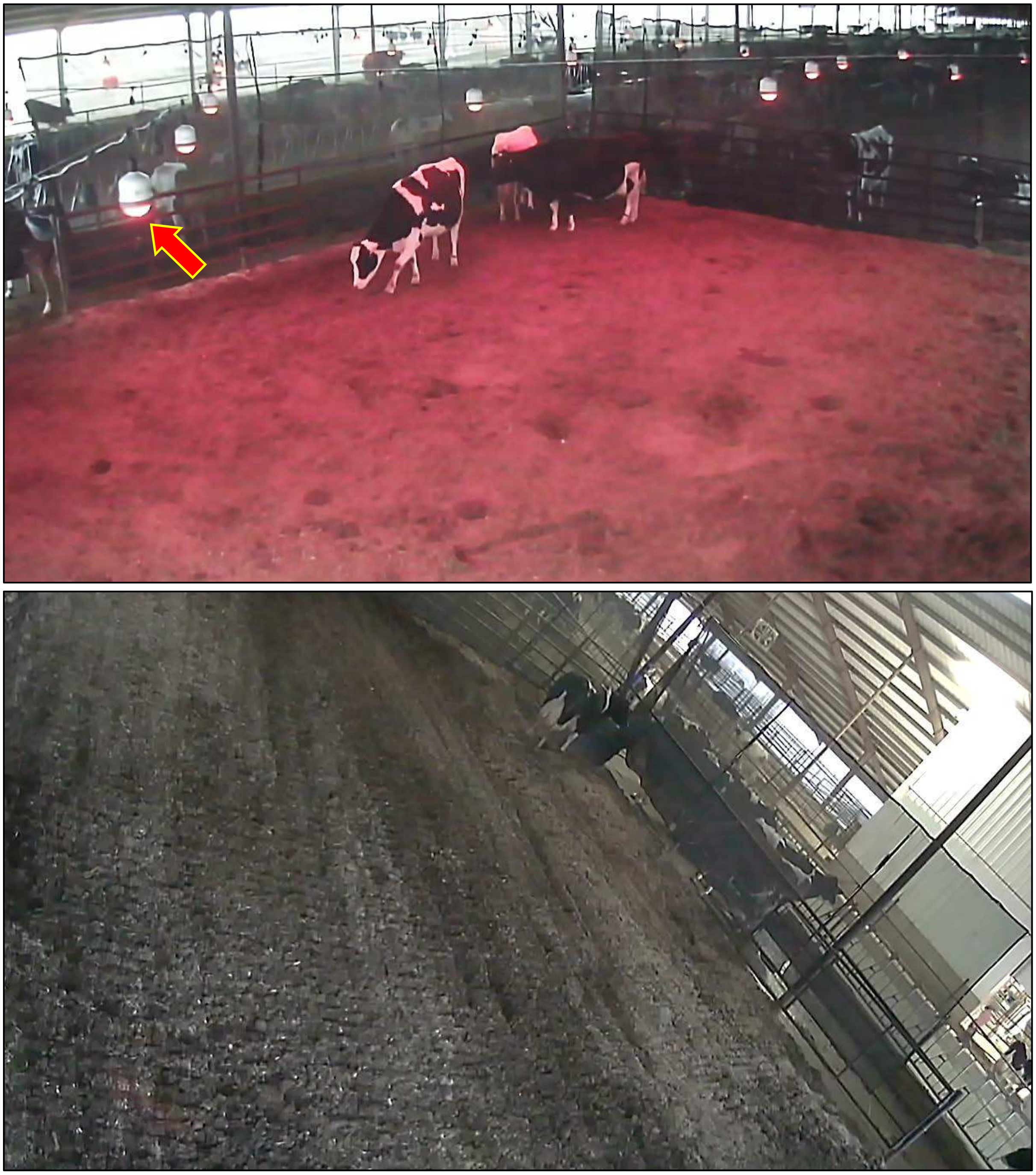

FIGURE 3.2: PAWS lamps (arrow) affixed to wires on the roof for the pen of cows exposed to PAWS (up), and the pen of unexposed CON group (down).

REFERENCES

Aleri J.W., Hine B.C., Pyman M.F., Mansell P.D., Wales W.J., Mallard B., Fisher A.D. (2016). Periparturient immunosuppression and strategies to improve dairy cow health during the periparturient period. Res Vet Sci. 108:8-17. DOI: 10.1016/j.rvsc.2016.07.007.

Bonmati-Carrion M.A., Arguelles-Prieto R., Martinez-Madrid M.J., Reiter R., Hardeland R., Rol M.A., Madrid J.A. (2014). Protecting the melatonin rhythm through circadian healthy light exposure. Int J Mol Sci. 15(12):23448-500. DOI: 10.3390/ijms151223448.

Caixeta, L. S., Herman, J. A., Johnson, G. W., & McArt, J. A. (2018). Herd-level monitoring and prevention of displaced abomasum in dairy cattle. Veterinary Clinics: Food Animal Practice, 34(1), 83-99.

Carlomagno G., Minini M., Tilotta M., Unfer V. (2018). From Implantation to Birth: Insight into Molecular Melatonin Functions. Int J Mol Sci. 19(9). pii: E2802. DOI: 10.3390/ijms19092802.

Collier R. J., Dahl G. E., VanBaale M.J. (2006). Major advances associated with environmental effects on dairy cattle. J Dairy Sci. 2006 Apr;89(4):1244-53. DOI: https://doi.org/10.3168/jds.S0022-0302(06)72193-2

Dahl G.E., Auchtung T.L., Reid E.D. (2004). Manipulating milk production in early lactation through photoperiod changes and milking frequency. Vet Clin North Am Food Anim Pract. 2004 Nov;20(3):675-85. DOI: https://doi.org/10.1016/j.cvfa.2004.06.004

Esposito, G., Irons, P. C., Webb, E. C., Chapwanya, A. (2014). Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. Animal Reproduction Science, 144 (3-4), 60-71. Doi: 10.1016/j.anireprosci.2013.11.007.

Funnell, B. J., & Hilton, W. M. (2016). Management and prevention of dystocia. Veterinary Clinics: Food Animal Practice, 32(2), 511-522.

Hardeland R., Pandi-Perumal S.R., Cardinali D.P. (2006). Melatonin. Int J Biochem Cell Biol.;38(3):313-6. Epub 2005 Sep 27.

Hendriks S.J., Phyn C.V.C., Turner S-A., Mueller K.M., Kuhn-Sherlock B., Donaghy D.J., Huzzey J.M., Roche J.R. (2019). Lying behavior and activity during the transition period of clinically healthy grazing dairy cows. J Dairy Sci. 2019 Aug;102(8):7371-7384. DOI: https://doi.org/10.3168/jds.2018-16045

Hernandez L.L (2018). ADSA Foundation Scholar Award: A role for serotonin in lactation physiology-Where do we go from here? J Dairy Sci. 101(7):5671-5678. DOI: 10.3168/jds.2018-14562.

Hernández-Castellano L.E., Hernandez L.L., Bruckmaier R.M. (2019). Review: Endocrine pathways to regulate calcium homeostasis around parturition and the prevention of hypocalcemia in periparturient dairy cows. Animal. 24:1-9. DOI: 10.1017/S1751731119001605.

Ho Mien I., Chua E.C.P., Lau P., Tan L.C., Lee I.T.G., Yeo S.C., Tan S.S., Gooley J.J. (2014) Effects of Exposure to Intermittent versus Continuous Red Light on Human Circadian Rhythms, Melatonin Suppression, and Pupillary Constriction. PLOS ONE 9(5): e96532. DOI: https://doi.org/10.1371/journal.pone.0096532

Jin-Ryong P., Nam-Jin Y., Shah-Ahmed B., Kwan-Seob S. (2018). Effect of LED Lighting Time on Productivity, Blood Parameters and Immune Responses of Dairy Cows. Korean Journal of Organic Agriculture Vol.26 No.3 pp.515-532. DOI: https://doi.org/10.11625/KJOA.2018.26.3.515

Lacasse P., Ollier S, Lollivier V, Boutinaud M. (2016). New insights into the importance of prolactin in dairy ruminants. J Dairy Sci. 2016 Jan; 99(1):864-74. DOI: https://doi.org/10.3168/jds.2015-10035.

Lacasse P., Zhao X., Ollier S. (2019). Effect of stage of lactation and gestation on milking-induced hormone release in lactating dairy cows. Domestic Animal Endocrinology, Volume 66, Pages 72-85, ISSN 0739-7240. DOI: https://doi.org/10.1016/j.domaniend.2018.10.003.

Laporta J., Moore S.A., Peters M.W., Peters T.L., Hernandez L.L. (2013). Short communication: Circulating serotonin (5-HT) concentrations on day 1 of lactation as a potential predictor of transition-related disorders. J Dairy Sci. 96(8):5146-50. DOI: 10.3168/jds.2013-6718.

Mahjoubi, E., Zahmatkesh D, Hossein-Yasdi M, Khabbazan MH, Samadian MR (2018). Short communication: Test of the relationship between prepartum milk leakage and blood calcium concentration in Holstein cows—An observational study." Journal of Dairy Science 101(4): 3579-3582. DOI: 10.3168/jds.2017-13767.

Martinez, N., Risco, C. A., Lima, F. S., Bisinotto, R. S., Greco, L. F., Ribeiro, E. S., Maunsell, F. Galvão, K., Santos, J. E. P. (2012). Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. Journal of Dairy Science, 95(12), 7158-7172. DOI: https://doi.org/10.3168/jds.2012-5812.

National Organic Farming Handbook, National Organic Program (NOP). (2015). United States Department of Agriculture.

National Research Council (2001). Nutrient requirements of dairy cattle (7th rev. ed. Natl. Acad. Sci., Washington, DC.).

Oetzel, G. R. (2017). Diagnosis and management of subacute ruminal acidosis in dairy herds. Veterinary Clinics: Food Animal Practice, 33(3), 463-480.

Ospina, P. A., Nydam, D. V., Stokol, T., & Overton, T. R. (2010). Evaluation of nonesterified fatty acids and β-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. J. Dairy Sci. 93 :546–554. doi: 10.3168/jds.2009-2277

Özçelik R., Bruckmaier R.M., Hernández-Castellano L.E. (2017). Prepartum daylight exposure increases serum calcium concentrations in dairy cows at the onset of lactation. J Anim Sci. 95(10):4440-4447. DOI: 10.2527/jas2017.1834.

Penev T., Radev V., Slavov T., Kirov V., Dimov D.K., Atanassov A., Marinov I. (2014). Effect of lighting on the growth, development, behaviour, production and reproduction traits in dairy cows. Int. J. Curr. Microbiol. App. Sci (2014) 3(11) 798-810.

Pinedo P, Manriquez D, Solano G., Paudyal S., Velasquez A., Velez J. (2019). Effect of pulsed alternating wavelengths on melatonin, cortisol, and serotonin concentrations in Holstein heifers. American Dairy Science Association Annual Meeting, June 23–26, 2019 Cincinnati, Ohio. Journal of Dairy Science, Volume 102, Supplement 1.

Sordillo L.M. (2016). Nutritional strategies to optimize dairy cattle immunity. J Dairy Sci. 99(6):4967-4982. DOI: 10.3168/jds.2015-10354.

Weaver S.R., Laporta J., Moore S.A., Hernandez L.L. (2016). Serotonin and calcium homeostasis during the transition period. Domest Anim Endocrinol. 56 Suppl:S147-54. DOI: 10.1016/j.domaniend.2015.11.004.

World Health Organization & Joint FAO/WHO Expert Committee on Food Additives. Meeting (78th : 2013 : Geneva, Switzerland). (2014). Toxicological evaluation of certain veterinary drug residues in food / prepared by the seventy-eighth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). World Health Organization. https://apps.who.int/iris/handle/10665/128550.

Yang Y., Pan C., Zhong R., Pan J. (2018). Artificial light and biological responses of broiler chickens: dose-response. J Anim Sci. 96(1):98-107. doi: 10.1093/jas/skx044.

Zaborski D., Grzesiak W., Szatkowska I., Dybus A., Muszynska M., Jedrzejczak M. (2009). Factors Affecting Dystocia in Cattle. Reproduction in Domestic Animals; Volume 44, Issue 3. DOI: https://doi.org/10.1111/j.1439-0531.2008.01123.x