Heat stress and uterine disease: Stressors influencing reproduction of dairy cattle

by

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B.S., University of Wisconsin – River Falls, 2012
M.S., Mississippi State University, 2014

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2017

Abstract

Reproduction is an important part of a dairy operation that directly affects milk production and profitability. Fertility of high-producing dairy cows is less than desired. Reproductive efficiency is further impaired during summer months and after cows develop postpartum uterine disease. Heat stress and uterine disease act as stressors that negatively influence fertility of dairy cattle through a variety of mechanisms. This dissertation further investigates the negative effects of heat stress and uterine disease on reproduction, as well as examines two potential markers for predicting risk of developing uterine disease. Study 1 investigated the treatment of lactating dairy cows with gonadotropin-releasing hormone (GnRH) before first insemination during heat stress. Two experiments were performed to compare ovarian responses, pregnancy per artificial insemination (AI), and patterns of insemination of two estrus detection-based presynchronization protocols before first AI during summer heat stress. Treatment of cows with GnRH during summer heat stress altered ovarian response and pattern of insemination, however, did not improve pregnancy per AI. Study 2 evaluated ovarian response to treatment with GnRH and the odds of bearing a corpus luteum or being inseminated in cows with or without purulent vaginal discharge (PVD). Furthermore, hazard of insemination after administration of prostaglandin $F_{2\alpha}$ was evaluated in dairy cows with or without PVD. Ovarian response was altered in cows with PVD compared with cows without PVD. Odds of bearing a corpus luteum or being inseminated was not associated with PVD in primiparous cows, whereas it was associated with PVD in multiparous cows. Hazard of insemination after prostaglandin $F_{2\alpha}$ was not associated with PVD. Study 3 investigated arginase and matrix metalloproteinase-8 (MMP-8) as potential markers for metritis. Activity of arginase 7 days before parturition has been identified as a potential marker for the risk of developing metritis in dairy cows. In contrast, MMP-8 was not associated with the risk of

developing metritis, therefore, MMP-8 is not a good candidate as a marker for metritis. Further research is warranted in the areas of reproductive physiology, heat stress, and uterine disease because several unanswered questions still exist. Improving fertility during times of heat stress and after the occurrence of postpartum uterine disease will improve milk production, animal welfare, and profitability of dairy farms across the world.

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Acknowledgments

I want to thank:

My family, especially my parents and sisters, for all the love and support during the previous 3 years. The time away from home has been difficult, but also fulfilling. My goal has been to make you all proud and I hope that this is just another step in that process.

Dr. Barry Bradford and Dr. Deryl Troyer for being a part of my Ph.D. committee and helping me grow and learn as an animal scientist.

Dr. Jeffrey Stevenson for serving on my committee, but most important being a great mentor and resource whenever I had questions.

Dr. Luis Mendonça for being an outstanding advisor and mentor. I cannot say enough great things about the time and effort you put into shaping me as a student.

My fellow graduate students and lifelong friends. Your assistance with research and after-hours camaraderie have helped me get to where I am today.

Kansas State University for an excellent opportunity to learn and grow as a scholar.

Thank you!

Dedication

"You miss one hundred percent of the shots you don't take"

–Wayne Gretzky

This dissertation is dedicated to all of my family and friends. Only with their love and support was this dissertation possible, and for that I say thank you!

Chapter 1 - Review of the Literature – Heat Stress and Uterine Disease: Stressors Influencing Reproduction of Dairy Cattle

B. E. Voelz

Abstract

Reproduction is one of the most important components of a dairy operation, however, reproductive efficiency of high-producing dairy cattle is less than desired. Most notably, reproductive efficiency is poor during summer months. In addition, cows that develop postpartum uterine disease have impaired fertility. Heat stress and uterine disease act as stressors that negatively impact fertility through a variety of mechanisms. Heat stress has been demonstrated to be associated with decreased duration of estrus expression, impaired steroidogenesis, compromised oocyte quality and embryonic development, and increased pregnancy loss in dairy cattle. Similarly, uterine disease has been shown to have negative effects on the hypothalamus, ovary, and uterine environment; therefore, increasing the proportion of anovular cows and days to first service, and decreasing pregnancy per artificial insemination. Mitigation of heat stress through cooling systems may improve milk yield, however, a limited number of studies have demonstrated improvement in fertility. Furthermore, treatments for uterine diseases have been ineffective at repairing fertility. Efforts to identify an effective marker for predicting cows at an increased risk of uterine disease would be beneficial for preventing the disease or early treatment with therapeutics. Immune-derived enzymes such as arginase and matrix metalloproteinase-8 are two potential candidates for markers to predict increased risk of uterine disease. Identifying strategies to improve reproductive performance of cows under heat stress or afflicted with uterine disease will have an enormous impact on the profitability of the dairy industry.

Introduction

Reproductive performance of high-producing dairy cattle has been decreasing since the 1960's (Butler, 1998), however, a recent upward trend in daughter pregnancy rate might support

the hypothesis that fertility is improving (Wiltbank and Pursley, 2014). Improved reproductive efficiency of dairy cattle is desired and associated with greater profitability because of increased milk yield, increased calf sales, decreased replacement costs, and reduced reproductive costs (Britt, 1985; Cabrera, 2014). Nevertheless, reproductive efficiency continues to be less than desired in a majority of dairy herds, especially during summer.

Heat stress and postpartum uterine disease are two of the major problems associated with impaired fertility of dairy cattle. Dairy cows suffering from heat stress or postpartum uterine disease are predisposed to poor reproductive performance with limited options to improve fertility. In spite of current trends in fertility, dairy producers could achieve outstanding reproductive performance by decreasing the negative effects of heat stress and uterine disease. Genetic selection for increased milk production, greater metabolic and nutritional demand, increased heat stress because of global climate warming, and increased disease prevalence are all factors associated with decreased fertility of dairy cattle (Lucy, 2001). These factors act as stressors that can negatively influence reproductive performance of high-producing dairy cows. Moberg (2000) defined stress as a biological response elicited when an individual perceives a threat to its homeostasis. Stressors can be a chemical, biological agent, environmental condition, or any form of external stimulus on an organism that disrupts homeostasis (Moberg, 2000). Stress over extended periods of time (chronic stress) can negatively influence basic physiological processes such as behavior and performance. In the hierarchy of the stress response, reproductive performance seems to be one of the first physiological processes extinguished in mammals. This review of the literature will highlight and discuss the influence of heat stress and uterine disease on reproductive performance of dairy cattle.

Heat Stress

It is well documented that summer heat stress decreases reproductive efficiency of dairy cattle. The negative impacts of heat stress on reproduction in dairy cattle have been reviewed comprehensively (Thatcher, 1974; Fuquay, 1981; Wolfenson et al., 2000; De Rensis and Scaramuzzi, 2003; Jordan, 2003). Bernabucci et al. (2014) defined heat stress as a condition when an animal cannot dissipate adequate amounts of body heat in order to maintain thermal balance. In the United States, an estimated $897 million to $1.5 billion per year is lost in the dairy industry because of heat stress (St-Pierre et al., 2003). Decreased milk production, impaired fertility, increased culling rates, and increased mortality account for a majority of the estimated losses accrued by dairy producers (St-Pierre et al., 2003). Despite research efforts to understand and increase reproductive efficiency during times of heat stress, minimal gains in reproductive performance have been achieved during the past 40 years.

Heat stress has been demonstrated to decrease dry matter intake (DMI; West, 1994), with the most drastic decrease in DMI being observed in multiparous cows compared with primiparous cows (Holter et al., 1997). Decreased DMI and milk production are commonly observed when the temperature-humidity index (THI) reaches 72 (du Preez et al., 1990; Armstrong 1994), however, milk yield in high-producing dairy cows has been reported to decrease when minimum THI is as low as 65 or average THI is 68 (Zimbelman et al., 2009, Collier et al., 2011). For every unit increase in THI, milk yield has been estimated to decrease by 0.32 kg (Ingraham, 1979). In a recent study, milk yield was estimated to decline 0.2 kg for every unit increase in THI when THI was greater than 72 (Ravagnolo et al., 2000). Mitigating heat stress with the use of shade, fans, and sprinklers has improved milk yield during times of heat stress (Collier et al., 1981; Wolfenson et

al., 1988; Armstrong, 1994), whereas reproductive performance during heat stress has not been consistently improved by cooling systems (Hansen, 1997).

Detrimental Effects of Heat Stress on Reproduction

Heat stress has negative effects on expression and duration of estrus (Younas et al., 1993; Cartmill et al., 2001; Hansen et al., 2001), follicle development (Badinga et al., 1993; Wilson et al., 1988a,b), oocyte competence (Wolfenson et al., 2000), fertilization risk (Sartori et al., 2002), embryonic development (Biggers et al., 1987; Edwards and Hansen, 1996; Drost et al., 1999), and pregnancy loss (Cartmill et al., 2001; Santos et al., 2004). Cows exposed to heat stress have altered growth and function of ovarian follicles as observed by reduced size of the first- and second-wave dominant follicles (Badinga et al., 1993; Wilson et al., 1998a,b). In contrast, during heat stress, an increased incidence of large, persistent follicles has been observed (Badinga et al., 1993, 1994; Wolfenson et al., 2000). Decreased steroidogenic capability of follicles has been demonstrated in cows (Wilson et al., 1998b) and heifers (Wilson et al., 1998a) that were exposed to heat stress. Bridges et al. (2005) performed an *in vitro* study utilizing theca interna and granulosa cells (follicular cells) cultured with luteinizing hormone (LH) and follicle stimulating hormone (FSH). Follicular cells were exposed to different temperatures (37, 39, and 41°C) during the culture process to imitate heat stress conditions. Follicular cells exposed to 41°C produced less androstenedione and estradiol compared with follicular cells exposed to 37°C and 39°C (Bridges et al., 2005). Furthermore, *in vivo* studies also have demonstrated decreased concentrations of estradiol in follicular fluid (Badinga et al., 1994) and decreased activity of aromatase in granulosa cells of heat-stressed dairy cows (Badinga et al., 1993).

Decreased steroidogenesis, especially estradiol, could explain some of the observed decreases in intensity and duration of expression of estrus (Younas et al., 1993; Cartmill et al., 2001; Hansen et al., 2001). Another negative effect of heat stress on the hormonal profile of dairy cows is a decrease in magnitude of the LH surge in cows expressing estrus. Secretion of LH has been shown to fluctuate with season. The greatest secretion of LH occurs during the spring equinox and gradually decreases until the autumn equinox (Day et al., 1986). Day et al. (1986) reported a tendency to increase amplitude of LH pulses in cows treated with estradiol compared with non-treated cows; therefore, increasing mean concentrations of LH. Heat stress could be a contributing factor to decreased LH secretion during summer, however, it is unknown whether the impact of heat stress on steroidogenesis and season of the year have additive effects on decreased LH secretion. Nonetheless, GnRH-induced LH release has been demonstrated to be reduced in heat-stressed cows (Gilad et al., 1993). Considering there is evidence of impaired response to GnRH during summer, it is possible that efficacy of reproductive management programs used in the United States may be affected during summer because the majority of these programs utilize GnRH to induce ovulation.

Regardless of steroidogenesis, quality and competence of the oocyte is also a vital concern in heat-stressed dairy cattle. Oocytes collected from cows exposed to summer heat stress have impaired development to the blastocyst stage when fertilized *in vitro* (Rocha et al., 1998; Al-Katanani et al., 2002). Oocytes exposed to heat shock during an *in vitro* maturation process also have decreased cleavage rates and impaired development to the blastocyst stage (Edwards and Hansen, 1997; Roth and Hansen, 2005). Compromised oocyte quality explains some of the carryover effects on fertility observed in the autumn after summer heat stress has subsided (Wolfenson et al., 2000).

Besides potential problems with oocyte quality, fertilization risk during summer is decreased in lactating dairy cows compared with heifers (Satori et al., 2002). In contrast, Ryan et al. (1993) reported no difference in fertilization risk during the hot and cool seasons, whereas proportion of embryos recovered from d 7 to 14 during the warm season was significantly decreased (59 vs. 27%). Edwards and Hansen (1996) demonstrated reduced number of embryos cleaving and developing to the blastocyst stage when exposed to heat shock. Embryos exposed to heat shock had increased production of heat shock protein 70 (Edwards and Hansen, 1996), a protein believed to increase the thermotolerance of oocytes and embryos (Hendrey and Kola, 1991). Furthermore, heat shock can induce apoptosis and caspase activity in ≥16 cell embryos (Paula-Lopes and Hansen, 2002). The previous authors suggested that apoptosis could be induced in preimplantation embryos of cows exposed to heat stress (Paula-Lopes and Hansen, 2002). Because of the detrimental effects of heat stress on the embryo, early embryonic loss seems to be greater during times of heat stress (Santos et al., 2004). Furthermore, fetal loss is increased during times of heat stress compared with cows not exposed to heat stress (Cartmill et al., 2001; Santos et al., 2004). Development of strategies to improve fertility during times of heat stress, specifically if the oocyte or embryo is compromised, presents difficult challenges for researchers and the dairy industry.

Strategies to Improve Fertility in Heat-stressed Cows

Various strategies have been implemented in an attempt to improve fertility in heat-stressed dairy cattle, most notably heat abatement techniques and use of exogenous reproductive hormones for estrous cycle manipulation (Jordan, 2001). Use of estrus synchronization protocols has become more common since the introduction of Ovsynch (Pursley et al., 1995). Inclusion of

presynchronization in synchronization protocols has drastically improved the effectiveness of these protocols (Souza et al., 2008; Stevenson and Pulley, 2012). In recent years, estrous synchronization protocols have been investigated as a possible strategy to improve fertility of first AI during heat stress (Friedman et al., 2011; Stevenson and Pulley, 2012; Dirandeh et al., 2015).

Stevenson and Pulley (2012) performed a study that investigated presynchronization with PG–3–G ($PGF_{2\alpha}$ – 3 d – GnRH) or Presynch-10 ($PGF_{2\alpha}$–10 d–$PGF_{2\alpha}$) before an Ovsynch protocol. Cows treated with PG–3–G during summer (June through September) had greater pregnancy per artificial insemination (P/AI) at 32 to 38 d after artificial insemination (AI) compared with Presynch-10 treated cows (35.9 vs. 26.7%). During the cool months of the year (October through May), no difference in P/AI was detected between PG–3–G and Presynch-10 treated cows (46.8 vs. 44.3%, respectively; Stevenson and Pulley, 2012).

In a more recent study, Dirandeh et al. (2015) evaluated three estrous synchronization protocols, Double-Ovsynch (DO), Presynch-GnRH-Ovsynch (PGO), and Presynch-Ovsynch (PO), in lactating dairy cows during summer heat stress. Cows treated with DO had a greater proportion of cows with a corpus luteum at the initiation of Ovsynch and a greater response to the first GnRH injection of the Ovsynch protocol compared with PGO and PO treated cows. Pregnancy per AI at 32 d after AI was greater for cows treated with DO compared with PGO and PO cows (23.2, 16.7, and 12.4% respectively; Dirandeh et al., 2015). Furthermore, P/AI differed among treatments at 60 d after AI. Cows submitted to the DO protocol had greater P/AI than cows submitted to the PGO and PO protocols (21.6, 15.6, and 11.4%, respectively; Dirandeh et al., 2015). When considering only cows that were synchronized after submission to the protocols, P/AI differed at 32 and 60 d after AI. Double-Ovsynch cows had greater P/AI than both PGO and PO cows (Dirandeh et al., 2015).

Using a unique approach, Friedman et al. (2011) utilized a combination of PGF2α and GnRH to induce 3 successive follicular waves in intervals of approximately 9 d. After the third follicular wave, cows were administered PGF2α and inseminated after detection of estrus. Induction of successive follicular waves improved fertility of primiparous cows and cows with body condition score >2 during summer. Turnover of the follicle pool using GnRH during heat stress could be a possible strategy to improve fertility (Friedman et al. 2011).

Previous research supports the idea that GnRH–$PGF_{2\alpha}$–based presynchronization protocols applied before first AI are more effective at improving fertility during summer than presynchronization protocols that rely soley on $PGF_{2\alpha}$ (Friedman et al., 2011; Stevenson and Pulley, 2012; Dirandeh et al., 2015). Presynchronization protocols using GnRH likely induce ovulation, therefore, possibly increasing concentrations of progesterone via an accessory corpus luteum. Dirandeh et al. (2015) speculated that greater fertility with DO during heat stress could be because of an increased concentration of progesterone during the development of the ovulatory follicle. Ayres et al. (2013) demonstrated that concentration of progesterone was increased during the growth of the preovulatory follicle of cows treated with DO compared with Presynch-Ovsynch. Oocyte and embryo quality are associated with the concentration of progesterone during final follicular development. Super-stimulated cows that had greater concentration of progesterone before AI produced a greater proportion of excellent, good, and fair embryos 6.5 d after AI compared with cows with low concentration of progesterone (Rivera et al., 2011). Denicol et al. (2012) demonstrated that P/AI was greater in cows with follicles growing in a high progesterone environment before timed AI compared with cows with follicles growing in a low progesterone environment (Denicol et al., 2012). The effects of the progesterone environment on oocyte quality and P/AI during heat stress conditions has not been investigated in dairy cattle.

Presynchronization protocols that utilize GnRH may induce ovulation in anovular cows, potentially explaining some of the improved fertility observed in heat–stressed dairy cows. During summer months, dairy cows are at a greater risk of being anovulatory (López–Gatius, 2003). Cows that are anovular before exposure to first AI have decreased fertility (Bisinotto et al., 2010), therefore, presynchronization programs that use GnRH to induce ovulation may improve fertility. Because improved fertility has been reported in cows that are inseminated based on detection of estrus compared with timed AI (Kasimanickam et al., 2005), investigating presynchronization programs that use GnRH but also depend on detection of estrus for facilitating AI could be of interest.

Wolfenson et al. (2000) suggested that heat stress might reduce peripheral concentration of progesterone. Because progesterone is essential for the establishment and maintenance of pregnancy, concentration of progesterone after AI during heat stress also might impact fertility. Treatment of GnRH agonists or human chorionic gonadotropin (**hCG**) have been utilized to induce accessory corpus lutea in an attempt to increase peripheral progesterone (Schmitt et al., 1996). In cows exposed to mild heat stress, injection of GnRH 5 or 11 d after insemination increased total luteal volume and concentration of progesterone on d 17 after insemination compared with cows that were not treated with GnRH (Willard et al., 2003). Pregnancy per AI tended to be increased in cows treated with GnRH on d 5 or 11 after insemination compared with untreated controls (Willard et al., 2003). Nevertheless, an inadequate sample size (approximately 34 cows per treatment) limits the ability to elucidate the effects of GnRH after AI during heat stress (Willard et al., 2003). Intravaginal placement of a controlled internal drug-release (**CIDR**) insert can supplement progesterone after insemination. Friedman et al. (2012) demonstrated that insertion of a CIDR 5 d after AI during summer improved P/AI of a subpopulation of fertility-impaired cows

(Friedman et al., 2012, 2014). Cows with low BCS (≤2.25) and uterine disease had decreased time to pregnancy when treated with a CIDR 5 d after AI when compared with untreated controls (Friedman et al., 2014). Use of hormonal treatments to increase concentration of progesterone after AI might have benefits during summer heat stress, however, the benefit seems to only be in a subpopulation of cows with predisposed infertility not caused by heat stress.

Uterine Diseases

Postpartum uterine disease is a major stressor that contributes to impaired and reduced fertility in dairy cattle (Crowe and Williams, 2012). Retained fetal membranes (RFM), metritis, and clinical endometritis are the three major diseases encompassing uterine disease after calving. Several risk factors are associated with the development of uterine diseases. Parity, dystocia, abortion, gender of calf, twinning, stillbirths, prolapsed uterus, body condition score, negative energy balance, hypocalcemia, and other stressors can predispose cows to a greater risk of developing uterine disease (Dubuc et al., 2010). Uterine disease has been shown to have negative effects on fertility and reproductive performance of dairy cattle (Leblanc et al., 2002; Gilbert et al., 2005; McDougall et al., 2007). Besides reduced fertility, uterine diseases also are associated with decreased milk production (Rajala and Gröhn, 1998; Dubuc et al., 2011) and an increased risk of being culled from the herd (Dubuc et al., 2011). Understanding the pathophysiology of uterine diseases, as well as the mechanisms that influence fertility, are important for developing interventions to improve reproductive efficiency.

Retained Fetal Membranes

Several accepted definitions for RFM exist, however, the majority of studies define RFM as the failure to expel the fetal membranes by 12 to 24 h after calving (Stevenson and Call, 1988; Kelton et al., 1998; Van Werven et al., 1992). The most common on-farm definition of RFM is failure to expel fetal membranes within 24 h of parturition. Using the 24 h timeframe is acceptable because 95% of cows that expel the fetal membranes by 24 h have already done so by 12 h (Van Werven et al., 1992); therefore, it is widely accepted to use 24 h as the reference time point for research purposes.

The pathophysiology of RFM is multifaceted. Dairy cattle have a placenta that consists of fetal cotyledons attached to the maternal caruncles that together form placentomes (Beagley et al., 2010). The cotyledon and caruncle are connected by collagen and the connection likely requires the breakdown of collagen for separation (Eiler and Hopkins, 1993), with failure of separation resulting in RFM. Several factors during the process of parturition might be associated with the pathophysiology of RFM (Beagely et al 2010). Regression of the corpus luteum during the process of parturition causes secretion of relaxin and decreased concentration of progesterone (Musah et al., 1987). Relaxin softens the cervix, relaxes the pelvic ligaments, and promotes breakdown of collagen (Musah et al., 1987). Decrease in progesterone promotes collagenase activity that is believed to be vital for fetal membrane detachment (Maj and Kankofer, 1997). Researchers also have suggested serotonin as a potential player in fetal membrane detachment (Eiler and Hopkins, 1993; Fecteau and Eiler, 2001). Increased concentrations of fetal and placental serotonin could promote cell proliferation of the placenta (Fecteau and Eiler, 2001) and inhibit collagenase activity (Eiler and Hopkins, 1993). In addition, immune function is important for fetal membrane separation in dairy cows. During pregnancy and the periparturient period, the immune system of

cows is suppressed (Kehrli et al., 1989). Kimura et al. (2002) demonstrated that decreased function of neutrophils is associated with RFM in dairy cattle. Leukocyte chemotaxis also has been shown to be increased in cows that do not develop RFM (Gunnink, 1984; Heuwieser and Grunert, 1987). Interleukin-8, an important chemokine involved in the chemotaxis process, is decreased in cows with RFM compared with cows without RFM (Kimura et al., 2002). Furthermore, cows with RFM have been shown to have decreased counts of peripheral neutrophils at the time of parturition compared with healthy control cows (Moretti et al., 2015, 2016).

Successful treatment options for RFM to improve fertility of dairy cows have not yet been discovered. Manual removal of RFM is a practice that has been investigated as a possible treatment option, however, previous research has failed to demonstrate an increase in milk production or fertility (Beagley et al., 2010). Furthermore, treatment with intrauterine antibiotics did not improve milk production or fertility in cows with RFM compared with cows without RFM (Drillich et al., 2006, 2007). Treatment with selenium and vitamin E has been shown to decrease the incidence of RFM (Julien et al., 1976; Bourne et al., 2007), however, incidence of RFM seems to only benefit cows that might be deficient in selenium (Gupta et al., 2005) or vitamin E (Bourne et al., 2007) before supplementation. Because fertility is impaired in cows that develop RFM creating management practices to prevent or reduce its occurrence are warranted.

Metritis

Puerperal metritis is defined as a cow with an abnormally enlarged uterus accompanied by fetid reddish-brown discharge during 21 d after parturition (Sheldon et al., 2006). Sheldon et al. (2006) proposed that clinical systemic signs of illness also are associated with puerperal metritis including fever >39.5°C, decreased milk production, and lethargic behavior. Clinical metritis is

defined as a cow free from visual and systemic signs of illness, however, has an enlarged uterus and fetid reddish-brown vaginal discharge during 21 d postpartum (Sheldon et al., 2006).

After parturition, bacteria invade the uterus while the cervix is dilated, with as high as 93% of cows having bacteria present during 2 wk postpartum (Sheldon et al., 2002). The main uterine pathogens isolated from the uterus are *E. coli* and *A. pyogenes* (Sheldon et al., 2002; Williams et al., 2005). The majority of cows spontaneously resolve the bacterial infection, however, approximately 10 to 20% of cows will develop metritis (Williams et al., 2008). The first defense against these pathogens is the innate immune system. The innate immune system relies on receptors, such as Toll-like receptors, to recognize pathogen-associated molecular patterns and activate the immune response. As mentioned previously, immune function and peripartum responses are impaired in dairy cows and believed to be one of the main causes of metritis (Hammon et al., 2006). Martinez et al. (2012) demonstrated that cows with subclinical hypocalcemia had decreased concentration of peripheral neutrophils in blood and decreased neutrophil function. Furthermore, cows with subclinical hypocalcemia had a greater risk of developing metritis (Martinez et al., 2012). Martinez et al. (2016) evaluated the effects of oral calcium supplementation after parturition on milk production and reproductive performance. Primiparous cows supplemented with oral calcium after partition had decreased P/AI and increased days to pregnancy compared with controls (Martinez et al., 2016). In contrast, multiparous cows supplemented oral calcium after parturition had increased P/AI and decreased days to pregnancy compared with controls (Martinez et al., 2016). Blanket treatment with oral calcium after parturition seems to have some benefits on milk yield and reproduction for a subpopulation of cows, whereas it has a detrimental effect on reproduction in another subpopulation of cows (Martinez et al., 2016).

The ability to detect cows at greater risk for metritis would be important for the development of interventions aimed at preventing the disease. Several factors are associated with the development of metritis, but no markers have been found at this time. Several studies have demonstrated an association between increased concentrations of haptoglobin after parturition and a greater risk of metritis (Smith et al., 1998; Sheldon et al., 2001; Huzzey et al., 2009). Huzzey et al. (2009) reported that cows with concentrations of haptoglobin ≥1 g/L at 3 d postpartum were 6.7 times more likely to develop metritis. When using haptoglobin ≥1 g/L as a cut-off, the test had a sensitivity of 50% and a specificity of 87% for predicting cases of metritis (Huzzey et al., 2009). Decreased DMI and feeding time are associated with an increased risk of developing metritis in dairy cows (Huzzey et al., 2007). Decreased DMI may result in a greater level of negative energy balance because it has been shown to be associated with metritis (Hammon et al., 2006). Increased concentrations of non-esterified fatty acids, which are associated with negative energy balance, are associated with risk of metritis (Hammon et al., 2006). Discovery of a specific biomarker that can better predict risk of metritis before parturition would be ideal. Because immune function is impaired and is believed to be the main cause of metritis, finding an immune-associated marker seems to be a logical approach to identify cows with greater risk of having metritis. Identifying this subpopulation of cows may allow the use of strategies to improve the cows' immune system, which may decrease the incidence of metritis, and consequently, minimize the negative effects of metritis on fertility of a dairy herd.

Clinical Endometritis

Clinical endometritis is defined as the presence of purulent or mucopurulent discharge in the vagina after 21 d postpartum (Sheldon et al., 2006). Diagnosis of clinical endometritis can be

performed using vaginoscopy, gloved-hand, or the Metricheck device to examine the presence of pus in the vagina (LeBlanc et al., 2002, Sheldon et al., 2006). Discharge obtained from the vagina can be classified using a 0 to 3 scale (Williams et al., 2005) based on characteristics of the vaginal mucus. For example, a score of 0 means the mucus is clear with no signs of pus, whereas a score of 1 classifies the mucus as mostly clear but contains small flecks of white. Scores of 2 represent that <50% of the discharge contains white pus and scores of 3 classify the discharge as being ≥50% purulent white pus (Williams et al., 2005). Cows with scores of 0 or 1 are commonly classified as being negative for clinical endometritis, whereas cows with scores of 2 or 3 are classified as being positive for clinical endometritis (Williams et al., 2005).

Presence of uterine disease during the early postpartum period, such as RFM and metritis, are associated with increased risk of clinical endometritis (LeBlanc et al., 2002; Sheldon et al., 2006). Of particular interest though are the large proportion of cows without a previous history of uterine disease that still develop clinical endometritis. (Sheldon et al., 2006). A recent study identified RFM, dystocia, stillbirths, parity, calf gender, and vulva angle as risk factors for clinical endometritis, but cleanliness of the environment and animal were not associated with risk of clinical endometritis (Potter et al., 2010). The previous authors concluded that risk factors of clinical endometritis seem to be associated with trauma of the reproductive tract or damage to the physical barriers that prevent infection instead of the cleanliness of the environment or animal (Potter et al., 2010).

Galvão et al. (2011) investigated the association between endometritis and inflammatory markers of the uterine endometrium between parturition to 7 wk after calving. At parturition, expression of tumor necrosis factor-α was decreased in cows that developed endometritis compared with control cows (Galvão et al., 2011). Expression of interleukin-1β tended to be

decreased 1 wk after parturition, but tended to be increased 5 and 7 wk after parturition in cows with endometritis compared with controls (Galvão et al., 2011). Interleukin-6 expression was increased at parturition and 7 wk after parturition in cows that developed endometritis compared with controls (Galvão et al., 2011). Cows with endometritis had increased expression of interleukin-8 7 wk after parturition compared with controls, whereas expression of interleukin-10 was not associated with endometritis (Galvão et al., 2011). Expression of cytokines in the uterine endometrium from parturition to 7 weeks after parturition seem to be associated with the risk of developing endometritis (Galvão et al., 2011).

Detrimental Effects of Uterine Diseases on Reproduction

The negative effects of uterine disease on reproduction are immense. Uterine disease increases proportion of anovular cows (Ribeiro et al., 2016), days to first service (LeBlanc et al., 2002), decreases P/AI (Holt et al., 1989; LeBlanc et al., 2002; McDougall et al., 2007), and increases days to pregnancy (Holt et al., 1989; LeBlanc et al., 2002; Gilbert et al., 2005; McDougall et al., 2007). Uterine infection is associated with impaired ovarian function, prolonged anestrous, and cystic follicles (Mateus et al., 2002). Cows with uterine disease have decreased rate of follicle growth, decreased plasma concentration of estradiol (Sheldon et al., 2002), and altered lifespan of the corpus luteum after ovulation (Mateus et al., 2003; Lüttgenau et al., 2016). Furthermore, exposure to lipopolysaccharide (LPS) has been shown to alter endocrine function at the level of the ovary (Battaglia et al., 2000; Herath et al., 2007) and the hypothalamus (Peter et al., 1989; Battaglia et al., 2000). Ewes (Battaglia et al., 2000) and heifers (Peter et al., 1989) exposed to LPS had decreased LH secretion and impaired ovulatory response. Herath et al. (2007) demonstrated that granulosa cells express immune mediator genes such as Toll-like receptor-4 and CD14.

Furthermore, granulosa cells exposed to LPS produce less estradiol compared with untreated controls (Herath et al., 2007).

Ribeiro et al. (2016) conducted several experiments investigating the carryover effects of inflammatory diseases, such as uterine diseases, on reproduction. Cows diagnosed with uterine disease have decreased ovulatory response, greater risk of being anovular, and decreased fertility (Ribeiro et al., 2016). Uterine disease was associated with fewer numbers of cleaved and live embryos, and decreased quality of embryos collected 6 d after AI (Ribeiro et al., 2016). Furthermore, uterine disease impaired conceptus elongation and decreased secretion of interferon-τ in the lumen of the uterus (Ribeiro et al., 2016). The negative effects of uterine disease persists for extended periods of time, even after the disease has subsided.

Estrous synchronization protocols are commonly used in the dairy industry to facilitate AI and improve reproductive efficiency. Synchronization protocols use exogenous reproductive hormones to induce ovulation and cause luteolysis. As previously discussed, uterine disease may have negative effects on the release of LH (Peter et al., 1989), follicular growth (Battaglia et al., 2000), and uterine environment (Ribeiro et al., 2016). Effects of uterine disease that impact the efficacy of estrus-synchronization protocols is not completely clear. Bittar et al. (2014) investigated the effect of treatment with GnRH during the early postpartum period on uterine health and fertility of dairy cows. Cows were treated with GnRH at 17 ± 3 and 20 ± 3 days in milk (**DIM**) or received no treatment and remained as controls. Treatment with GnRH did not improve P/AI or hazard of pregnancy. In contrast, an interaction between treatment with GnRH and ovulation was detected. Cows that ovulated in response to GnRH had increased hazard of pregnancy by 300 DIM compared with controls and cows treated with GnRH that did not ovulate (Bittar et al., 2014). One could speculate that cows with uterine disease were less likely to ovulate

in response to GnRH and could possibly represent a proportion of the sub-fertile cows identified. Nevertheless, more research is required to investigate how uterine disease influences the success of estrus-synchronization protocols.

Candidates for Markers to Predict Uterine Disease

Attempts to counteract the negative effects of uterine disease on fertility have been unsuccessful (Galvão et al., 2009; Lima et al., 2013). With that in mind, the most logical approach to improve reproductive efficiency would be to prevent or reduce uterine disease altogether. In order to prevent uterine disease, it is important to be able to identify cows at greater risk for developing the disease. Discovering a biomarker that can accurately predict risk of uterine disease would be the first step in the process of improving reproductive performance of dairy cows. Immune function has a vital role in the pathophysiology of uterine diseases (Kehrli et al., 1989; Kimura et al., 2002; Hammon et al., 2006), therefore, markers associated with the immune function could be possible candidates for biomarkers.

Arginase is an enzyme that converts L-arginine to L-ornithine and urea during the urea cycle. In addition, arginase is expressed by various cells of the immune system, specifically neutrophils (Munder, 2009). Depletion of L-arginine and increased expression of arginase has been associated with immunosuppression in humans (Bronte and Zanovello, 2005). The immune system is suppressed during gestation and the periparturient period in dairy cattle (Goff and Horst, 1997). Immunosuppression during these critical times periods is not completely understood. Furthermore, arginase has not been investigated in dairy cattle. Level of immunosuppression could play a role in the development of uterine disease, which makes arginase an intriguing candidate to be evaluated during the periparturient period.

Matrix metalloproteinase-8 (MMP-8), which is commonly referred to as neutrophil collagenase, is another enzyme that may be associated with the development of uterine disease. Members of the matrix metalloproteinase family have been investigated and linked to the pathophysiology of RFM (Gross et al., 1985; Fecteau and Eiler, 1996; Maj and Kankofer, 1997; Walters and Boos, 2001). Fecteau and Eiler (1996) suggested that collagenase is required for the release, separation, and expulsion of the fetal membranes in cattle. Little is known about MMP-8 and its potential role in the development of RFM in dairy cattle. Because MMP-8 is secreted by neutrophils and previous research indicates a possible role of MMP-8 in placental detachment, further research is warranted to investigate MMP-8 as a possible marker for uterine disease.

Summary

Several stressors negatively impact reproduction of dairy cattle. As research in this area continues, focus should be placed on understanding the mechanisms of stressors that decrease fertility and identifying markers that can accurately predict disease. Identifying subpopulations of cows at greater risk for developing uterine disease may allow the use of strategies to decrease incidence of disorders in order to improve fertility in the most cost-effective manner. In addition, blanket treatments or interventions to mitigate the negative effects of uterine disease may be detrimental to non-diseased cows in some instances, which increases the value of targeting interventions to a specific subpopulation of cows.

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Chapter 2 - Treatment of Lactating Dairy Cows with Gonadotropin-Releasing Hormone before First Insemination during Summer Heat Stress

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2016. J Dairy Sci 99: 7612–7623.

Abstract

The objectives of the experiments were to compare ovarian responses, pregnancy per AI (**P/AI**), and pattern of insemination of 2 estrus detection-based presynchronization protocols before first AI during heat stress. In experiment 1, primiparous lactating dairy cows (n = 1,358) from 3 dairies were assigned randomly to 2 treatments at 60 ± 3 DIM (study d 0): 1) treatment with 100 µg of GnRH on study d 0 (**Gpresynch**); or 2) no treatment on study d 0 (**Control**). In experiment 2, multiparous lactating dairy cows (n = 1,971) from 3 dairies were assigned randomly to 2 treatments at 49 ± 3 DIM (study d 0), similar to experiment 1. In both experiments, $PGF_{2\alpha}$ injections were administered 14 d apart starting on study d 7 for all cows. Cows not inseminated after detection of estrus were submitted to a timed AI protocol at study d 35. In a subgroup of cows from 2 dairies, concentrations of progesterone were determined from blood samples collected on study d 0 and 7. Furthermore, ovaries were examined by ultrasonography on study d −14, 0, and 7 to determine cyclic status and ovulation in response to GnRH treatment. In experiment 1, progesterone concentration was not different on d 0, but progesterone was increased for Gpresynch compared with control cows on study d 7 (3.6 ± 0.3 vs. 2.7 ± 0.4 ng/mL), respectively. Ovulation risk from study d 0 to d 7 was increased for Gpresynch compared with control (50.6 vs. 15.2%). Control cows were inseminated at a faster rate than Gpresynch cows (adjusted hazard ratio [AHR] = 0.89, 95% CI = 0.80 to 1.00) and the interaction between treatment and dairy affected P/AI at 36 and 94 d post AI. In experiment 2, concentrations of progesterone did not differ on study d 0 or 7, despite ovulation risk from study d 0 to 7 being greater in Gpresynch than control cows (46.9 vs. 23.8%). The interaction between treatment and dairy affected hazard of insemination with Gpresynch cows from dairy 1 (AHR = 1.21; 1.05 to 1.41) being inseminated faster than control cows. Hazard of pregnancy was affected by treatment because Gpresynch cows became pregnant

at a faster rate than control cows (AHR = 1.25; 1.04 to 1.50). In conclusion, GnRH-based presynchronization protocols initiated before the end of the voluntary waiting period may have benefits in reproductive efficiency of estrus detection-based programs during heat stress. In addition, treatment with GnRH decreased the prevalence of anovular cows at the initiation of $PGF_{2\alpha}$ injections.

Introduction

Reproductive efficiency in dairy cattle has steadily declined during the past 45 years (Lucy, 2001), with declines in reproductive performance greatest during summer because of heat stress (De Rensis and Scaramuzzi, 2003). Poor oocyte and embryo quality, decreased fertilization rates (Sartori et al., 2002), and increased embryonic and fetal mortality (Santos et al., 2004) partly explain the decreased fertility in cows exposed to heat stress during summer. The direct effect of heat stress on the gonads is one mechanism by which heat stress impacts fertility. Heat-stressed dairy cows have altered hormone production and metabolism, which induce changes in the follicular micro-environment (Leroy et al., 2011).

The progesterone environment in which a follicle develops is believed to affect the quality of oocyte, and consequently, embryo quality (Wiltbank et al., 2014). Rivera et al. (2011) demonstrated that super-stimulated cows that had greater progesterone concentration before AI had a greater percentage of excellent/good and fair embryos recovered 6.5 days after AI, compared with cows with lesser concentration of progesterone. Furthermore, increased pregnancy per AI (**P/AI**) was detected in cows with follicles growing under greater concentration of progesterone before AI (Denicol et al., 2012). Although greater concentration of progesterone before AI has been studied in cows submitted to timed-AI (**TAI**) protocols, little is known about greater

concentration of progesterone in cows inseminated based on estrus (no TAI). Furthermore, others (Rivera et al., 2011; Denicol et al., 2012) did not investigate whether greater concentration of progesterone during dominant follicle selection had an effect during periods of summer heat stress, a time in which improving oocyte and embryo quality would be desired.

Several studies demonstrated that GnRH-$PGF_{2\alpha}$-based presynchronization protocols for first TAI increased P/AI compared with only using $PGF_{2\alpha}$ in lactating dairy cows (Stevenson et al., 2012; Ayres et al., 2013; Dirandeh et al., 2015). Cows exposed to presynchronization with $PGF_{2\alpha}$ followed in 3 d by GnRH (**PG-3-G**), 7 d before Ovsynch, had greater P/AI compared with $PGF_{2\alpha}$-based presynchronization (Stevenson et al., 2012). The percentage of cows bearing a corpus luteum (**CL**) and progesterone concentration ≥ 1 ng/mL was greater in the PG-3-G cows than the $PGF_{2\alpha}$-based presynchronized controls at the time of the first GnRH injection of the TAI protocol (Stevenson and Pulley, 2012), indicating improved synchrony of the estrous cycle. Improved fertility of cows synchronized with a GnRH-$PGF_{2\alpha}$-based presynchronization protocol compared with a $PGF_{2\alpha}$-based presynchronization protocol was partly related to the increased concentration of progesterone during dominant follicle selection (Ayres et al., 2013).

A study conducted during summer demonstrated that synchronizing the first TAI with Double-Ovsynch, a GnRH-$PGF_{2\alpha}$-based presynchronization protocol, improved fertility in dairy cows compared with the Presynch-Ovsynch, $PGF_{2\alpha}$-based presynchronization protocol (Dirandeh et al., 2015). Although concentrations of progesterone were not evaluated, a greater proportion of Double-Ovsynch cows had a CL present at the first GnRH of the TAI protocol, which may have resulted in greater concentration of progesterone during dominant follicle selection before AI (Dirandeh et al., 2015). The reported improved fertility of cows submitted to PG-3-G (Stevenson and Pulley, 2012) and Double-Ovsynch (Dirandeh et al., 2015) during summer may be a result of

increased concentration of progesterone as previously suggested (Ayres et al., 2013). In contrast, the improved fertility may be consequence of increased fertility of anovular cows. The additional and multiple injections of GnRH in the PG-3-G and Double-Ovsynch protocols, respectively, are likely to induce ovulation during the protocol, which is believed to be beneficial for anovular cows. Because anovulation before first AI is associated with poor fertility (Bisinotto et al., 2010), inducing ovulation with an injection of GnRH during presynchronization protocols may have an additional benefit to anovular cows compared with protocols based only on $PGF_{2\alpha}$. Despite the potential benefits of using GnRH in presynchronization protocols, herds that strive to inseminate cows based on estrus detection may choose to use $PGF_{2\alpha}$-based presynchronization protocols in lieu of GnRH-$PGF_{2\alpha}$-based protocols because GnRH suppresses signs of estrus (Mendonça et al., 2012; Bruno et al., 2013; Chebel et al., 2013). Thus, a presynchronization protocol that induces estrus expression and ovulation of anovular cows, and increases concentration of progesterone during dominant follicle selection may improve reproductive efficiency during summer heat stress in herds that submit cows to AI largely based on estrus detection.

We hypothesized that cows treated with GnRH before an estrus detection-based presynchronization protocol would induce ovarian cyclicity in anovular cows, increase concentration of progesterone, and improve P/AI of heat-stressed cows. The objectives of the experiments were to compare ovarian response, P/AI, and pattern of insemination of 2 estrus detection-based presynchronization protocols for first insemination during summer heat stress.

Materials and Methods

Cows, Housing, and Feeding

These experiments were conducted in 3 commercial dairy herds. Dairy 1 was located in the panhandle of Oklahoma, and dairies 2 and 3 were located in southwest Kansas. Lactating Holstein dairy cows in the 3 herds were enrolled in the experiments from June 2014 through September 2014. Cows from dairy 1 were housed in dry-lot corrals without shade and were milked thrice daily with a projected 305-d milk yield of 8,400 kg. Cows from dairy 2 were housed in dry-lot corrals with shade and were milked twice daily with a projected 305-d milk yield of 8,904 kg. Cows from dairy 3 were housed in free-stall barns equipped with fans and sprinklers, with access to a dirt exercise lot, and milked thrice daily with a projected 305-d milk yield of 9,017 kg. The 3 herds were fed a TMR once daily with *ad libitum* access to feed and water. Milk yields were recorded at each milking using a parlor management software program (DairyPlan C21, GEA Farm Technologies, Naperville, IL). Average daily milk yield during the week before enrollment was extracted from the on-farm software.

Temperature and Relative Humidity Measurements

Temperature and relative humidity measurements were collected from the closest official meteorological stations to the dairy farms. Temperature and relative humidity measurements for dairy 1 was collected from a meteorological station located in Clayton, New Mexico, and measurements for dairy 2 and 3 were collected from a meteorological station located in Liberal, Kansas. Using the measurements collected, hourly temperature-humidity index (**THI**) was calculated by the following formula (NOAA, 1976): T - (0.55 - (0.55 RH/100) x (T-58)); where T and RH are dry bulb temperature (°F) and relative humidity, respectively.

Experiment 1 – Primiparous Cows

For experiment 1, weekly cohorts of primiparous cows were enrolled in the study at 60 ± 3 DIM (study d 0). Cows (n = 1,358) were assigned randomly based on ear tag identification number to 2 treatments: 1) treatment with 100 µg of GnRH (Factrel; Zoetis Inc., Florham Park, NJ; **Gpresynch**); or 2) no treatment (**Control**) at study d 0. At study d 7, all cows were presynchronized with two 25 mg injections of $PGF_{2\alpha}$ (dinoprost tromethamine; 5 mL Lutalyse, Zoetis Inc.) injections given 14 d apart. Estrus detection was performed once daily based on tail paint removal. Cows detected in estrus were eligible to be inseminated after 53 DIM. Once inseminated, cows received no further injections of the synchronization protocol. Cows not inseminated by study d 35 were enrolled in the Cosynch-72 protocol for TAI (Figure 2.1).

Experiment 2 – Multiparous Cows

Weekly cohorts of multiparous cows were enrolled in the study at 49 ± 3 DIM (study d 0). Cows (n = 1,971) were assigned randomly based on ear tag identification number to 2 treatments: 1) treatment with 100 µg of GnRH (**Gpresynch**); or 2) no treatment (**Control**) at study d 0. At study d 7, all cows received two 25 mg $PGF_{2\alpha}$ injections given 14 d apart. Estrus detection was performed once daily based on tail paint removal. Cows detected in estrus were eligible to be inseminated after 53 DIM. Once inseminated, cows received no further injections of the synchronization protocol. Cows not inseminated by study d 35 were enrolled in the Cosynch-72 protocol for TAI (Figure 2.1).

Blood Sampling, Progesterone, and Ovarian Ultrasonography

In a subgroup of cows (experiment 1 = 225 and experiment 2 = 394) from dairies 2 and 3, ovaries were examined by ultrasonography on study d −14, 0, and 7. Number of follicles ≥ 10 mm in diameter and presence of a CL ≥ 20 mm in diameter in each ovary were recorded. Cyclic status was determined by the presence of a CL at study d −14 or 0. Cows were considered to have ovulated to the GnRH injection if they had a follicle ≥ 10 mm on d 0 and a new CL present in the same ovary on d 7.

In a subgroup of cows (experiment 1 = 224 and experiment 2 = 394) from dairies 2 and 3, blood samples were collected on d 0 and 7 to determine concentration of progesterone. Blood samples were collected from a coccygeal vessel into evacuated tubes containing K2 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Following collection, blood samples were placed on ice until centrifugation at 1,200 x *g* for 15 min for plasma separation. Plasma samples were frozen and stored at -20 °C until analysis. Concentration of progesterone was determined using a solid-phase RIA kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA) previously validated (Stevenson et al., 2012). Samples were assayed in duplicate with an intra-assay and inter-assay coefficient of 8.0 and 8.8%, respectively. Cows were classified as having concentration of progesterone ≥ 1 ng/mL or as having concentration of progesterone < 1 ng/mL at study d 0 and 7 for statistical analysis.

Pregnancy Diagnosis

Cows in both experiments were submitted for pregnancy diagnosis at 36 ± 3 d after AI by transrectal ultrasonography based on detection of fluid, and presence of an embryo with a

heartbeat. At dairies 1 and 2, pregnancy was reconfirmed 94 ± 3 d after AI. At dairy 3, cows diagnosed pregnant were not re-examined to determine pregnancy loss.

Statistical Analyses

The experiment was a completely randomized design with randomization based on ear tag identification number. Cows with even ear tag identification number were assigned to the Gpresynch treatment and cows with odd ear tag identification number were assigned to the control. In experiment 1, a sample size of 650 experimental units in each treatment was expected to detect a statistical difference with a 6.5% unit difference in P/AI, when P/AI ranges from 30 to 37% (α = 0.05; β = 0.20; one-tailed test). The same number of experimental units was expected to detect a difference in 1.7 days difference in days to AI (SD = 12.0; α = 0.05; β = 0.20; one-tailed test). In experiment 2, a sample size of 950 experimental units per treatment was needed to detect a statistical difference with a 5% unit difference in P/AI when P/AI ranges from 25 to 30% (α = 0.05; β = 0.20; one-tailed test). In addition, it was expected to detect a statistical difference in 1.3 days difference in days to AI (SD = 11.7; α = 0.05; β = 0.20; one-tailed test). Dichotomous outcomes were analyzed using the LOGISTIC procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Continuous outcomes were analyzed by ANOVA using the GLM procedure of SAS. Models for analysis of P/AI and pregnancy loss included the following fixed effects in the models: treatment, dairy, and the interactions between treatment and dairy. The study timeline was categorized into 4 time periods to investigate potential treatment differences in P/AI and percentage of cows inseminated at specific time points: Period 1 = study d 0 to 7; Period 2 = study d 8 to 21; Period 3 = study d 22 to 35; and Period 4 = study d 36 to 45.

Variables included in the models for analysis of progesterone concentration, percentage of cows with progesterone ≥ 1 ng/mL, and ovulation risk were: treatment, dairy, cyclic status, and interactions between treatment and dairy and treatment and cyclic status. Backward stepwise elimination was performed to remove variables ($P > 0.10$). Treatment and dairy were forced to remain in the models.

The rate at which cows were inseminated or became pregnant were analyzed by the Cox proportional hazard ratio using the PHREG procedure of SAS. Variables were removed using the stepwise backward elimination function based on the Wald statistic criterion when $P > 0.10$. Survival analysis for time to insemination and pregnancy were performed using the LIFETEST procedure of SAS. Statistical significance was determined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

Results

In experiment 1, 57 primiparous cows were culled or deemed not eligible to be inseminated before AI, and 24 primiparous cows were culled before pregnancy diagnosis. In experiment 2, 103 and 40 multiparous cows were culled or deemed not eligible to be inseminated before AI, and culled before pregnancy diagnosis, respectively.

Milk Production and Temperature-Humidity Index

In experiment 1, projected 305-d milk (7,597 ± 28 kg; $P = 0.30$) and average milk yield one week before enrollment (30.0 ± 0.2 kg/d; $P = 0.51$) did not differ between treatments. Furthermore, in experiment 2, projected 305-d milk (9,638 ± 29 kg; $P = 0.34$) and average milk yield one week before enrollment (43.4 ± 0.2 kg/d; $P = 0.42$) did not differ between treatments.

Average of daily maximum THI from June 2014 to September 2014 for dairy 1 was 88.9, whereas for dairies 2 and 3 was 94.7. Furthermore, the average of daily minimum THI for dairy 1 was 60.9 and the average of daily minimum THI was 66.4 for dairies 2 and 3. Average of daily maximum and minimum temperature were 29.2 and 15.6°C for dairy 1, and 31.5 and 17.5°C for dairies 2 and 3, respectively. Average of daily maximum and minimum relative humidity were 80.5 and 30.4% for dairy 1, and 79.2 and 35.7% for dairies 2 and 3, respectively. Mean daily percentage of hours with THI ≥ 72 was 50.4% for dairy 1, and 70.9% for dairies 2 and 3.

Experiment 1 – Primiparous Cows

Concentration of Progesterone and Ovarian Response. Concentration of progesterone and percentage of cows with progesterone concentration ≥ 1 ng/mL on d 0 did not ($P \geq 0.57$) differ between treatments (Table 2.1). Furthermore, the percentage of noncyclic cows ($P = 0.17$) and cows with a CL present on d 0 ($P = 0.12$) did not differ between control and Gpresynch cows (Table 2.1).

Concentration of progesterone on d 7 was greater ($P < 0.01$) in Gpresynch than control cows (3.6 ± 0.3 vs. 2.7 ± 0.4 ng/mL). In addition, the percentage of cows with progesterone concentration ≥ 1 ng/mL on d 7 was greater ($P < 0.01$) in Gpresynch than control cows (79.1 vs. 58.0%). The Gpresynch cows had a greater ($P < 0.01$) percentage of cows with a CL present on d 7 (91.7 vs. 70.9%). Risk of ovulation from d 0 to 7 was increased ($P < 0.01$) for Gpresynch cows compared with control cows (50.6 vs. 15.2%). Furthermore, treatment with GnRH decreased ($P = 0.02$) the percentage of cows that did not have a CL present at any of the ultrasound examinations conducted on study d −14, 0, and 7 (2.7 vs. 10.3%). No interactions ($P = 0.74$) were detected

between dairy and concentration of progesterone, dairy and percentage of cows with progesterone concentration ≥ 1 ng/ml, and dairy and percentage of cows with a CL present on d 7 (Table 2.1).

Pregnancy per AI and Pregnancy Loss. Treatment did not ($P = 0.48$) affect overall P/AI 36 d after AI; however, an interaction was detected ($P = 0.01$) between treatment and dairy for P/AI (Table 2.2). Furthermore, the interaction between treatment and dairy affected ($P = 0.01$) P/AI at d 94 after AI. In dairy 1, P/AI was greater ($P = 0.03$) for Gpresynch cows than control cows at 36 d after AI. In contrast, in dairy 2, control cows had greater ($P = 0.05$) P/AI at d 36 post AI compared with Gpresynch cows. In dairy 3, no difference ($P = 0.37$) between treatments was detected in P/AI at 36 d after AI. Similar results were observed for cows inseminated during period 2, in which an interaction between treatment and dairy affected ($P = 0.04$) P/AI 36 d after AI. In contrast, no differences ($P > 0.50$) were detected in other periods (Table 2.3). Pregnancy loss from d 36 to 94 post AI did not differ ($P = 0.72$) between treatments (7.6%; Table 2.2).

Pattern of Insemination and Hazard of Pregnancy. During period 1, a greater ($P < 0.01$) percentage of control cows were inseminated than Gpresynch cows (25.5 vs. 9.4%). In contrast, during period 2, a greater ($P < 0.01$) percentage of Gpresynch cows were inseminated compared with control cows (58.3 vs. 43.1%). During periods 3 and 4, no differences ($P > 0.45$) in percentage of cows inseminated were detected between control and Gpresynch cows (Table 2.3).

Hazard of insemination was affected ($P = 0.05$) by treatment because control cows were inseminated at a faster rate (adjusted hazard ratio [**AHR**] = 0.89, 95% CI = 0.80 to 1.00) than Gpresynch cows. Despite the difference observed in hazard of insemination, hazard to pregnancy did not ($P = 0.35$) differ between treatments (AHR = 0.91, CI = 0.74 to 1.11). The interaction

between treatment and dairy did not ($P \geq 0.29$) affect the hazard of insemination or hazard to pregnancy. According to the survival analysis, the interval from study d 0 to insemination was greater ($P \leq 0.01$) for Gpresynch cows compared with control cows in dairies 2 and 3. In contrast, in dairy 1 no difference ($P = 0.30$) was detected between treatments. Mean days to AI (± SEM) were 16.7 ± 0.8 d, 16.0 ± 1.0, and 18.0 ± 1.0 d for control cows from dairies 1, 2, and 3, respectively. Mean days to AI were 16.6 ± 0.7 d, 19.0 ± 1.1 d, and 20.0 ± 0.9 d for Gpresynch cows from dairies 1, 2, and 3, respectively. Median days to AI were 11 d for both control and Gpresynch cows from all dairies.

Experiment 2 – Multiparous Cows

Concentration of Progesterone and Ovarian Response. Concentration of progesterone, percentage of cows with concentration of progesterone ≥ 1 ng/mL, and percentage of cows bearing a CL did not ($P \geq 0.30$) differ between treatments on study d 0 (Table 2.1). Furthermore, percentage of cows classified in the noncyclic status at study d 0 did not ($P = 0.15$) differ between treatments.

Risk of ovulation from d 0 to d 7 was greater ($P < 0.01$) for Gpresynch cows than control cows (46.9 vs. 23.8%). Although percentage of cows with concentration of progesterone ≥ 1 ng/mL was greater ($P = 0.03$) in Gpresynch than control cows at study d 7, concentration of progesterone on d 7 did not ($P = 0.34$) differ between treatments (Table 2.1). Among cows not inseminated from study d 0 to 7, a greater ($P = 0.04$) percentage of control cows did not have a CL present at any of the 3 ultrasound examinations performed on study d −14, 0, and 7 compared with Gpresynch cows (5.8 vs. 1.5%).

Pregnancy per AI and Pregnancy Loss. Overall P/AI at d 36 (P = 0.33) and d 94 (P = 0.47) after AI did not differ between treatments (Table 2.4). In contrast, pregnancy loss from d 36 to 94 after AI was greater (P = 0.04) in Gpresynch than control cows (11.2 vs. 4.6%). Furthermore, control cows had greater (P < 0.04) P/AI during period 3 (36.8 vs. 26.0%) and period 4 (24.5 vs. 12.6%) compared with Gpresynch cows (Table 2.2). In contrast, no differences were detected between treatments during periods 1 (P = 0.32) and 2 (P = 0.59; Table 2.2).

Pattern of Insemination and Hazard of Pregnancy. During period 1, a greater (P < 0.01) percentage of control cows were inseminated than Gpresynch cows (Table 2.2). In contrast, during period 2, a greater (P < 0.01) percentage of Gpresynch cows were inseminated compared with control cows. Furthermore, the interaction between treatment and dairy affected (P < 0.01) the percentage of cows inseminated in period 2. Such an interaction was observed because in dairy 1, a greater (P < 0.01) percentage of Gpresynch cows were inseminated compared with control cows (71.6 vs. 53.4%); whereas in dairies 2 and dairy 3, no differences (P > 0.35) were detected between treatments during this time period. During period 3, an interaction of treatment and dairy affected (P = 0.03) the percentage of cows inseminated. This interaction was observed because a greater (P < 0.01) percentage of control cows from dairy 1 were inseminated than Gpresynch cows (21.7 vs. 13.5%). In contrast, no differences (P > 0.44) between treatments were detected in the percentage of cows inseminated at dairies 2 and 3.

Treatment did not (P = 0.41) affect hazard of insemination, but an interaction between treatment and dairy was detected (P = 0.03). In dairy 1, Gpresynch cows (AHR = 1.21; CI = 1.05 to 1.41) were inseminated at a faster (P = 0.01) rate than control cows, but no differences (P > 0.23) were detected in dairy 2 (AHR = 0.91: CI = 0.78 to 1.06), and dairy 3 (AHR = 1.02; CI =

0.85 to 1.21). The survival analyses revealed similar results, in which no differences ($P \geq 0.15$) were detected between treatments in the interval from study d 0 to insemination in dairies 2 and 3; however, in dairy 1, the interval from study d 0 to insemination was lesser ($P = 0.02$) for Gpresynch cows compared with control cows. Mean days (± SEM) from study d 0 to AI for control cows were 17.2 ± 0.6, 16.9 ± 0.6, and 20.6 ± 0.8 d for dairies 1, 2, and 3, respectively. Mean days from study d 0 to AI for Gpresynch cows were 15.2 ± 0.5, 17.9 ± 0.6, and 20.4 ± 0.8 d for dairies 1, 2, and 3, respectively. Median days to first insemination were 12, 12, and 16 d for control cows at dairies 1, 2 and 3, respectively. Median days to first insemination were 11, 11, and 17 d for Gpresynch cows at dairies 1, 2, and 3, respectively.

The interaction between treatment and dairy did not ($P = 0.31$) affect hazard of pregnancy; however, hazard of pregnancy was affected ($P = 0.02$) by treatment because Gpresynch cows became pregnant at a faster rate than control cows (AHR = 1.25; CI = 1.04 to 1.50). Mean days from study d 0 to pregnancy were 18.3 ± 0.7 and 15.9 ± 0.7 d for control and Gpresynch cows, respectively ($P = 0.02$; Figure 2.2). Furthermore, hazard of pregnancy was affected by dairy ($P = 0.05$). Cows at dairy 1 became pregnant faster ($P = 0.01$) than cows at dairy 3 (AHR = 1.33; CI = 1.06 to 1.67). In addition, cows at dairy 1 tended ($P = 0.10$) to become pregnant at a faster rate than cows at dairy 2 (AHR = 1.22; CI = 0.96 to 1.53). Mean days from study d 0 to pregnancy were 15.9 ± 0.7, 16.7 ± 0.8, and 19.6 ± 1.0 d for dairies 1, 2, and 3, respectively.

Discussion

In the U.S., 93% of dairy herds utilize estrus detection in their reproductive program as one of the methods to identify cows to be inseminated (NAHMS, 2007). Success of reproductive programs that predominantly utilize estrus detection for AI depends on facility design and accuracy

of detecting cows in estrus. In addition, presynchronization and synchronization programs tailored to maximize insemination based on estrus detection may affect AI submission rate, which influences reproductive efficiency of dairy herds. Presynchronization protocols based on $PGF_{2\alpha}$ before initiation of TAI programs induce luteolysis and ovulation without suppressing estrus expression (Chebel et al., 2013). Nevertheless, the advantage of using $PGF_{2\alpha}$-based presynchronization methods in programs tailored to inseminate cows in estrus is limited to cyclic cows. Considering that anovular cows may benefit from treatment with GnRH, one of the main purposes of the present experiments was to incorporate a GnRH injection in a $PGF_{2\alpha}$-based presynchronization strategy to maximize the response of the $PGF_{2\alpha}$ injections in a program focused to inseminate cows based on estrus detection. Furthermore, we hypothesized that treatment with GnRH before initiation of the presynchronization protocol with $PGF_{2\alpha}$ injections may increase P/AI as a result of increased concentration of progesterone before the first $PGF_{2\alpha}$ injection.

Even though overall P/AI did not differ between treatments, multiparous cows (experiment 2) treated with GnRH before the presynchronization protocol became pregnant at a faster rate compared with cows not treated with GnRH. In addition, Gpresynch cows in experiment 1 had greater P/AI than control cows in one of the herds (dairy 1 – primiparous cows). On the other hand, the opposite was observed at dairy 2. It is important to note that control cows from dairy 1 had decreased P/AI compared with control cows from dairies 2 and 3, indicating differences in fertility of primiparous cows among herds. The potential causes for differences in fertility of primiparous cows among herds also might have resulted in distinct responses to the GnRH and $PGF_{2\alpha}$ injections. Responses to the hormonal treatments might be influenced by several factors, including management practices, facilities, and percentage of anovular cows. Based on the findings from experiment 1, one could suggest that the treatment with GnRH in primiparous cows was only

beneficial in the herd with decreased fertility, potentially with increased prevalence of anovular cows. Unfortunately, cows from dairy 1 were not enrolled in the subgroup of cows in which ovaries were examined by ultrasonography, which limits our conclusions. Nonetheless, other authors have demonstrated that incorporating GnRH in presynchronization protocols for first TAI only benefited a subpopulation of cows (Souza et al., 2008). Only primiparous cows benefited from a GnRH-$PGF_{2\alpha}$-based presynchronization protocol, not multiparous cows (Souza et al., 2008), which are expected to be at a lesser risk for anovular condition. Indeed, Ribeiro et al. (2012) suggested that the lack of benefit of a GnRH-$PGF_{2\alpha}$-based protocol observed in grazing dairy herds was related to the low proportion of anovular cows (14.1%). Unfortunately, the 2 previous reports did not account for season in their statistical analysis (Souza et al., 2008; Ribeiro et al., 2012), limiting the understanding how cows responded to the protocols during summer.

The negative effect of GnRH treatment on overall P/AI of Gpresynch cows from dairy 2 is intriguing (experiment 1 – primiparous). Nevertheless, a previous study has shown evidence of a detrimental effect on fertility of cows treated with GnRH before a $PGF_{2\alpha}$-based presynchronization protocol (Bittar et al., 2014). Treating cows with GnRH at 17 ± 3 and 20 ± 3 DIM resulted in decreased fertility of cows bearing a CL at treatment that failed to ovulate to the GnRH injections (Bittar et al., 2014). Despite the fact that Bittar et al. (2014) administered GnRH earlier postpartum compared with the current study, the differences in P/AI between treatments for primiparous cows from dairy 2 could be explained by the potential poor fertility of cows that failed to ovulate to the GnRH injection.

The increased proportion of cyclic cows at the first $PGF_{2\alpha}$ injection in the Gpresynch treatment indicates that the GnRH treatment was effective in treating anovular condition in a subset of cows. Gumen et al. (2003) demonstrated that the first GnRH injection of the Ovsynch protocol

was effective in inducing ovulation of anovular cows; however, P/AI of anovular cows was decreased compared with cyclic cows. In the current study, it is likely that anovular cows that ovulated to the GnRH injection at study d 0, and had subsequent $PGF_{2\alpha}$-induced luteolysis and detected in estrus, had decreased P/AI compared with cows that were already cyclic. It is possible that we did not detect P/AI differences between treatments during period 2 because a greater proportion of Gpresynch cows that were in anovular condition were induced to ovulate before the first $PGF_{2\alpha}$ injection and were inseminated during period 2. Thus, these cows may have decreased overall P/AI of Gpresynch cows during period 2.

Despite the greater percentage of cows with a CL present at the first $PGF_{2\alpha}$ injection observed in both experiments, the increase in ovulation risk only resulted in an increased concentration of progesterone in experiment 1 (primiparous cows). A possible explanation for these results could be differences in concentration of progesterone at the time of GnRH treatment, which was lesser in experiment 1 compared with experiment 2. The decreased progesterone concentration might have promoted a greater response to the GnRH injection due to decreased negative feedback of progesterone on the hypothalamus and greater release of LH (Giordano et al. 2012; Pulley et al., 2015). A more plausible explanation for these findings may be related to the design of the experiments. Because treatment with GnRH in experiment 1 was administered after cows were eligible to be inseminated, cows that did not respond to the GnRH injection might have been inseminated between GnRH and $PGF_{2\alpha}$ treatments and a sample at study d 7 was not collected from these cows. Thus, it is likely that an increased proportion of Gpresynch cows from experiment 1 sampled at study d 7 responded to the GnRH injection compared with Gpresynch cows from experiment 2. The collaborating dairies in the current study desired to initiate the presynchronization protocol earlier for multiparous cows than primiparous, but maintain a similar

voluntary waiting period (**VWP**). Because of differences in initiation of protocols relative to the end of the VWP, the authors decided to allocate parity by experiment and conduct the statistical analysis separately in order to avoid the confounding effects of DIM at initiation of treatment.

Suppression of estrus after administration of GnRH has been well documented (Mendonça et al., 2012; Chebel et al., 2013). In experiment 1 (primiparous cows), GnRH was administered 4 to 10 d after the end of the VWP. Therefore, Gpresynch cows eligible to be inseminated had their expression of estrus suppressed resulting in treatment differences in the percentage of cows inseminated from study d 0 to 7. In experiment 2 (multiparous cows), GnRH was administered before cows were considered eligible to be inseminated by the farm personnel. Therefore, the pattern in which multiparous cows (experiment 2) were inseminated was not affected by the suppression of estrus from the GnRH treatment to the same extent as the insemination pattern was affected for primiparous cows (experiment 1). The increased percentage of Gpresynch cows having a CL at study d 7 explains the greater proportion of Gpresynch primiparous cows being inseminated after the first $PGF_{2\alpha}$ injection (period 2) compared with control cows. In contrast, in multiparous cows, the difference in the proportion of Gpresynch cows inseminated in period 2 compared with control cows was only observed in dairy 1. These findings support the premise that responses to GnRH and $PGF_{2\alpha}$ treatments were not similar in dairy 1 compared with dairies 2 and 3. As previously mentioned, cow-related factors might have influenced hormonal responses. Factors not assessed in this study (e.g., BCS and body core temperature) were likely associated to the observed treatment differences across herds. Friedman et al. (2011) had previously reported that the benefit of treating heat-stressed cows with GnRH and $PGF_{2\alpha}$ was limited to subpopulations of cows. Among cows with BCS > 2 at 50 to 60 DIM, treatments with GnRH and $PGF_{2\alpha}$ increased P/AI compared with no treatment. Furthermore, the treatments benefited primiparous, but not

multiparous cows (Friedman et al., 2011). The previous authors speculated that the lesser milk production of primiparous cows may have resulted in lower body temperature compared with multiparous cows, leading to a better response to the hormonal treatments.

Heat stress has been shown to decrease the expression and duration of estrus in dairy cattle (Gwazdausakas et al., 1981; Younas et al., 1993), justifying why we conducted this study during summer. We attempted to increase expression of estrus in the critical time of the year. The current experiments demonstrate that regardless of the possible decrease in estrus expression during summer caused by heat stress, lactating dairy cows may still be detected in estrus efficiently. The findings from the current study demonstrate that 86.3% and 89.3% of cows from experiment 1 and 2, respectively, were detected in estrus and inseminated before initiation of the TAI protocol. Cartmill et al. (2001) reported that fewer cows were detected in estrus when THI was ≥ 72. In the current study, the mean daily percentage of hours with THI ≥ 72 was lesser for dairy 1 than dairies 2 and 3, 50.4% and 70.9%, respectively. Although cows from the 3 herds were exposed to heat stress conditions during the experiments, cows from dairy 1 might have experienced a lesser extent of heat stress compared with cows housed in dairies 2 and 3, despite the differences in heat abatement strategies used in the dairies. It is possible that the increased severity of heat stress for cows located in dairies 2 and 3 might have further affected the response to GnRH treatment via altered GnRH-induced LH release. Cows under chronic heat stress conditions had lesser peaks of GnRH-induced LH (Gilad et al., 1993). In addition, responses to $PGF_{2\alpha}$ treatments may have been decreased in cows located in dairies 2 and 3 because of the exacerbated heat stress. Luteolysis after $PGF_{2\alpha}$ is less likely to occur during summer than during moderate or cold weather (Stevenson et al., 2012). Thus, luteolysis is presumably affected by the severity of heat stress.

In conclusion, results from the current experiments collectively suggest that using a GnRH-$PGF_{2\alpha}$-based presynchronization protocol has benefits in some herds compared with a $PGF_{2\alpha}$-based presynchronization protocol during summer heat stress. The current study demonstrated that treatment with GnRH induced ovulation of cows in anovular condition. In addition, treatment with GnRH 7 d before initiating a $PGF_{2\alpha}$-based presynchronization protocol results in a greater proportion of cows bearing a CL at the first $PGF_{2\alpha}$ injection, which may result in a greater percentage of cows to be inseminated earlier in lactation, and consequently, increased AI submission rate. Nevertheless, if GnRH is administered after cows become eligible to be inseminated, estrus is suppressed and it may negatively influence insemination rate. Herds with efficient estrus detection could benefit from a GnRH-$PGF_{2\alpha}$-based presynchronization protocol initiated before the end of the VWP, which can potentially decrease days open by inseminating a greater proportion of cows earlier in lactation. Differences in treatments across herds are an indication that cow-related factors potentially affect the responses of including a GnRH injection before starting the presynchronization protocol. Percentage of anovular cows likely influences the response to GnRH and $PGF_{2\alpha}$ injections and it should be considered before implementation in dairy herds. Further, the severity with which heat stress affects cows might affect responses to the hormonal treatments, thus affecting P/AI and insemination risk.

Acknowledgements

This research was partially funded by the Kansas Dairy Commission and Zoetis Animal Health. The authors thank the managers and owners of the collaborating dairies, Robert Briggeman, ABS Global, Gary Neubauer, and Zoetis Inc. (Florham Park, NJ). This article is contribution number 16-170-J from the Kansas Agricultural Experiment Station. This research

project was completed as part of the Multistate Research Project, W-2173, Impacts of Stress Factors on Performance, Health, and Well-Being of Farm Animals.

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Table 2.1 Concentration of progesterone, presence of corpus luteum (CL), and ovulation risk for primiparous and multiparous cows enrolled in experiment 1 and 2.

	Treatment (T)[1]		*P*-value		
Item	Control	Gpresynch	T	Dairy	T x dairy
Experiment 1 - primiparous cows	(n = 123)	(n = 102)			
Study d 0					
Non-cyclic status, %[2]	29.5	32.5	0.17	0.97	0.68
Cows bearing a CL, %	71.5	61.8	0.12	0.83	0.67
Progesterone concentration, ng/mL	2.3 ± 0.3	2.5 ± 0.3	0.57	0.83	0.16
Progesterone concentration ≥ 1 ng/mL, %	59.5	53.9	0.74	0.30	0.40
Study d 7					
Cows bearing a CL, %	70.9	91.7	< 0.01	0.92	0.77
Progesterone concentration, ng/mL	2.7 ± 0.4	3.6 ± 0.3	< 0.01	0.07	0.75
Progesterone concentration ≥ 1 ng/mL, %	58.0	79.1	< 0.01	0.20	0.74
Ovulation risk, %[3]	15.2	50.6	< 0.01	0.32	0.34
No CL present, %[4]	10.3	2.7	0.02	0.26	0.99
Experiment 2 - multiparous cows	(n = 198)	(n = 196)			
Study d 0					
Non-cyclic status, %[2]	12.8	19.0	0.15	< 0.01	0.87
Cows bearing a CL, %	74.8	73.0	0.99	0.41	0.40
Progesterone concentration, ng/mL	3.4 ± 0.3	3.8 ± 0.3	0.30	0.13	0.63
Progesterone concentration ≥ 1 ng/mL, %	63.2	66.8	0.43	< 0.01	0.07
Study d 7					
Cows bearing a CL	83.3	95.6	< 0.01	0.29	0.58
Progesterone concentration, ng/mL	4.4 ± 0.3	4.8 ± 0.3	0.34	0.42	0.63
Progesterone concentration ≥ 1 ng/mL, %	76.4	85.8	0.03	0.93	0.48
Ovulation risk, %[3]	23.8	46.9	< 0.01	0.85	0.50
No CL present, %[4]	5.8	1.5	0.04	0.14	0.88

[1]Cows were either treated (Gpresynch) or not treated (control) with GnRH before presynchronization with 2 injections of $PGF_{2\alpha}$ given 14 d apart

[2]Percentage of cows not bearing a CL on study d −14 to 0.

[3]Percentage of cows that had an ovulatory response from study d 0 to 7.

[4]Percentage of cows that did not have a CL present on study d −14, 0, and 7.

Table 2.2 The effect of treatment and dairy on pregnancy per AI (P/AI) at 36 ± 3 and 94 ± 3 d after AI and pregnancy loss of primiparous cows from experiment 1

	Treatment (T)[1]		*P*-value		
Item	Control	Gpresynch	T	Dairy	T x dairy
Overall	(n = 655)	(n = 622)			
P/AI on d 36 ± 3, %	32.1	31.8	0.48	0.69	0.01
P/AI on d 94 ± 3, %[2]	27.8	29.8	0.97	0.62	0.01
Pregnancy loss, %[2]	8.2	6.9	0.72	0.51	0.71
Dairy 1	(n = 283)	(n = 255)			
P/AI on d 36 ± 3, %	27.6	36.1	0.03	. . .	. . .
P/AI on d 94 ± 3, %	25.5	33.5	0.04	. . .	. . .
Pregnancy loss, %	6.6	6.6	0.99	. . .	. . .
Dairy 2	(n = 163)	(n = 156)			
P/AI on d 36 ± 3, %	35.6	25.6	0.05	. . .	. . .
P/AI on d 94 ± 3, %	31.9	23.9	0.11	. . .	. . .
Pregnancy loss, %	10.3	7.5	0.63	. . .	. . .
Dairy 3	(n = 209)	(n = 211)			
P/AI on d 36 ± 3, %	35.4	31.3	0.37	. . .	. . .
P/AI on d 94 ± 3, %	. . .	. . .	. . .	. . .	. . .
Pregnancy loss, %	. . .	. . .	. . .	. . .	. . .

[1]Primiparous cows were either treated (Gpresynch) or not treated (control) with GnRH before presynchronization with 2 injections of $PGF_{2\alpha}$ given 14 d apart.
[2]Data of P/AI at d 94 after AI and pregnancy loss only pertain to dairies 1 and 2.

Table 2.3 Percentage of primiparous and multiparous cows inseminated by period for experiment 1 and experiment 2

	Treatment (T)[1]		*P*-value		
Item	Control	Gpresynch	T	Dairy	T x dairy
Experiment 1 - primiparous cows					
Percentage inseminated	(n = 670)	(n = 631)			
Period 1, %	25.5 (171/670)	9.4 (59/631)	<0.01	0.34	0.43
Period 2, %	43.1 (289/670)	58.3 (368/631)	<0.01	<0.01	0.78
Period 3, %	17.2 (115/670)	19.0 (120/631)	0.45	0.30	0.40
Period 4, %	14.2 (95/670)	13.3 (84/631)	0.73	<0.01	0.19
Pregnancy per AI	(n = 655)	(n = 622)			
Period 1, %	38.8 (66/170)	42.4 (25/59)	0.50	0.64	0.42
Period 2, %	32.7 (91/278)	33.2 (121/364)	0.73	0.88	0.04
Period 3, %	27.4 (31/113)	30.3 (36/119)	0.71	0.85	0.65
Period 4, %	23.4 (22/94)	20.0 (16/80)	0.73	0.85	0.12
Experiment 2 - multiparous cows					
Percentage inseminated	(n = 936)	(n = 932)			
Period 1, %	12.6 (118/936)	7.9 (74/932)	<0.01	0.09	0.53
Period 2, %	54.9 (514/936)	62.5 (582/932)	<0.01	<0.01	<0.01
Period 3, %	21.4 (200/936)	19.2 (179/932)	0.24	<0.01	0.03
Period 4, %	11.1 (104/936)	10.4 (97/932)	0.45	<0.01	0.12
Pregnancy per AI	(n = 911)	(n = 917)			
Period 1, %	21.9 (25/114)	28.8 (21/73)	0.32	0.86	0.67
Period 2, %	25.5 (128/502)	26.9 (154/572)	0.59	0.44	0.93
Period 3, %	36.8 (71/193)	26.0 (46/177)	0.03	0.97	0.76
Period 4, %	24.5 (25/102)	12.6 (12/95)	0.04	0.09	0.35

[1]Cows were either treated (Gpresynch) or not treated (control) with GnRH before presynchronization with 2 injections of PGF2α given 14 d apart

Table 2.4 The effect of treatment and dairy on pregnancy per AI (P/AI) at 36 ± 3 and 94 ± 3 d after AI and pregnancy loss of multiparous cows from experiment 2

	Treatment (T)[1]		*P*-value		
Item	Control	Gpresynch	T	Dairy	T x dairy
Overall	(n = 911)	(n = 917)			
P/AI on d 36 ± 3, %	27.3	25.4	0.33	0.49	0.84
P/AI on d 94 ± 3, %[2]	22.9	21.1	0.47	0.38	0.13
Pregnancy loss, %[2]	4.6	11.2	0.04	0.42	0.11
Dairy 1	(n = 341)	(n = 335)			
P/AI on d 36 ± 3, %	28.2	27.8	0.91	…	…
P/AI on d 94 ± 3, %	20.0	21.8	0.59	…	…
Pregnancy loss, %	7.5	9.2	0.71	…	…
Dairy 2	(n = 327)	(n = 333)			
P/AI on d 36 ± 3, %	26.9	24.0	0.39	…	…
P/AI on d 94 ± 3, %	25.7	20.4	0.11	…	…
Pregnancy loss, %	2.4	13.2	0.01	…	…
Dairy 3	(n = 243)	(n = 249)			
P/AI on d 36 ± 3, %	26.8	24.1	0.50	…	…
P/AI on d 94 ± 3, %	…	…	…	…	…
Pregnancy loss, %	…	…	…	…	…

[1]Multiparous cows were either treated (Gpresynch) or not treated (control) with GnRH before presynchronization with 2 injections of $PGF_{2\alpha}$ given 14 d apart.
[2]Data of P/AI at 94 d after AI and pregnancy loss only pertain to dairy 1 and 2.

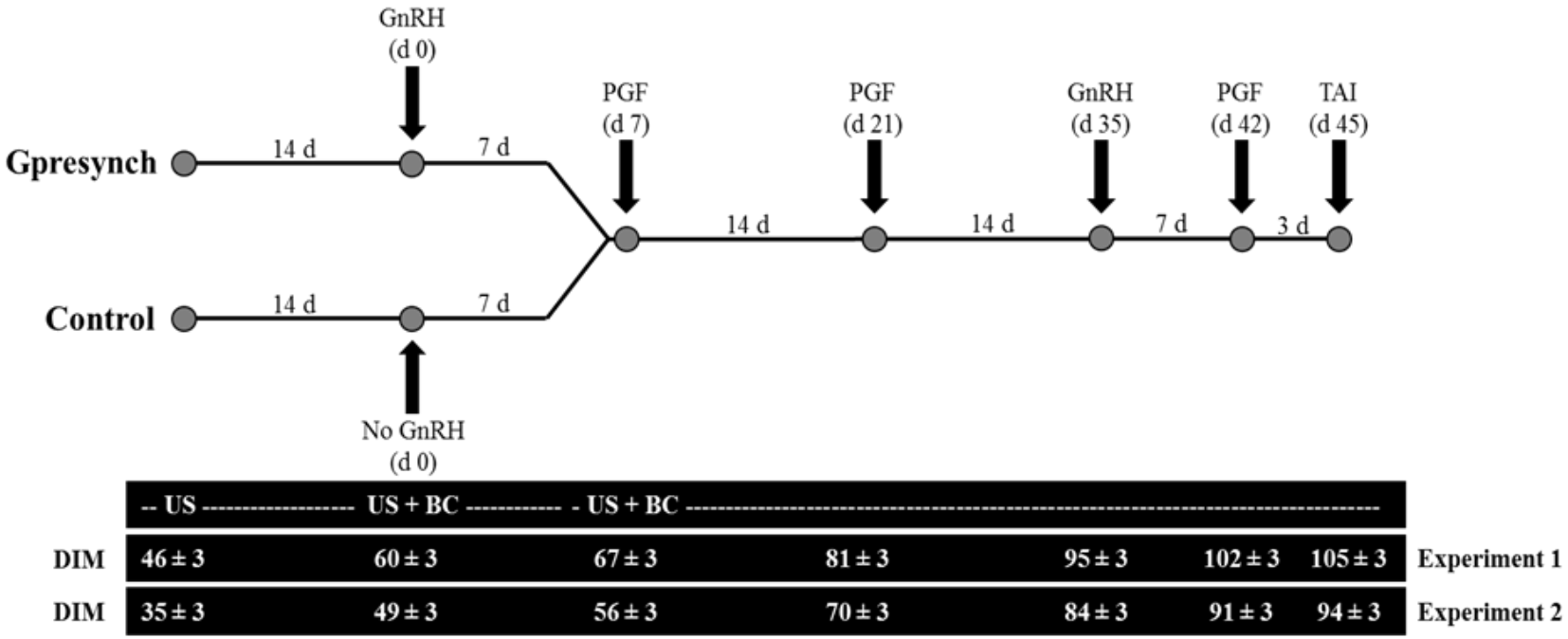

Figure 2.1 Schematic representation of experimental procedures for experiments 1 and 2.

Primiparous cows in experiment 1 were enrolled in the study at 60 ± 3 DIM (study d 0). Multiparous cows in experiment 2 were enrolled at 49 ± 3 DIM (study d 0). In both experiments cows became eligible to be inseminated at 53 DIM. On study d 0, cows were assigned randomly to receive either 100 µg of GnRH (Gpresynch) or no GnRH (control). Cows were submitted to a presynchronization with $PGF_{2\alpha}$ (PGF) 14 d apart at study d 7. Cows inseminated received no further treatment whereas cows not inseminated by study d 35 were enrolled in the Cosynch-72 protocol (GnRH -7 d -$PGF_{2\alpha}$ -3 d - GnRH+TAI) for TAI. Ovarian ultrasonography examinations (US) were performed on study d –14, 0, and 7, and blood collection (BC) was performed on study d 0 and 7.

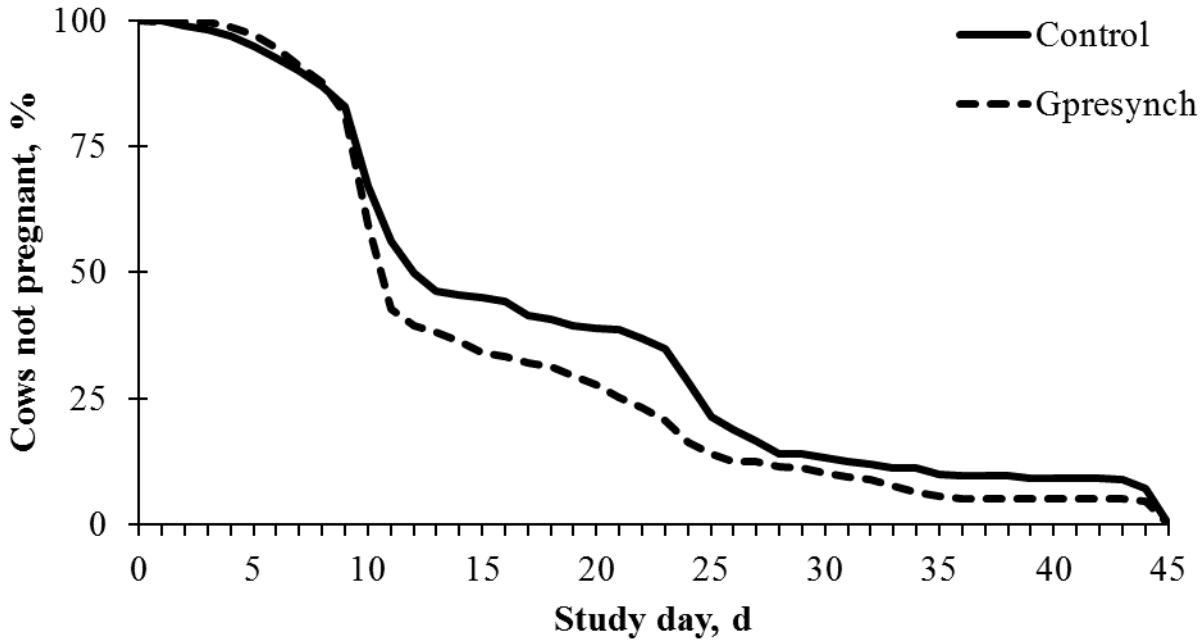

Figure 2.2 Survival analysis for days to pregnancy for multiparous cows treated with GnRH (Gpresynch) or control before presynchronization in experiment 2.

Median days to pregnancy were 12 and 11 d for control and Gpresynch cows, respectively. Mean days to pregnancy (±SEM) were 18.3 ± 0.7 and 15.9 ± 0.7 d for control and Gpresynch cows, respectively ($P = 0.02$).

Chapter 3 - Response of Dairy Cows with or without Purulent Vaginal Discharge to Gonadotropin-Releasing Hormone and Prostaglandin $F_{2\alpha}$

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ABSTRACT

Purulent vaginal discharge (**PVD**) is a common uterine disease in dairy cattle that has negative effects on reproductive performance. Reproductive management programs that synchronize ovulation use GnRH to induce ovulation and $PGF_{2\alpha}$ to induce luteolysis. The objectives of the study were to evaluate ovarian response to treatment with GnRH and the odds of bearing a corpus luteum or being inseminated in dairy cows with or without PVD. Another objective was to determine the hazard of insemination after administration of $PGF_{2\alpha}$ in dairy cows with or without PVD. Primiparous (n = 291) and multiparous (n = 402) cows were evaluated for PVD using a Metricheck device at 46 ± 3 and 35 ± 3 DIM (study d 0), respectively. On study d 14, primiparous (n = 83) and multiparous (n = 178) cows were treated with GnRH and subsequent ovulation was recorded. Primiparous (n = 178) and multiparous (n = 368) cows not inseminated by study d 21 were administered $PGF_{2\alpha}$ and response to $PGF_{2\alpha}$ treatment was determined by detection of estrus. Furthermore, cows were categorized by the presence of a CL or being inseminated by study d 14, 21, and 35. Overall prevalence of PVD was 28.5 and 13.4% for primiparous and multiparous cows, respectively. Projected 305-d milk yield was less ($P < 0.01$) in PVD+ multiparous cows compared with PVD– multiparous cows, however, no ($P = 0.26$) difference was detected between primiparous PVD+ and PVD– cows. Ovulatory response to GnRH treatment was 51.8 and 47.8% for primiparous and multiparous cows, respectively. Primiparous PVD– cows tended ($P = 0.06$) to be less likely to ovulate in response to GnRH than primiparous PVD+ cows, whereas multiparous PVD+ cows were less ($P = 0.04$) likely to ovulate to GnRH than PVD– multiparous cows. The odds of bearing a corpus luteum or being inseminated by study d 14, 21, or 35 was not associated with PVD in primiparous cows. In contrast, the odds of bearing a corpus luteum or being inseminated by study d 14 and 21 was ($P < 0.03$) associated

with PVD in multiparous cows, but not (P = 0.11) on study d 35. Hazard of insemination after $PGF_{2\alpha}$ was not ($P \geq 0.38$) associated with PVD in primiparous or multiparous cows. Purulent vaginal discharge is associated with response to treatment with GnRH in dairy cattle. Purulent vaginal discharge might negatively affect reproductive management programs that use GnRH to induce ovulation.

Introduction

Clinical endometritis is a common uterine disorder in dairy cows, ranging from 5 to 26% prevelance (LeBlanc et al., 2002). Clinical endometritis in dairy cows is defined as the presence of purulent vaginal discharge (**PVD**) after d 26 postpartum or having a cervical diameter ≥7.5 cm determined by transrectal palpation after d 20 postpartum (LeBlanc et al., 2002). Endometritis is associated with bacterial infection in the reproductive tract consisting of *Escherichia coli* and *Arcanobacterium pyogenes*, among other gram–negative and gram–positive bacteria (Williams et al., 2005). Clinical endometritis has negative effects on overall reproductive performance of dairy cows including increased days to first service (LeBlanc et al., 2002), decreased pregnancy per artificial insemination (**AI**; LeBlanc et al., 2002; McDougall et al., 2007), and increased median days to pregnancy (LeBlanc et al., 2002; McDougall et al., 2007; Dubuc et al., 2010).

Bacterial infection of the uterus is associated with impaired ovarian function including prolonged anestrus and cystic ovarian follicles (Mateus et al., 2002). Large uterine bacteria load is associated with decreased odds of ovulating the first dominant follicle (Sheldon et al., 2002); therefore, possibly increasing the risk of anovulation. Furthermore, large uterine bacterial contamination is associated with decreased follicle growth and plasma concentration of estradiol (Sheldon et al., 2002). If ovulation occurs, bacterial infection of the uterus can influence the

function and life span of the corpus luteum (**CL**). Intrauterine infusion of lipopolysaccharide (**LPS**) reduced secretion of progesterone from the CL and induced early luteolysis in dairy heifers (Lüttgenau et al., 2016a). In contrast, researchers have observed extended luteal phases in cows exposed to LPS or bacterial infection of the uterus (Mateus et al., 2002, 2003). Duration of the estrous cycle is controlled by the CL and the uterus via secretion of prostaglandin $F_{2\alpha}$ (**PGF**) from endometrial epithelial cells (Horton and Poyser, 1976). In an *in vitro* study, Herath et al. (2009) demonstrated a shift from secretion of PGF to prostaglandin E_2 (**PGE**) by endometrial epithelial cells stimulated with LPS. In cows with uterine bacterial infection, the luteotropic action of PGE may prolong the interval from estrus to luteolysis.

Reproductive management programs commonly used in dairy farms depend on inducing ovulation with GnRH and causing luteolysis with PGF in order to synchronize ovulation. The influence of clinical endometritis on the success of reproductive management strategies is not completely understood, except that fertility is decreased. Previous studies investigated the physiological effects of exposure of LPS on ovulation in the periestrual stage of the cycle (Peter et al., 1989; Lavon et al., 2008) and CL life span (Lüttgenau et al., 2016a,b). Exposure to LPS alters endocrine function at the level of the ovary (Battaglia et al., 2000; Herath et al., 2007) and hypothalamus (Peter et al., 1989). Ovarian responses to treatment with GnRH and PGF of dairy cows with PVD have not been fully elucidated. More research is warranted to better understand the effects of PVD on physiological responses to treatments with GnRH and PGF, which may partly explain the decrease in reproductive efficiency observed for cows with clinical endometritis (LeBlanc et al., 2002; McDougall et al., 2007; Dubuc et al., 2010).

We hypothesized that cows with PVD would be less likely to ovulate in response to GnRH treatment and to be detected in estrus after treatment with PGF. The objectives of the current study

were to evaluate: (1) GnRH-induced ovulation risk; and (2) the odds of bearing a CL or being inseminated in dairy cows with or without PVD. A third objective was to determine the hazard of insemination after administration of PGF in dairy cows with or without PVD.

Materials and methods

Animals

Cows utilized in this retrospective observational study were a subset of cows from a previous study (Voelz et al., 2016). The current study was conducted at two commercial dairy farms located in southwest Kansas from June through August 2014. Cows from dairy A were housed in dry-lot corrals with shade and milked twice daily. Cows from dairy B were housed in a sand-bedded free-stall barn with fans and sprinklers. Furthermore, cows from dairy B had access to a dirt exercise lot and were milked thrice daily. Both dairies fed a TMR once daily with ad libitum access to feed and water. Complete reproductive management procedures for primiparous and multiparous cows have previously been described (Voelz et al., 2016).

Experimental procedures

Experimental procedures are illustrated in Figure 3.1. Weekly cohorts of primiparous (n = 291) and multiparous (n = 402) cows were evaluated for clinical endometritis at 46 ± 3 and 35 ± 3 days in milk (**DIM**; study d 0), respectively. Clinical endometritis was determined using the Metricheck device (Simcro, Hamilton, New Zealand) as previously described (McDougall et al., 2007). Vaginal discharge was scored by a single technician on a 4-point scale (0 = clear mucus; 1 = clear mucus with flecks of white pus; 2 = ≤ 50% white or off-white exudate; 3 = > 50% purulent white, yellow; or 4 = blood-colored exudate; Williams et al., 2005). Cows with vaginal discharge

score ≥ 2 were categorized as being clinical endometritis positive (PVD+) and cows with a vaginal discharge score < 2 were classified as being endometritis negative (PVD−). Furthermore, BCS (0.25-point increments; 1 = thin and 5 = fat; Ferguson et al., 1994) was assessed at enrollment (study d 0). Body condition score was categorized as high (≥ 3.00) or low (< 3.00). For primiparous cows, ultrasound examinations of ovaries were performed at 46 ± 3 (study d 0), 60 ± 3 (study d 14), and 67 ± 3 (study d 21) DIM. Ovaries of multiparous cows were examined at 35 ± 3 (study d 0), 49 ± 3 (study d 14), and 56 ± 3 (study d 21) DIM. Number of CL and ovarian follicles ≥ 10 mm in diameter were recorded for each ovary. Cyclic status (yes vs. no) was determined by the presence of an ultrasonically detected CL on either study d 0 or 14. Cows without a CL on study d 0 and study d 14 were considered to be noncyclic at study d 14. Furthermore, projected 305-d milk yield (P305M) was extracted from the on-farm record keeping software (DairyComp 305, Valley Agriculture Software, Tulare, CA) at enrollment (study d 0).

In a subgroup of primiparous (n = 83) and multiparous (n = 178) cows, 100 µg im GnRH (2 mL Factrel, Zoetis Inc., Florham Park, NJ) was administered on study d 14, respectively. Ovulation to GnRH (yes vs. no) was determined on study d 21 by the presence of a new CL in the same ovary where a previous follicle ≥ 10 mm was identified on study d 14.

Estrus detection was performed once daily and estrus was determined by removal of tail paint. All cows were eligible to be inseminated after 53 DIM. Primiparous (n = 178) and multiparous (n = 368) cows not inseminated by study d 21 (67 ± 3 or 56 ± 3 DIM, respectively), were administered 25 mg PGF i.m. (dinoprost tromethamine, 5 mL Lutalyse, Zoetis Inc.). Response to PGF (yes vs. no) was determined by the detection of estrus and insemination of cows by 14 d after receiving PGF.

Cows were further categorized by the presence of a CL or being inseminated by study d 14 (**CL + AI14**), 21 (**CL + AI21**), and 35 (**CL + AI35**). At each time point (study d 14, 21, and 35), cows bearing a CL or receiving AI were classified as a success (yes or no).

Statistical analyses

Data for primiparous and multiparous cows were analyzed in separate models because of differences in DIM at enrollment. Descriptive statistics were performed using the FREQ procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC) with Chi-square analysis. Continuous variables were analyzed using the GLM procedure of SAS. Dichotomous data were analyzed using the LOGISTIC procedure of SAS. For analysis of the ovulatory response to GnRH treatment, models included PVD (positive vs. negative), cyclic status (cyclic vs. noncyclic), BCS category (high vs. low), and the 2-way interactions between PVD, cyclic status and BCS category. Models for evaluating odds of success at study d 14 (CL + AI14) included PVD, BCS category, and the interaction between PVD and BCS category. For analysis of being a success at study d 21 (CL + AI21) and d 35 (CL + AI35), models included PVD, GnRH treatment at study d 14 (yes vs. no), BCS category, and the 2-way interactions between PVD, GnRH treatment at study d 14, and BCS category. Dairy was included as a fixed effect in all models. Variables were removed from the model using a backward elimination ($P > 0.10$). Dairy was forced to remain in the final model. Analysis of the Cox proportional hazard of insemination after PGF was performed using the PHREG procedure of SAS. Models included PVD, GnRH treatment at study d 14, cyclic status, BCS category, and dairy. Models for the hazard of insemination also included interactions between PVD and GnRH treatment at study d 14, PVD and BCS category, and PVD and cyclic status. Variables were removed using backward elimination based on the Wald criterion when $P > 0.10$

with dairy being forced to stay in the final model. Survival analysis for time to insemination after PGF were performed using the LIFETEST procedure of SAS. Statistical significance was determined as $P \leq 0.05$ and statistical tendencies as $0.05 < P \leq 0.10$.

Results

Descriptive results

Descriptive results are presented in Table 3.1. Overall prevalence of PVD was 28.5 and 13.4% for primiparous and multiparous cows, respectively. Prevalence of PVD in dairy A was 26.2 and 9.9%, whereas prevalence of PVD in dairy B was 30.3 and 18.2% for primiparous and multiparous cows, respectively. Proportion of multiparous PVD– cows cyclic at study d 14 was greater ($P = 0.01$) than multiparous PVD+ cows (Table 3.1). No difference ($P = 0.24$) was detected in proportion of cows cyclic at study d 14 between primiparous PVD+ and PVD– cows. Projected 305-d milk yield was less ($P < 0.01$) in PVD+ multiparous cows compared with PVD– multiparous cows (Table 3.1). In contrast, projected 305-d milk yield did not ($P = 0.26$) differ between primiparous PVD+ and PVD– cows.

Ovarian responses to GnRH treatment

Ovulatory response to GnRH treatment was 51.8 and 47.8% for primiparous and multiparous cows, respectively. Primiparous PVD– cows tended ($P = 0.06$) to be less likely to ovulate after GnRH treatment than primiparous PVD+ cows (Table 3.2). In contrast, multiparous PVD+ cows had a reduced ($P = 0.04$) ovulatory response to GnRH treatment than multiparous PVD– cows (Table 3.2). Both primiparous and multiparous noncyclic cows were more likely ($P < 0.01$) to ovulate to GnRH treatment than cyclic cows (Table 3.2). Primiparous cows with low BCS

had decreased ($P = 0.02$) odds of ovulating after GnRH treatment compared with high BCS cows, whereas in multiparous cows, BCS was not associated ($P = 0.96$) with ovulation after GnRH (Table 3.2). Ovulatory response to GnRH did not differ ($P > 0.64$) between dairies. The two-way interactions between PVD, cyclic status and BCS category were not significant ($P > 0.10$) for ovulatory response to GnRH treatment.

Percentage of cows bearing a CL or inseminated by study d 14 (CL + AI14), 21 (CL + AI21), or 35 (CL + AI35)

For primiparous cows, CL + AI14 was not ($P > 0.28$) associated with PVD or dairy, however, was associated ($P < 0.01$) with BCS (Table 3.3). Primiparous cows with high BCS were ($P < 0.01$) more likely to be classified as a success on study d 14 than low BCS cows (85.3 vs. 62.7%; Table 3.3). For multiparous cows, CL + AI14 was associated ($P < 0.03$) with PVD and BCS. Multiparous PVD+ cows were ($P = 0.03$) less likely to be classified as a success on study d 14 than PVD– cows (Table 3.3). Furthermore, multiparous cows with low BCS had decreased ($P < 0.01$) odds of being classified a success on study d 14 than high BCS cows (Table 3.3). Dairy was associated ($P < 0.01$) with CL + AI14. Multiparous cows from dairy A were ($P < 0.01$) more likely to be classified a success than multiparous cows from dairy B (90.1 vs. 79.4%; Table 3.3). The interaction between PVD and BCS category was not significant ($P > 0.10$) for CL + AI14.

Among primiparous cows, CL + AI21 was not ($P > 0.14$) associated with PVD, treatment with GnRH at study d 14, or dairy (Table 3.4). Body condition score was associated ($P < 0.01$) with CL + AI21. Similar to study d 14, cows with high BCS were more likely to be classified a success on study d 21 compared with low BCS cows (94.6 vs. 79.1%; Table 3.4). For multiparous cows, CL + AI21 was associated ($P \leq 0.01$) with PVD, treatment with GnRH at study d 14, and

BCS. Low BCS and PVD+ cows were ($P \leq 0.01$) less likely to be classified as a success on study d 21 compared with high BCS and PVD– cows, respectively (Table 3.4). Multiparous cows treated with GnRH on study d 14 had increased ($P < 0.01$) odds of being classified as a success on study d 21 than multiparous cows not treated with GnRH (Table 3.4). Dairy tended ($P = 0.10$) to be associated with CL + AI21 for multiparous cows (Table 3.4). Two-way interactions between PVD, GnRH treatment at study d 14, and BCS category were not significant ($P > 0.10$) for CL + AI21 for primiparous and multiparous cows.

For primiparous cows, CL + AI35 was not associated ($P = 0.29$) with PVD (Table 3.5). In contrast, GnRH and BCS were associated ($P \leq 0.03$) with CL + AI35 for primiparous cows (Table 3.5). Primiparous cows treated with GnRH or with high BCS were ($P < 0.04$) more likely to be a success on study d 35 than primiparous cows not treated with GnRH or with low BCS. Dairy tended to be associated with CL + AI35 for primiparous cows (Table 3.5). For multiparous cows, CL + AI35 was not associated ($P \geq 0.11$) with PVD or dairy (Table 3.5). Treatment with GnRH at study d 14 and BCS were associated ($P \leq 0.03$) CL + AI35 for multiparous cows (Table 3.5). Two-way interactions between PVD, GnRH treatment at study d 14, and BCS category were not significant ($P > 0.10$) for CL + AI35 for primiparous and multiparous cows.

Hazard of insemination after PGF

For primiparous cows, hazard of insemination after PGF was not ($P = 0.38$) associated with PVD. Hazard of insemination after PGF tended ($P = 0.06$) to be associated with cyclic status because noncyclic cows tended to be inseminated at a slower rate [adjusted hazard ratio (**AHR**) = 0.65, 95% CI = 0.41 to 1.03] than cyclic cows. Furthermore, BCS, treatment with GnRH, dairy, and all interactions were not ($P > 0.38$) associated with hazard of insemination after PGF for

primiparous cows. The survival analyses for primiparous cows revealed that PVD was not (P = 0.40) associated with the interval from PGF to insemination (Figure 3.2). Median and mean (± SE) days to insemination after PGF were 4 and 7.9 ± 0.5 d for PVD– primiparous cows, and 11 and 8.6 ± 0.7 d for PVD+ primiparous cows. Interactions between PVD and GnRH treatment at study d 14, PVD and BCS category, and PVD and cyclic status were not significant (P > 0.10) for hazard of insemination of primiparous cows.

Hazard of insemination after PGF was not (P = 0.76) associated with PVD for multiparous cows. In addition, treatment with GnRH, BCS, and all interactions were not (P > 0.15) associated with hazard of insemination. Hazard of insemination after PGF for multiparous cows was associated (P = 0.02) with cyclic status. Noncyclic multiparous cows were inseminated at a slower rate (AHR = 0.58, CI = 0.37 to 0.90) than cyclic cows. Survival analyses for multiparous cows revealed that PVD was not (P = 0.37) associated with the interval from PGF to insemination (Figure 3.3). Median and mean (± SE) days to insemination after PGF were 6 and 8.2 ± 0.3 d for PVD– multiparous cows, and 10 and 6.7 ± 0.5 d for PVD+ multiparous cows. Interactions between PVD and GnRH treatment at study d 14, PVD and BCS category, and PVD and cyclic status were not significant (P > 0.10) for hazard of insemination of multiparous cows.

Discussion

Previous studies demonstrated a negative association between PVD and reproductive efficiency in dairy cattle (LeBlanc et al., 2002; McDougall et al., 2007; Dubuc et al., 2010). Decreased reproductive efficiency of cows with PVD can be a result of several factors, including delayed resumption of cyclicity (Galvão et al., 2010), decreased pregnancy per AI (LeBlanc et al., 2002; McDougall et al., 2007), and altered uterine microbiota (Bicalho et al., 2017). Although

several reports demonstrated a negative effect of PVD on fertility of dairy cows, a paucity of evidence exists for PVD influencing ovarian responses to GnRH or PGF treatments. The current study provides evidence that PVD is associated negatively with ovarian responses to hormonal treatments, which may potentially influence success of reproductive management programs. In the United States, ovulation synchronization programs are dependent on exogenous GnRH and PGF to synchronize successfully ovulation for timed AI. Ovulation response to the initial GnRH of the Ovsynch protocol is important for synchronizing ovulation at the time of AI and improving fertility (Bello et al., 2006). The current study raises concern that PVD might decrease synchronization of cows submitted to reproductive programs by decreasing the ovulatory response to GnRH in multiparous cows, but not in primiparous cows.

The observation that primiparous PVD+ cows were more likely to ovulate to GnRH than primiparous PVD– cows is bewildering, especially because the interaction between PVD and cyclic status was not significant. In dairy cattle, PVD is associated with bacterial infection of the uterus, with the most common pathogens being *E. coli* and *A. pyogenes* (Williams et al., 2005). Increased bacterial load of the uterus can result in the presence of LPS in follicular fluid (Herath et al., 2007). In an *in vitro* study, exposure of granulosa cells to LPS decreased mRNA expression of aromatase and decreased production of estradiol from granulosa cells isolated from medium (4 to 8 mm) and larger follicles (> 8 mm; Herath et al., 2007). Decreased concentrations of estradiol because of LPS exposure could attenuate the magnitude of LH release or block the LH surge; therefore, preventing ovulation. Infusion of LPS into the uterus has been shown to suppress the preovulatory surge of LH in estrus-synchronized Holstein heifers (Peter et al., 1989). The previous authors suggested that an increased concentration of cortisol in LPS-infused heifers resulted in suppressed LH secretion (Peters et al., 1989), however, a direct effect on the ovary and follicle

cannot be ignored (Herath et al., 2007). Administration of GnRH during ovulation-synchronization programs causes release of LH in all cows (Stevenson and Pulley. 2015), however, magnitude of LH release is dependent on the concentration of progesterone (Giordano et al., 2012; Stevenson and Pulley, 2015; Pulley et al., 2015). Of particular interest, ewes infused with LPS had greater concentration of progesterone compared with non-infused controls (Battaglia et al., 1997, 1999, 2000). The observed LPS-induced increase in concentration of progesterone was attributed to adrenal secretion (Battaglia et al., 1997). Therefore, differences in GnRH-induced ovulatory responses of PVD+ and PVD– cows between parities could be related to differences in DIM at diagnosis of PVD and GnRH treatment, direct effects of PVD on the follicle, or differences in concentrations of progesterone and cortisol at the time of GnRH administration.

In addition to demonstrating that multiparous PVD+ cows have decreased GnRH-induced ovulatory responses, the current study presents evidence that multiparous PVD+ cows have decreased spontaneous ovulation. Spontaneous ovulation was indirectly assessed in the analyses that evaluated percentages of cows bearing a CL or inseminated by study d 14, 21, or 35. Fewer successes were recorded in cows diagnosed with PVD, based on presence of a CL or insemination, when controlling for BCS. It is important to note that these time points (study d 14, 21, and 35) may coincide with the end of the voluntary period in some dairy herds. Decreased ovulatory responses may influence DIM at first AI and pregnancy outcomes, thus impacting reproductive efficiency of dairy herds. These findings indicate that PVD in multiparous cows may have carryover effects on ovulatory responses. Ribeiro et al. (2016) demonstrated evidence that uterine disease has carryover effects on fertility by altering uterine environment. Indeed, the negative impact of uterine disease on fertility is independent of anovular condition or low BCS after calving (Ribeiro et al., 2016). Collectively, results from the present study and those reported by Ribeiro et

al. (2016) demonstrate that cows diagnosed with uterine disease have decreased ovulatory responses and fertility, respectively, regardless of cyclic status or BCS. Furthermore, these data demonstrate uterine disease has a prolonged impact in the reproductive tract because of the observed negative effects in the ovary and uterus several days after diagnosis of uterine disease. Nevertheless, it is unclear why ovarian responses differed in PVD+ and PVD– cows between parities. Similar rates of success were recorded in primiparous PVD+ cows, based on presence of a CL or insemination, compared with PVD– cows at study d 14, 21, and 35.

Although PVD was determined and categorized at greater DIM for primiparous than multiparous cows, prevalence of PVD was greater in primiparous than multiparous cows. Multiparous cows are more likely to have metabolic diseases, such as hyperketonemia and hypocalcemia, compared with primiparous cows (Markusfeld, 1987). Despite PVD being less prevalent in multiparous cows, uterine disease and other disorders might have an additive effect in altering ovarian responses. In fact, Ribeiro et al. (2016) demonstrated an additive negative effect of uterine and non-uterine disease on pregnancy per AI.

In the present study, PVD was associated with decreased P305M in multiparous cows. In contrast, no differences were detected in P305M between PVD+ and PVD– primiparous cows. Other researchers have not observed an effect of PVD on milk production (Fourichon et al., 1999; Dubuc et al., 2011). It is possible that multiparous PVD+ cows from the present study experienced other disorders besides uterine disease. The hypothesis that PVD affects multiparous cows more than primiparous cows is interesting, however, one should be cautious in drawing any inferences from the present study because of differences in DIM at PVD diagnosis.

Peripheral concentration of PGE has been shown to be increased in cows with bacterial infection of the uterus during the first 2 wk postpartum (Herath et al., 2009). Using an *in vitro*

model, Herath et al. (2009) also demonstrated a shift from production of PGF to PGE by epithelial cells of the bovine endometrium treated with LPS. In contrast, repeated intrauterine infusion of LPS in heifers did not modulate mRNA expression of PGE synthase in the endometrium of the uterus on d 6 of the estrous cycle, whereas mRNA expression of PGE synthase was increased in d 6 CL compared with controls (Lüttgenau et al., 2016a). Although these studies evaluated the effects of LPS on prostaglandin secretion and mRNA modulation of the endometrium, limited experiments exist that have evaluated responses to exogenous PGF in PVD+ cows. Even though the current study did not detect differences in hazard of insemination between cows with or without PVD in response to PGF, caution should be exercised when interpreting these results. One limitation of the current study was the extended time (21 d) between classification of PVD and administration of PGF. Endometrial inflammation or decreased bacteria load of the uterus in PVD+ cows may have been partly resolved before PGF administration. Furthermore, the small sample size of cows with PVD receiving PGF also should be considered. A large proportion of cows were inseminated before PGF treatment, resulting in a small number of cows treated with PGF. Experiments with a shorter interval between PVD diagnosis and PGF treatment with an adequate sample size are required to fully elucidate the role of PVD on ovarian responses to PGF.

Conclusions

Purulent vaginal discharge is associated with GnRH-induced ovulatory response and spontaneous ovulation in dairy cattle. Purulent vaginal discharge in multiparous cows had a more pronounced detrimental effect on ovarian responses than in primiparous cows. Dairy herds with a high prevalence of PVD, especially in multiparous cows, might observe decreased reproductive efficiency and responses to reproductive management programs. Response to PGF was not

associated with PVD in primiparous or multiparous cows, however, more research is required to replicate and verify these findings.

Acknowledgments

This research was partially funded by Zoetis Animal Health (Florham Park, NJ) and the Kansas Dairy Commission (Hays, KS). The authors thank the owners and managers of the collaborating dairies, Gary Neubauer of Zoetis Animal Health, and Robert Briggeman of ABS Global (DeForest, WI).

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Table 3.1 Projected 305-d milk yield (P305M), BCS and proportion of cows with BCS > 3 at enrollment, and proportion of cows cyclic at study d 14 based on parity for cows with or without purulent vaginal discharge (PVD).

	PVD		
Item	Positive	Negative	P-value
Primiparous	n = 83	n = 208	
P305M, kg (± SE)	7,808 ± 131	7,979 ± 80	0.26
BCS (± SE)	3.06 ± 0.04	3.06 ± 0.02	0.85
BCS ≥ 3, %	75.9	77.4	0.78
Cyclic at study d 14, %	75.3	81.5	0.24
Multiparous	n = 54	n = 348	
P305M, kg (± SE)	9,455 ± 200	9,994 ± 68	<0.01
BCS (± SE)	2.87 ± 0.06	2.95 ± 0.02	0.16
BCS ≥ 3, %	57.4	58.3	0.90
Cyclic at study d 14, %	73.6	87.1	0.01

Table 3.2 Odds of ovulation from study d 14 to 21 after treatment with GnRH for primiparous and multiparous cows positive or negative for purulent vaginal discharge (PVD).

Item	Level	Ovulation, %	OR (95% CI)	P-value
Primiparous				
PVD	negative	40.8	Referent	0.06
	positive	67.6	2.75 (0.95 – 7.93)	
Cyclic Status[1]	cyclic	37.5	Referent	<0.01
	noncyclic	81.5	18.82 (3.76 – 94.13)	
BCS[2]	high	57.6	Referent	0.02
	low	37.5	0.15 (0.03 – 0.74)	
Dairy	A	51.6	Referent	0.95
	B	51.9	1.04 (0.35 – 3.06)	
Multiparous				
PVD	negative	49.7	Referent	0.04
	positive	37.0	0.35 (0.13 – 0.95)	
Cyclic Status[1]	cyclic	39.6	Referent	<0.01
	noncyclic	82.4	8.75 (3.17 – 24.15)	
BCS[2]	high	45.9	Referent	0.96
	low	50.0	1.02 (0.53 – 1.96)	
Dairy	A	44.1	Referent	0.64
	B	51.8	1.17 (0.61 – 2.21)	

[1] Cows with a corpus luteum present on study d 0 or 14 were classified as cyclic.
[2] Body condition score category: high (≥ 3.00) or low (< 3.00).

Table 3.3 Odds of bearing a corpus luteum or being inseminated by study d 14 for primiparous and multiparous cows positive or negative for purulent vaginal discharge (PVD).

Item	Level	Success, %	OR (95% CI)	P-value
Primiparous				
PVD	negative	81.7	referent	0.28
	positive	75.9	0.70 (0.37 – 1.32)	
BCS[1]	high	85.3	referent	<0.01
	low	62.7	0.29 (0.16 – 0.55)	
Dairy	A	78.6	referent	0.87
	B	81.2	1.05 (0.57 – 1.91)	
Multiparous				
PVD	negative	87.5	referent	0.03
	positive	74.1	0.46 (0.22 – 0.94)	
BCS[1]	high	90.6	referent	<0.01
	low	78.6	0.35 (0.19 – 0.62)	
Dairy	A	90.1	referent	<0.01
	B	79.4	0.41 (0.23 – 0.74)	

[1] Body condition score category: high (≥ 3.00) or low (< 3.00).

Table 3.4 Odds of bearing a corpus luteum or being inseminated by study d 21 for primiparous and multiparous cows with or without purulent vaginal discharge (PVD).

Item	Level	Success, %	OR (95% CI)	P-value
Primiparous				
PVD	negative	91.8	referent	0.40
	positive	89.2	0.68 (0.28 – 1.67)	
GnRH	no	89.7	referent	0.14
	yes	93.5	2.00 (0.79 – 5.10)	
BCS[1]	high	94.6	referent	<0.01
	low	79.1	0.21 (0.09 – 0.48)	
Dairy	A	91.3	referent	0.61
	B	90.9	0.80 (0.35 – 1.87)	
Multiparous				
PVD	negative	97.4	referent	<0.01
	positive	87.0	0.17 (0.06 – 0.51)	
GnRH	no	93.7	referent	<0.01
	yes	98.5	5.81 (1.54 – 22.22)	
BCS[1]	high	97.9	referent	0.01
	low	93.5	0.23 (0.07 – 0.73)	
Dairy	A	97.4	referent	0.10
	B	94.1	0.40 (0.13 – 1.20)	

[1] Body condition score category: high (≥ 3.00) or low (< 3.00).

Table 3.5 Odds of bearing a corpus luteum or being inseminated by study d 35 for primiparous and multiparous cows with or without purulent vaginal discharge (PVD).

Item	Level	Success, %	OR (95% CI)	P-value
Primiparous				
PVD	negative	95.2	referent	0.29
	positive	92.6	0.55 (0.18 – 1.66)	
GnRH	no	92.4	referent	0.03
	yes	98.1	5.43 (1.18 – 24.39)	
BCS[1]	high	96.4	referent	<0.01
	low	87.7	0.18 (0.08 – 0.66)	
Dairy	A	96.8	referent	0.08
	B	92.7	0.35 (0.10 – 1.14)	
Multiparous				
PVD	negative	98.3	referent	0.11
	positive	94.4	0.29 (0.07 – 1.32)	
GnRH	no	96.1	referent	0.03
	yes	99.5	10.42 (1.25 – 90.91)	
BCS[1]	high	99.2	referent	0.02
	low	95.8	0.15 (0.03 – 0.78)	
Dairy	A	98.7	referent	0.12
	B	96.5	0.31 (0.07 – 1.33)	

[1] Body condition score category: high (≥ 3.00) or low (< 3.00).

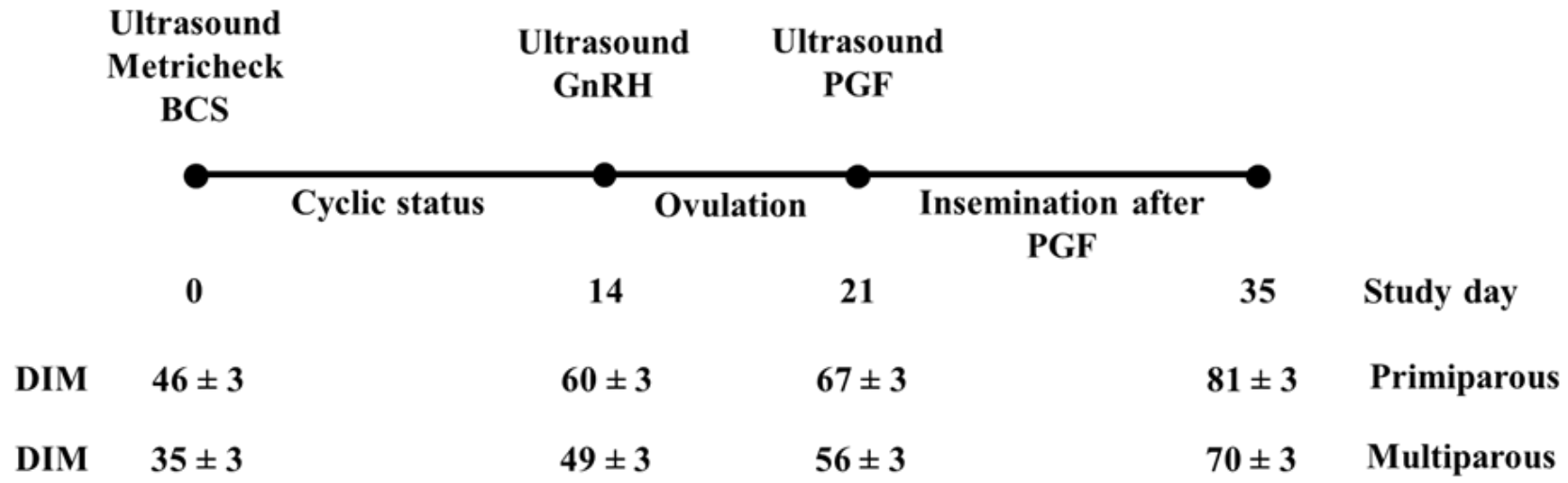

Figure 3.1 Schematic of the experimental design.

DIM = days in milk; BCS = body condition score; GnRH = gonadotropin-releasing hormone; PGF = prostaglandin $F_{2\alpha}$.

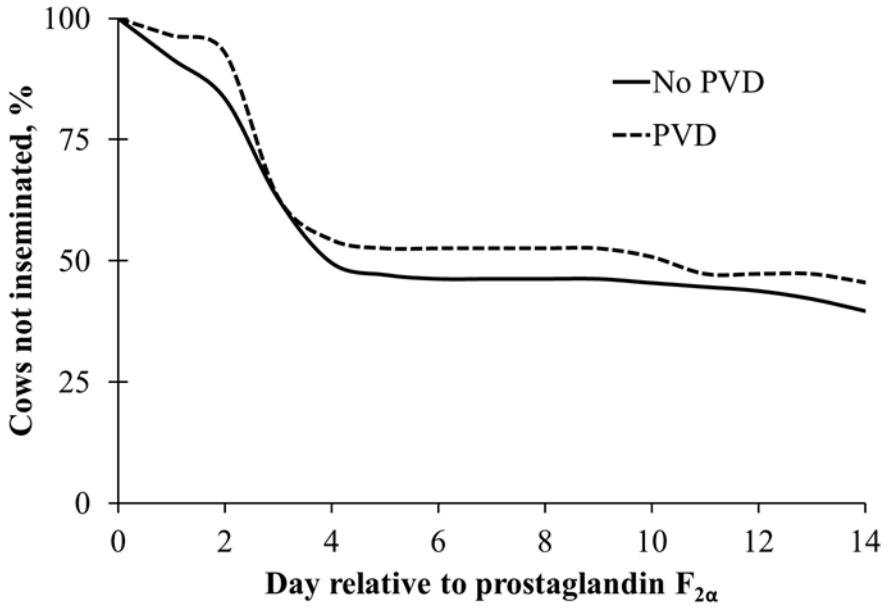

Figure 3.2 Survival analysis for days to insemination for primiparous cows positive (PVD+) or negative (PVD–) for purulent vaginal discharge.

Median days to insemination of primiparous cows were 11 and 4 d for PVD+ or PVD– cows, respectively ($P = 0.40$).

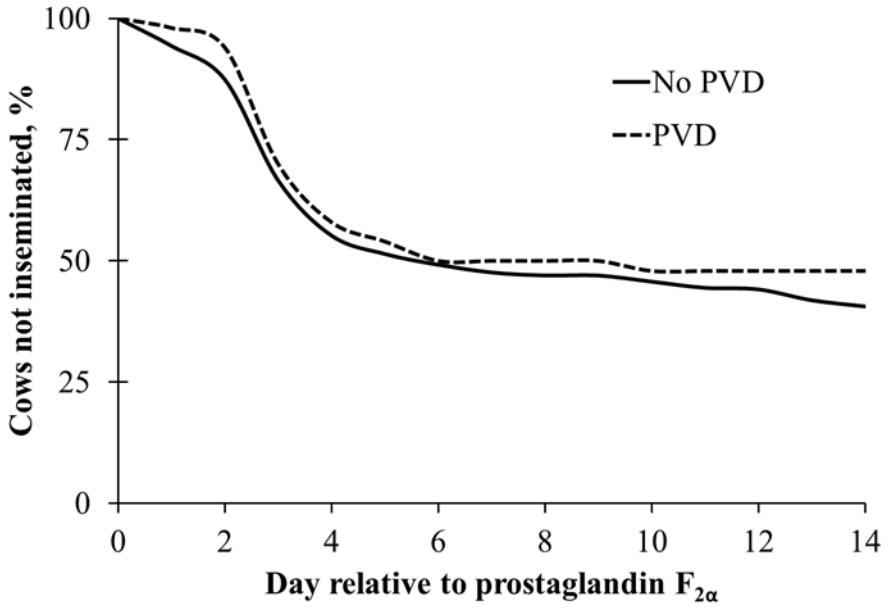

Figure 3.3 Survival analysis for days to insemination for multiparous cows positive (PVD+) or negative (PVD–) for purulent vaginal discharge.

Median days to insemination of multiparous cows were 10 and 6 d for PVD+ or PVD– cows, respectively ($P = 0.37$).

Chapter 4 - Associations between Activity of Arginase or Matrix Metalloproteinase-8 (MMP-8) and Metritis in Periparturient Dairy Cattle

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2017. Theriogenology 97: 83–88.

Abstract

Metritis, a uterine disease caused by bacterial infection, is highly prevalent in dairy cattle after parturition. Uterine disease has negative effects on milk production and reproductive efficiency. Finding markers or indicators that can predict cows at greater risk for uterine disease could be beneficial to mitigating these deleterious effects. This study investigates the immune-derived enzymes arginase and matrix metalloproteinase-8 (**MMP-8**) as potential markers for development of metritis in dairy cows. In a retrospective matched case-control study, 53 lactating Holstein cows diagnosed with metritis were matched and paired to 53 lactating Holstein control cows. In addition to examining cows for diagnosis of metritis on d 4, 7, 10, and 14 after parturition, occurrence of retained fetal membranes, gender of the calf, and the event of a stillbirth were recorded. Blood samples were collected 7 ± 3 d before calving, on the day of calving, and 7 ± 3 d after calving and were assayed for activity of arginase and MMP-8. Associations between metritis and activity of arginase or MMP-8 were determined by conditional logistic regression at each individual sampling time point. An interaction between activity of arginase, before and on the day of parturition, and retained fetal membranes tended ($P \leq 0.13$) to be associated with metritis. After parturition, activity of arginase and the interaction between activity of arginase and retained fetal membranes were not ($P \geq 0.22$) associated with metritis. Activity of MMP-8 was not ($P \geq 0.20$) associated with metritis in the periparturient period. Retained fetal membranes were associated with the odds of developing metritis. Activity of arginase before and at the time of parturition might be a potential marker for occurrence of metritis, especially in cows that develop retained fetal membranes. MMP-8 does not seem to be a potential indicator for metritis.

Introduction

Dairy cattle that have uterine disease after parturition have decreased milk production (Rajala and Gröhn, 1998; Huzzey et al., 2007) and reproductive performance (McDougall, 2001). The two major uterine diseases during the early postpartum period are retained fetal membranes and metritis. Retained fetal membranes is usually defined as the failure to expel fetal membranes by 24 h after parturition (Stevenson and Call, 1988; Kelton et al., 1998). Considering that 95% of cows that pass fetal membranes by 24 h had passed membranes by 12 h postpartum, defining the disorder at 12 or 24 h after calving is not relevant (Van Werven et al., 1992; LeBlanc, 2008). Puerperal metritis is defined as a cow having an enlarged uterus with the presence of foul red-brown uterine discharge, with decreased milk production, dullness, and fever within 21 d after parturition (Sheldon et al., 2006). Difficult calving or dystocia increases the risk of retained fetal membranes and uterine disease in cows (Dubuc et al., 2010). Factors that can increase the likelihood of cows having dystocia include gender of the calf, stillbirth event (death within 24 h of birth), and twin births (Mee, 2008).

Predicting whether cows will have postpartum uterine disorders may allow implementation of prevention strategies to decrease the negative effects caused by uterine disease on performance. Prepartum dry matter intake (Huzzey et al., 2007) and postpartum concentrations of the acute phase protein haptoglobin (Huzzey et al., 2009) are associated with metritis risk in dairy cattle. Decreased dry matter intake as early as 2 wk prepartum is associated with an increased risk of metritis (Huzzey et al., 2007). Furthermore, cows with concentration of haptoglobin ≥1 g/L 3 d after parturition have 6.7 times greater risk of developing metritis than cows with a concentration <1 g/L (Huzzey et al., 2009). Nevertheless, dry matter intake and concentration of haptoglobin are associated with metritis risk, but are poor predictors despite these observed associations.

It is well documented that dairy cows undergo immunosuppression during the periparturient period (Kehrli et al., 1989). Level of immunosuppression could be a factor that influences postpartum health in dairy cattle because neutrophil function is associated with postpartum uterine diseases. In dairy cattle, retained fetal membranes is believed to be a result of decreased neutrophil function and decreased serum concentrations of interleukin-8 (Kimura et al., 2002). In addition, the cotyledonary placenta of cattle requires release of collagenase from neutrophils to breakdown the microvilli interaction of the cotyledon and caruncle and allow release of the fetal membranes (Eiler and Hopkins, 1993). In dairy cattle, reduced myeloperoxidase activity of neutrophils (Hammon et al., 2006) and increased serum concentrations of tumor necrosis factor-α and interleukin-6 (Kasimanickam et al., 2013) have been reported to be associated with metritis. Arginase and matrix metalloproteinase-8 (MMP-8) are two enzymes produced by polymorphonuclear neutrophils and are released at sites of inflammation (Weiss et al., 1985; Munder, 2009). Expression of arginase and depletion of L-arginine are signs of immunosuppression (Bronte and Zanovello, 2005). In mice, MMP-8 is essential for a lipopolysaccharide-induced inflammatory response, chemokine production, and is an indicator of neutrophil function (Tester et al., 2007). In cattle, gram negative bacteria invade the uterus within 2 wk of calving (Sheldon et al., 2008). Gram negative bacteria can cause metritis via a lipopolysaccharide-induced inflammatory response via the TLR4/CD14/MD-2 receptor complex (Herath et al., 2006; Sheldon et al., 2008). Because neutrophil function is associated with uterine disorders, activity of enzymes produced by neutrophils, such as arginase and MMP-8, might be potential candidates for indicators or predictors of uterine disease.

The hypothesis of the current study was that activity of arginase and MMP-8 during the periparturient period would be associated with development of metritis in dairy cattle. The

objectives of the current study were to: (1) quantify activity of arginase and MMP-8 in maternal blood plasma samples during the periparturient period in dairy cattle and (2) determine whether activity of arginase and MMP-8 during the periparturient period are associated with the risk of developing metritis.

Materials and Methods

Study Design and Sampling

This study was a retrospective matched case-control study. Blood samples collected from cows in a previous experiment (Liboreiro et al., 2015) were used in this study. The experiment (Liboreiro et al., 2015) was conducted in one commercial dairy herd located in northwestern Wisconsin. Holstein animals housed in naturally ventilated free-stall barns were enrolled in the study at 258.3 ± 0.2 d of gestation (mean ± SEM). Nulliparous heifers were housed in separate pens from primiparous and multiparous cows before calving. Animals were fed the same prepartum TMR, except that nulliparous heifers were not supplemented with anionic salts. Animals demonstrating signs of calving (e.g., discomfort, restlessness, tail twitching, and visualization of the allantoic sac through the vulva) were moved to a loose housing pen. After parturition cows were moved to one free-stall pen regardless of parity and were fed the same postpartum TMR. Fifty-three cows diagnosed with metritis were matched and paired to control cows by parity and date of calving. Cows (n = 106) were examined on d 4, 7, 10, and 14 after calving for diagnosis of metritis. In addition, cows were examined during the first 24 h for occurrence of retained fetal membranes. Furthermore, gender of the calf and the event of a stillbirth were recorded. Blood samples were collected 7 ± 3 d before parturition, by 24 h after calving (study d 0), and 7 ± 3 d after parturition. Blood samples were collected from a coccygeal vessel

into evacuated tubes containing K2 EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) and placed on ice after collection until centrifugation (1,200 × *g* for 15 min at 4°C). Blood plasma was separated, aliquoted to microcentrifuge tubes, and stored at –32 °C or –20 °C until analysis. Body condition score was assessed 21 d before and at calving on a scale of 1 (severe under conditioned) to 5 (severe over conditioned) with 0.25 increments (Ferguson et al., 1994).

Arginase and MMP-8 Assay

Enzymatic activities of arginase and MMP-8 were quantified using two nanoplatforms that were previously developed by researchers at Kansas State University (Manhattan, KS; Wang et al., 2014; Udukala et al., 2016).

Briefly, dopamine-coated Fe/Fe_3O_4 core/shell nanoparticles with covalently attached fluorescent ligands were synthesized for detecting MMP-8 (consensus sequence: GAGPSG-LRGAG) and arginase I+II (tether: GRRRRRRRG) in aqueous buffer solutions. Each nanoplatform featured a Foerster donor-acceptor pair. The donor was attached to Fe/Fe_3O_4 via a tether, whereas the acceptor was directly bound to dopamine. This design enabled a sub-femtomolar limit of detection of MMP-8, whereas arginase could be detected in the picomolar range. The upper bound for both enzymes was in the micromolar range. Fe/Fe_3O_4 – based nanoplatforms were dispersed in HEPES buffer solution to form the assay solution (0.3 mg/mL). Next, 5 µL of sample plasma and 125 µL of assay solution were added to a 96-well plate in triplicate. Furthermore, a sample control consisting of 5 µL of sample plasma and 125 µL of HEPES buffer was also added to the 96-well plate for each sample. Plates were analyzed using a Synergy H1 Plate Reader (BioTek Instruments Inc., Winooski, VT). A spectral scan from 600 nm to 700 nm with 2 nm step increment was performed, in accordance with standard procedures.

Activities of arginase and MMP-8 were quantified by calculation of the area under the curve from 620 nm to 680 nm for each sample and subtracting the area under the curve of the sample control. All samples were then divided by an assay control to standardize the values across assay. Activities of arginase and MMP-8 are reported in relative florescence units (RFU). Fold-change in activity of arginase and MMP-8 were calculated by dividing the activity before parturition by the activity after parturition, as well as dividing the activity at parturition by the activity after parturition. Fold-change in activity was calculated in order to evaluate differences in activity of arginase and MMP-8 over time.

Statistical Analyses

Descriptive statistics for the activity of arginase and MMP-8 for cows with and without metritis were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Individual models were created for arginase and MMP-8 that included retained fetal membranes (yes vs. no), calf gender (male vs. female), twinning (yes vs. no), stillbirth (yes vs. no), parity (nulliparous, primiparous, or multiparous), and body condition score of cows 21 d before and at calving. Blood sample day was treated as the repeated measure and covariance structure (unstructured, autoregressive, or compound symmetry) was determined by the Akaike information criterion. A manual backward elimination procedure was used to build the final models. Variables and interactions with $P < 0.15$ were retained in the model. Results for activity of arginase and MMP-8 are reported as least square means (±SEM).

Associations between metritis and arginase, and metritis and MMP-8 were analyzed by conditional logistic regression using the LOGISTIC procedure of SAS at each time point. Cows were conditionally stratified by the matched pairs, and event of metritis was used as the outcome variable. Separate analysis was conducted for each enzyme (arginase and MMP-8). The full

models included activity of enzyme or activity fold-change, retained fetal membranes (yes vs. no), calf gender (male vs. female), twinning (yes vs. no), stillbirth (yes vs. no), and body condition score of cows before and at calving. Furthermore, the models included the interaction between the activity of the enzyme and retained fetal membranes. The conditional logistic regression models removed variables and interactions by a backward elimination based on the Wald criterion when $P > 0.15$. Statistical significance was defined as $P \leq 0.05$ and tendency was considered if $0.05 < P \leq 0.15$.

Results

Descriptive Analysis for Activity of Arginase and MMP-8

Among cows with metritis, activity of arginase on d -7 tended to be ($P = 0.08$) and was ($P < 0.01$) greater than on d 0 and 7, respectively (Table 4.1). Nulliparous heifers had greater ($P < 0.01$) activity of arginase than multiparous cows, but nulliparous heifers did not differ ($P = 0.16$) from primiparous cows (Table 4.1). Multiparous cows tended ($P = 0.07$) to have decreased activity of arginase than primiparous (Table 4.1). Cows that had stillbirth tended ($P = 0.15$) to have lesser activity of arginase than cows without stillbirth (4.86 ± 0.29 vs. 3.68 ± 0.74 RFU; Table 4.1). In addition, cows diagnosed with retained fetal membranes tended ($P = 0.07$) to have greater activity of arginase compared with cows without retained fetal membranes (4.78 ± 0.51 vs. 3.76 ± 0.46 RFU; Table 4.1).

Among cows without metritis, activity of arginase did not ($P = 0.40$) differ between d -7 and 0; however, activity on d 7 was decreased ($P < 0.01$) compared with d -7 and 0 (Table 4.2). Similar to cows with metritis, no difference ($P = 0.37$) was detected between nulliparous heifers and primiparous cows (Table 4.2). Primiparous cows had greater ($P = 0.02$) activity of arginase

compared with multiparous cows (Table 4.2). Stillbirth was not ($P = 0.34$) associated with activity of arginase (Table 4.2). Cows diagnosed with retained fetal membranes tended ($P = 0.10$) to have lesser activity of arginase than cows without retained fetal membranes (5.03 ± 0.41 vs. 3.08 ± 1.17 RFU; Table 4.2).

In cows diagnosed with metritis, activity of MMP-8 was lowest ($P < 0.01$) on d 7 compared with d -7 and 0, but did not ($P = 0.27$) differ between d -7 and 0 (Table 4.1). Furthermore, activity of MMP-8 was associated ($P = 0.05$) with parity (nulliparous = 108.65 ± 7.84, primiparous = 114.99 ± 6.31, and multiparous = 132.27 ± 6.31 RFU; Table 4.1).

Activity of MMP-8 was lowest ($P < 0.04$) on d 7, and tended ($P = 0.12$) to be decreased between d -7 to 0 in cows that did not have metritis (Table 4.2). Activity of MMP-8 was associated ($P = 0.01$) with parity (nulliparous = 124.73 ± 11.15, primiparous = 129.68 ± 8.59, and multiparous = 150.68 ± 10.05 RFU; Table 4.2). Furthermore, cows that had twins had greater ($P = 0.05$) activity of MMP-8 compared with cows that did not have twins (151.02 ± 15.58 vs. 119.04 ± 5.00 RFU, Table 4.2).

Association between Arginase and Metritis

Activity of arginase on study d -7 tended ($P = 0.14$) to be associated with metritis. In addition, the interaction between activity of arginase on study d -7 and retained fetal membranes tended ($P = 0.12$) to be associated with metritis (Table 4.3). Among cows that were diagnosed with retained fetal membranes, the odds of having metritis increased by 295% for each one-unit increase in activity of arginase (adjusted odds ratio [AOR] = 3.95; 95% CI = 0.74 to 21.03; Table 4.3). In contrast, among cows not diagnosed with retained fetal membranes, the odds of having metritis

increased by 2% for every unit increase in activity of arginase (AOR = 1.02; 95% CI = 0.55 to 1.90; Table 4.3).

Activity of arginase on study d 0 was not (P = 0.42) associated with metritis. The interaction between activity of arginase on study d 0 and retained fetal membranes tended (P = 0.13) to be associated with metritis (Table 4.3). Similar to study d -7, the effect of one-unit increase in activity of arginase on study d 0 was greater for cows that had retained fetal membranes compared with cows that did not have retained fetal membranes (Table 4.3).

The interaction between activity of arginase on d 7 and retained fetal membranes was not associated (P = 0.22) with the odds of cows having metritis. Furthermore, activity of arginase on d 7 was not associated (P = 0.96) with occurrence of metritis (Table 4.3); however, retained fetal membranes were associated (P < 0.01) with metritis. Cows that did not have retained fetal membranes had lesser odds of developing metritis than cows with retained fetal membranes. Fold-change in the activity of arginase from d -7 to d 0 was not associated (P = 0.73) with development of metritis. Furthermore, fold-change in activity of arginase from d 0 to d 7 was not associated (P = 0.98) with metritis. Odds of developing metritis was not ($P \geq 0.62$) affected by the interactions between retained fetal membranes and fold-change in activity of arginase from d -7 to d 0 and from d 0 to d 7.

Association between MMP-8 and Metritis

Activity of MMP-8 before parturition (d -7), at parturition (d 0), and after parturition (d 7) were not associated (P > 0.20) with the odds of developing metritis (Table 4.4). Retained fetal membranes was associated (P < 0.01) with the odds of developing metritis. Cows that were not diagnosed with retained fetal membranes had lesser odds of developing metritis (Table 4.4).

Fold-change in activity of MMP-8 from d -7 to d 0 and from d 0 to d 7 was not associated ($P \geq 0.25$) with occurrence of metritis. Similar to the findings in the arginase analysis, calf gender, twinning, occurrence of stillbirth, and body condition score before and at calving were not associated ($P > 0.15$) with the odds of developing metritis in any of the MMP-8 models.

Discussion

Recent studies provide evidence of the role of arginase in modulating inflammatory responses, neutrophil function, and immunosuppression (Munder et al., 2005, Munder et al., 2006; Munder, 2009). Nevertheless, the importance of arginase in the interplay of inflammation and immunity has not been well investigated in dairy cattle. Upregulation of MMP enzymes during inflammatory responses has been reported previously in experiments involving dairy cows (Raulo et al., 2002; Hanthorn et al., 2014). Nonetheless, experiments evaluating the role of MMP-8 in cattle are limited. Based on the potential of these enzymes being used as markers of inflammation or immune responses, one of the objectives of the current study was to evaluate plasma activity of arginase and MMP-8 during the periparturient period of dairy cows. In addition, we intended to investigate whether these enzymes were associated with metritis, a uterine disorder that commonly affects postpartum dairy cows.

Other researchers have investigated the activity of proteases in placentomes from cows that were diagnosed with retained fetal membranes (Gross et al., 1985). The majority of the MMP family enzymes investigated include the gelatinases (MMP-2 and MMP-9), but the collagenase MMP-8 has not been investigated (Maj and Kankofer, 1997; Walter and Boos, 2001). Cows diagnosed with retained fetal membranes have decreased activity of MMP-9, and may have deficiency of MMP-2, as suggested by Maj and Kankofer (1997). Furthermore, cows with retained

fetal membranes have decreased concentrations of interleukin-8 (**IL-8**; Kimura et al., 2002), whereas, Osmers et al. (1995) speculated that IL-8 increased collagenase secretion. Researchers demonstrated that therapeutic treatment with collagenase into the umbilical artery after cesarean section or in cows with retained fetal membranes decreased the amount of force required to separate cotyledons from caruncles (Fecteau and Eiler, 1996). The potential association between IL-8 and secretion of collagenases, especially MMP-8, garners further research and attention in dairy cattle, despite the fact that the current study did not demonstrate an association between MMP-8 and uterine disease.

Previous research demonstrated an increase in MMP-8 gene expression of neutrophils isolated during the periparturient period of dairy cattle (Burton et al., 2005). Expression of MMP-8 was greatest during parturition, but decreased by 6 h after calving (Burton et al., 2006). In the current study, it is important to note that blood samples were collected within 24 h of parturition. It is possible that activity of MMP-8 was increased around the time of parturition but cleared from circulation before blood samples were collected. Frequent blood sampling of periparturient cows may provide a better understanding of the association between MMP-8 and postpartum uterine health of dairy cows.

L-arginine is competitively utilized by both arginase and nitric oxide synthase for the production of either L-ornithine during the urea cycle or L-citrulline during the nitric oxide cycle, respectively (Bratt et al., 2011). In humans, nitric oxide has been shown to be important for vasodilation and normal function of the placenta (Noris et al., 1996), whereas arginase expression has been shown to be increased in the placenta of women with preeclampsia (Noris et al., 2004). Preeclampsia is a hypertensive and multiple system disease that occurs during pregnancy in humans resulting in insufficient blood flow to the uterus. Depletion of L-arginine resources

because of increased activity of arginase might be the mechanism that triggers preeclampsia, but arginase is not the only enzyme that is associated with preeclampsia. Several MMP genes are involved in blood vessel collagen breakdown of women with preeclampsia, including MMP-8. Women with preeclampsia had decreased MMP-8 gene methylation compared with women without preeclampsia (Mousa et al., 2012), indicating a greater activity of MMP-8. Preeclampsia greatly increases the risk of newborn morbidity and mortality. In cattle, risk of stillbirths is most influenced by parity, with primiparous cows having greater incidence of stillbirths than multiparous cows (Meyer et al., 2000). Despite the fact that preeclampsia does not affect cattle, this study reveals that arginase may also be linked to uterine disorders in cattle. A novel and intriguing finding from the current study is that as activity of arginase increased, the odds of metritis increased in cows diagnosed with retained fetal membranes. Further research is needed to investigate how arginase and MMP-8 might influence the development of uterine disease across different species.

Activity of arginase was lowest on d 7, regardless of the incidence of metritis; therefore, activity of arginase might be associated with pregnancy in cattle. Research in humans has shown that arginase is associated with pregnancy and is one mechanism that contributes to the suppression of the maternal immune system (Kropf et al., 2007). Arginase is able to regulate and suppress T cell function in healthy human pregnancies (Kropf et al., 2007) by depleting L-arginine that is essential for T cell activation (Bronte and Zanovello, 2005). More recently, researchers isolated arginase-expressing cells in peripheral mononuclear cells and in the term placenta of women (Ssemaganda et al., 2014). To our knowledge, the contribution of arginase to immunosuppression during pregnancy in cattle has not been demonstrated. Activity of arginase observed in the current

study suggests that arginase during pregnancy of cattle might be similar to that previously observed in humans.

Among cows with metritis, cows with retained fetal membranes had greater activity of arginase than cows without retained fetal membranes, whereas the opposite was observed among cows that did not develop metritis. Many different risk factors influence the occurrence of retained fetal membranes. The observed differences in activity of arginase and incidence of metritis could be associated with the different risk factors of retained fetal membranes. It is possible that the pathophysiology of metritis is different for cows that have retained fetal membranes compared with cows that do not. Retained fetal membranes is associated with an increased risk of developing metritis (Gröhn et al., 1990), which also was observed in the current study. The finding that cows with an increased risk of developing uterine disease (cows with retained fetal membranes) had greater odds of developing metritis as activity of arginase increased is intriguing. In contrast, the association was diminished among cows at low risk of developing uterine disease (cows not diagnosed with retained fetal membranes). It is possible that cows at high risk for metritis may experience an exacerbated degree of immunosuppression with increased activity of arginase. The interaction of magnitude between occurrence of retained fetal membranes and activity of arginase on metritis was observed on study d -7 and d 0, but not on study d 7. This interaction indicates that activity of arginase before and at calving may be a potential marker for metritis in cows at high risk for developing uterine diseases. In contrast, activity of arginase after calving is not indicative of occurrence of metritis, indicating it may not serve as a potential diagnostic tool. Metabolic markers have been identified as indicators for the development of metritis. Increased serum non-esterified fatty acids (**NEFA**) and beta-hydroxybutyrate during the periparturient period are associated with a greater level of negative energy balance (Grummer et al., 2004) and an increased

risk of metritis (Hammon et al., 2006). Negative energy balance is influenced by dry matter intake, which also has been shown to be associated with incidence of metritis (Huzzey et al., 2007). Furthermore, increased serum NEFA is associated with decreased neutrophil function, which is involved in the pathophysiology of retained fetal membranes (Kimura et al., 2002) and metritis (Hammon et al., 2006). Furthermore, concentration of haptoglobin, an acute phase protein, is associated with risk of developing metritis (Huzzey et al., 2009). In the current study, concentrations of NEFA, beta-hydroxybutyrate, and haptoglobin were not accounted for in the conditional logistic regression model; however, other risk factors associated with uterine disease were considered (e.g., body condition score, stillbirth, twinning, and calf gender). Even when controlling for these known risk factors, increased activity of arginase in high risk cows was associated with the development of metritis

Conclusions

Arginase may be a potential marker for metritis because cows at high risk for metritis had increased odds of developing the disease for each unit increase in activity of arginase. Although activity of arginase may be a plausible predictor of metritis, activity 7 d after calving does not seem to have diagnostic value for metritis. In addition, it does not seem that MMP-8 is a good indicator for development of metritis. Further research is vital to understanding the importance and role of arginase and MMP-8 during the periparturient period in dairy cattle.

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Table 4.1 Activity of arginase and matrix metalloproteinase-8 (MMP-8) in plasma of cows diagnosed with metritis

	Arginase		MMP-8	
Item	Mean ± SE[1]	P-value	Mean ± SE[1]	P-value
Day relative to calving		<0.01		<0.01
-7	4.65 ± 0.41		123.69 ± 4.08	
0	4.34 ± 0.41		121.06 ± 4.46	
7	3.83 ± 0.41		111.15 ± 4.24	
Parity		0.01		0.05
Nulliparous	5.28 ± 0.61		108.65 ± 7.84	
Primiparous	4.29 ± 0.49		114.99 ± 6.31	
Multiparous	3.24 ± 0.54		132.27 ± 6.31	
Retained fetal membranes		0.07		0.31
Yes	4.78 ± 0.51		110.39 ± 8.34	
No	3.76 ± 0.46		120.49 ± 9.31	
Stillbirth		0.15		0.92
Yes	3.68 ± 0.74		112.72 ± 17.33	
No	4.86 ± 0.29		111.14 ± 9.07	
Twins		0.93		0.49
Yes	4.96 ± 0.89		125.66 ± 10.66	
No	4.83 ± 1.06		117.56 ± 4.41	
Gender of calf		0.79		0.22
Male	4.99 ± 0.83		113.84 ± 5.54	
Female	4.82 ± 0.73		123.61 ± 5.67	

[1] Least squares means for activity of arginase and MMP-8 reported as relative fluorescence units (RFU)

Table 4.2 Activity of arginase and matrix metalloproteinase-8 (MMP-8) in plasma of cows not diagnosed with metritis

	Arginase		MMP-8	
Item	Mean ± SE[1]	P-value	Mean ± SE[1]	P-value
Day relative to calving		<0.01		0.01
-7	4.34 ± 0.67		139.83 ± 8.69	
0	4.19 ± 0.67		136.51 ± 8.59	
7	3.62 ± 0.67		128.74 ± 9.06	
Parity		0.02		0.01
Nulliparous	5.01 ± 0.85		124.73 ± 11.15	
Primiparous	4.34 ± 0.73		129.68 ± 8.59	
Multiparous	2.81 ± 0.77		150.58 ± 10.05	
Retained fetal membranes		0.10		0.51
Yes	3.08 ± 1.17		151.08 ± 18.25	
No	5.03 ± 0.41		138.11 ± 13.86	
Stillbirth		0.34		0.34
Yes	2.65 ± 1.60		151.69 ± 20.59	
No	4.10 ± 0.66		133.28 ± 8.59	
Twins		0.42		0.05
Yes	1.68 ± 1.55		151.02 ± 15.58	
No	3.10 ± 1.20		119.04 ± 5.00	
Gender of calf		0.78		0.18
Male	2.52 ± 1.20		129.67 ± 9.38	
Female	2.35 ± 1.11		138.70 ± 8.91	

[1] Least square means for activity of arginase and MMP-8 reported as relative fluorescence units (RFU)

Table 4.3 Adjusted odds ratios (AOR) for metritis from conditional logistic regression model controlling for the activity of arginase, retained fetal membranes, and its associations

Study day	AOR (95% CI)[1]	*P*-value
Day -7		
Arginase	---	0.14
Retained fetal membranes	---	0.35
Interaction	---	0.12
Arginase x retained fetal membranes	3.95 (0.74 to 21.03)	---
Arginase x no retained fetal membranes	1.02 (0.55 to 1.90)	---
Day 0		
Arginase	---	0.42
Retained fetal membranes	---	0.33
Interaction	---	0.13
Arginase x retained fetal membranes	2.98 (0.53 to 16.91)	---
Arginase x no retained fetal membranes	0.76 (0.37 to 1.57)	---
Day 7		
Arginase	1.01 (0.70 to 1.45)	0.96
Retained fetal membranes	Referent	<0.01
No retained fetal membranes	0.11 (0.03 to 0.48)	
Interaction	---	0.22

[1] 95% confidence interval

Table 4.4 Adjusted odds ratios (AOR) for metritis from conditional logistic regression model controlling for the activity of matrix metalloproteinase-8 (MMP-8), retained fetal membranes, and its associations.

Study day	AOR (95% CI)[1]	*P*-value
Day -7		
MMP-8	0.98 (0.94 to 1.03)	0.50
Retained fetal membranes	Referent	<0.01
No retained fetal membranes	0.10 (0.02 to 0.43)	
Interaction	---	0.44
Day 0		
MMP-8	1.03 (0.99 to 1.07)	0.20
Retained fetal membranes	Referent	<0.01
No retained fetal membranes	0.11 (0.03 to 0.45)	
Interaction	---	0.71
Day 7		
MMP-8	1.00 (0.98 to 1.02)	0.85
Retained fetal membranes	Referent	<0.01
No retained fetal membranes	0.11 (0.03 to 0.48)	
Interaction	---	0.27

[1] 95% confidence interval

Chapter 5 - Summary

B. E. Voelz

Final Remarks

The studies presented herein have provided novel and valuable information in the areas of reproductive physiology, animal health, and dairy cattle management. Information gained from these studies can be utilized to create new management practices to improve reproductive performance during summer heat stress, but also potentially explain some of the negative effects of uterine disease on fertility. Nevertheless, many unanswered questions remain regarding heat stress and uterine disease. These studies, however, create a solid foundation for additional research. Overall, this dissertation has highlighted and further investigated the negative effects of heat stress and uterine disease on reproduction in dairy cattle, as well as explore two potential markers (arginase and matrix metalloproteinase-8) for predicting risk of developing uterine disease.

Chapter 2 demonstrated that a GnRH-$PGF_{2\alpha}$-based presynchronization protocol during summer heat stress might have benefits for some herds compared with a $PGF_{2\alpha}$-based protocol. The added benefit seems to be exaggerated in herds that have exceptional estrus detection risk. Herds with a high prevalence of anestrous cows might benefit most from presynchronization protocols that employ GnRH. Treatment with GnRH in presynchronization protocols, however, should only be administered before the end of the voluntary waiting period to prevent suppression of estrus and delayed insemination. Although the GnRH-$PGF_{2\alpha}$-based presynchronization protocol investigated in this dissertation did not improve pregnancy risk, the treatment scheme increased the proportion of cows with a corpus luteum at $PGF_{2\alpha}$, therefore, increasing the effectiveness of the first $PGF_{2\alpha}$ injection. In herds that emphasize detection of estrus during presynchronization protocols and have excellent estrus detection risk, opportunities exist to improve reproductive efficiency during summer heat stress with a GnRH-$PGF_{2\alpha}$-based presynchronization protocol.

Chapter 3 investigated the ovarian response to treatment with GnRH and the odds of bearing a corpus luteum or being inseminated in dairy cows with or without purulent vaginal discharge. Furthermore, response to $PGF_{2\alpha}$ was examined in cows with or without purulent vaginal discharge. This study demonstrated that purulent vaginal discharge is associated with altered ovarian responses to GnRH, however, differences in response to $PGF_{2\alpha}$ between cows with or without purulent vaginal discharge were not detected. This observation is especially important for herds with high prevalence of purulent vaginal discharge because the efficiency of reproductive management programs might be impaired if ovulatory response to GnRH is altered, thus, decreasing reproductive performance. Understanding the underlying mechanisms that alter ovarian response to GnRH of cows with purulent vaginal discharge will be important to ameliorate future reproductive management programs.

Chapter 4 investigated arginase and matrix metalloproteinase-8 as potential markers for the development of metritis in dairy cattle. Activity of arginase 7 d before calving was identified as a possible marker for metritis, specifically in high risk dairy cows that developed retained fetal membranes before developing metritis. In contrast, matrix metalloproteinase-8 was not associated with the development of metritis. Further research that investigates the role of arginase during pregnancy and leading up to parturition is needed to fully understand how arginase might influence immune function and the pathophysiology of uterine diseases.

In conclusion, many unanswered questions remain with respect to heat stress and uterine disease and how these stressors can be mitigated to improve reproductive efficiency. As the population of the world continues to increase and demand for food also increases, answers to these pivotal questions will become even more important. Improving fertility during times of heat stress

and after the occurrence of uterine disease will help fulfill the food supply requirements, as well as improve animal welfare and increase the profitability of dairy farms across the world.

Appendix A - Kansas Dairy Producers' Needs Survey: Reproductive Management on Kansas Dairy Farms

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2017. Journal of Extension (Accepted)

Abstract

A section of the Kansas Dairy Producers' Needs Survey was designed to evaluate the most common reproductive management practices on Kansas dairy farms and needs of educational programs in the area of reproduction. Among the 312 mailed surveys to dairy producers, 70 were returned fully completed. The results indicate a need to educate producers on the topic of reproduction. In addition, they indicate that reproductive management practices and herd sizes are related to where the farms are located in the state. Planning of future reproductive management Extension programs should consider the diverse dairy industry in Kansas.

Introduction

The Kansas dairy industry is extremely diverse and has gradually grown during the past 20 years (University of Kansas, 2013). Kansas has become unique in its extreme variation in dairy farm sizes and management practices. The western portion of Kansas favors large dry-lot facilities because of dry climate conditions. In contrast, in other portions of the state, producers have traditionally chosen conventional free-stall and tie-stall facilities because of greater rainfall and humidity. Diversity among Kansas dairy farm facilities may result in a variety of reproductive management practices, which may warrant varying approaches when choosing topics for Extension programs in order to meet producers' needs. Mailed surveys can be a valuable tool for gathering information from producers, especially individuals who are not familiar with Extension programs or services (Kelsey and Mariger, 2003). Therefore, we conducted a survey that was partly intended to identify Kansas dairy producers' needs related to dairy cattle reproductive management and the most favorable topics for Extension programs to meet these needs.

Methods

In November 2014, the Kansas Dairy Producers' Needs Survey was mailed to 312 Kansas dairy producers to assess their management practices and interest in educational programs. The Committee on Research Involving Human Subjects and Institutional Review Board for Kansas State University reviewed the survey before it was mailed to producers. Surveys were sent in a hand-addressed envelope containing the survey itself, a cover letter, and a return-addressed envelope. Response to the survey was voluntary and anonymous with no reward incentive for completion. Producers were given a deadline of 45 days to return the completed survey.

Survey questions were created by our collaborating group and elicited general information about the dairy farm, including farm location, herd size, characteristics of employees, cattle reproductive management practices, and productivity traits. Furthermore, respondents were asked to provide information related to reproductive efficiency according to season (October to May and June to September). Survey responses were categorized by geographic region: northeast, southeast, central, and west. This article focuses particularly on responses related to the dairy producers' needs around reproductive management practices of their cows and heifers.

Results and Discussion

Demographics of Respondents

A total of 81 surveys were returned, of which 70 were fully completed. The 11 uncompleted surveys indicated that the producers were no longer dairy farming therefore, those surveys were discarded. Thus, the overall response rate of completed surveys was 22.4% (70/312).

Response rate by geographic region is described in Table A.1. Among the 70 responses, 76% of the responses were from the northeast (n = 29) and central (n = 24) regions of the state; the

remaining responses were from the west (n = 9) and southeast (n = 8) regions. It is important to note that differences in herd size were apparent by region. In the central and southeast region, 100% of the respondents had a herd size of less than 250 milking cows, whereas 75.9% of the respondents from the northeast had a herd size of less than 250 milking cows. In contrast, 77.8% of respondents from the west region had herds greater than 2,000 milking cows. These figures demonstrate the dramatic diversity in dairy herd size among Kansas's geographic regions (Table A.1). Differences in herd sizes based on region are important to consider when interpreting the results of this survey. In addition, these differences should be acknowledged when planning Extension programs.

Reproductive Management

One of the questions asked in the survey was "What is the average 21-day pregnancy rate in your herd?" Producers were asked to report the 21-day pregnancy rate from October to May and from June to September. We asked this question to evaluate reproductive efficiency of Kansas dairy herds during warm and cool months of the year. Producers reported an average 21-day pregnancy rate of 29.8% from October to May and 23.7% from June to September. The wide range of rates reported by producers (2% to 90%) included many values outside the expected pregnancy rates for dairy farms. The prevalence of unreasonable values for average 21-day pregnancy rate indicates that not all producers have a clear understanding of the definition of 21-day pregnancy rate and how the value is calculated. This information should be considered when developing Extension programs related to reproduction. Using 21-day pregnancy rate as a key performance indicator is advised for monitoring efficiency of reproductive performance of dairy herds (Mendonça, 2015).

Producers were asked which topics for educational programs would most benefit them and their employees. Sixty six percent (46/70) of producers indicated that reproduction would be a beneficial topic (Table A.2). In addition, producers were asked "Which management areas do you plan to improve in the next year?" Reproduction was the management area that the majority of the producers planned to improve (Table A.3). These results indicate that reproductive management is an important area for dairy producers in the state of Kansas.

In a survey of Wisconsin dairy producers, 30.3% of producers who were planning to expand and 36.0% of producers who were not planning to expand were interested in reproductive management programs (Cabrera and Janowski, 2011). Although the overall percentage of producers interested in these programs were lower in the Wisconsin survey than in the study reported here, the topic of reproductive management was ranked the highest among producers who were not planning to expand, and it was a top priority for producers who were planning to expand (Cabrera and Janowski, 2011). Results of the current survey agree with the survey of Wisconsin dairy producers, suggesting that the topic of reproduction is of extreme interest to dairy producers.

In addition, we attempted to evaluate which reproductive management practices were the most common among producers by including the following question: "Which of the following practices apply to your reproductive program? (Check all that apply)." The most common reproductive management practice reported by producers was the use of visual estrus detection, with 80.0% (56/70) of farms using this practice (Table A.4). Furthermore, 35.7% (25/70) of producers used tail paint or chalk as an estrus-detection aid, whereas only 11.4% (8/70) of producers had incorporated the use of accelerometers or pedometers as estrus-detection aids (Table A.4). All producers having herds with more than 1,000 lactating cows used tail paint or chalk. In contrast, only 26.2% of producers having herds with less than 1,000 lactating cows used this

method as an estrus-detection aid. Similar findings were reported in the National Animal Health Monitoring System (**NAHMS**) survey (NAHMS, 2007), which indicated that 93% of U.S. dairy operations used visual estrus detection as part of their reproductive management program. The NAHMS (2007) survey indicated a decreased percentage of producers performing visual estrus detection in the western U.S. states than in the eastern U.S. states (73 vs. 94.9%, respectively). Approximately 58 and 8% of U.S. dairy producers indicated that they used tail chalk and pedometers, respectively, to aid in estrus detection (Caraviello et al., 2006).

In the current survey, 51.4% (36/70) of respondents indicated that they employ timed artificial insemination programs (Table A.4). The NAHMS (2007) survey showed that timed artificial insemination protocols were being used on 58.2% of U.S. dairy farms; however, the same survey indicated that fewer dairy farms in western U.S. used timed artificial insemination protocols than eastern U.S. dairy farms (35.6 vs. 60.3%, respectively). Incorporating timed artificial insemination in reproductive programs may decrease days to first insemination, reduce days open, and, consequently, increase reproductive efficiency (Ribeiro et al., 2012). Upcoming Extension programs should focus on educating producers from Kansas about the benefits of using synchronization programs that incorporate timed artificial insemination. In addition, Extension programs should focus on how to apply these strategies to improve reproductive efficiency. Furthermore, educating producers about the importance of using estrus-detection aids is essential because only 36% of producers indicated that they use estrus-detection aids. Producers can increase reproductive efficiency of cows, and consequently, profitability by combining the use of timed artificial insemination programs with estrus detection (Galvão et al., 2013). Despite the fact that 80% of producers use visual estrus detection, this practice may not be the most effective way to detect cows in estrus considering that cows housed in facilities with concrete surfaces generally

have decreased mounting activity compared with cows housed on dirt lots (Vailes and Britt, 1990). Using estrus-detection aids may assist in detecting a greater percentage of cows in estrus.

The responding producers indicated that most of the inseminations are performed by on-farm employees. Only 8.6% (6/70) of respondents indicated use of professional artificial insemination technicians (Table A.4). Among the producers who used technicians from artificial insemination companies, 75% were located in the western part of the state and managed more than 2,000 cows. With regard to the use of sexed-sorted semen, 39% (27/70) and 7% (5/70) of producers in the current survey responded that it was used to inseminate heifers and cows, respectively (Table A.4). Khanal et al. (2010) reported that approximately 10.4% of U.S. dairy producers were using embryo transfer and/or sexed semen in their herds in 2005. Use of sexed semen in reproductive programs has become popular since it was introduced in 2003 for commercial application (DeJarnette et al., 2009; Stevenson, 2013). Use of sexed semen in combination with genomic testing to aid in breeding decisions has provided opportunities for additional revenue for dairy producers. In 2014, a genetic company reported a record high for beef semen sales to dairy producers (Cooperative Resources International, 2014). In the current survey, 12.9% (9/70) of producers indicated that they used beef semen for inseminations of cows, and 4.3% (3/70) used beef semen for inseminations of heifers (Table A.4).

Use of natural service was a common practice among respondents from this survey. Forty-four percent (31/70) of producers reported using bull breeding in their reproductive programs (Table A.4). Use of natural service was observed in all regions of the state. The northeast, central, and southeast regions accounted for 45.1% (14/31), 29.0% (9/31), and 12.9% (4/31) of the responses, respectively. The results from the NAHMS (2007) survey also indicated that producers relied on natural service in their reproductive programs. Natural service was used for first service

on 21.7% and 33.2% of operations for cows and heifers, respectively (NAHMS, 2007). Moreover, natural breeding for second and subsequent services was used on 22.2% and 35.1% of operations in the U.S. for cows and heifers, respectively (NAHMS, 2007). In addition to decreasing input costs of maintaining a bull on the farm, use of artificial insemination helps eliminate the hazard of housing bulls and venereal diseases they can spread to the cow herd. Despite the unnecessary risks associated with maintaining bulls, some producers may choose to house bulls on the farm in an attempt to generate pregnancies from less fertile subpopulations of cows in the herd (e.g., repeat-breeder cows).

Limitations

The majority of respondents to this survey were located in the central and northeast regions of Kansas. As previously mentioned, the dairy industry in Kansas is geographically diverse; thus, the findings of this survey should be interpreted with caution to avoid potential biases.

Conclusions

The Kansas Dairy Producers' Needs Survey demonstrated that dairy farms in Kansas are diverse in herd size and reproductive management practices, partly related to the geographic locations of the farms. As the dairy industry in Kansas continues to grow, more opportunities will be available for Extension programs and research projects to be conducted. This survey has identified reproductive management as an important topic that Kansas dairy producers desire to improve. Ultimately, Extension professionals should tailor future Extension activities to improve producers' understanding of successful reproductive management practices, which should result

in increased efficiency on Kansas dairy farms. In addition, findings of this survey provide important insights to allied industries that support and service Kansas dairy businesses.

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Table A.1 Geographic distribution of responses and herd size variation by region.

Region	Overall response rate (no.), %	Proportion of completed surveys (no.), %	Herds with ≤250 milking cows (no.), %	Herds with >250 milking cows (no.), %
Northeast	25.9 (29/112)	41.4 (29/70)	75.9 (22/29)	24.1 (7/29)
Central	19.2 (24/125)	34.3 (24/70)	100 (24/24)	0.0 (0/24)
Southeast	19.0 (8/42)	11.4 (8/70)	100 (8/8)	0.0 (0/8)
West	27.3 (9/33)	12.9 (9/70)	22.2 (2/9)	77.8 (7/9)

Table A.2 Proportion of responding Kansas dairy producers interested in various topics for educational programs

Topic	Response (no.), %
Reproduction	65.7 (46)
Cow health	55.7 (39)
Milk quality	51.4 (36)
Nutrition	48.6 (34)
Calf/heifer management	47.1 (33)
Lameness	45.7 (32)
Cow comfort	44.3 (31)
Transition cow management	38.6 (27)
Waste management	24.3 (17)
Technology	21.4 (15)
Record management	21.4 (15)
Employee leadership skills	14.0 (20)

Table A.3 Proportion of responding Kansas dairy producers desiring to make improvements in specific management areas

Management area	**Response (no.), %**
Reproduction	52.9 (37)
Milk quality	44.3 (31)
Cow health	41.4 (29)
Cow nutrition	35.7 (25)
Waste management	34.3 (24)
Calf/heifer management	32.9 (23)
Transition cow management	32.9 (23)
Risk management	30.0 (21)
Record keeping	30.0 (21)
Employee management and training	21.4 (15)
Parlor management	21.4 (15)

Table A.4 Proportion of responding Kansas dairy producers using specific reproductive management practices

Management practice	**Response (no.), %**
Visual heat detection	80.0 (56)
Timed artificial insemination protocols	51.4 (36)
Natural service by herd bulls	44.3 (31)
Sexed semen in dairy heifers	38.6 (27)
Estrus-detection aids (chalk or paint)	35.7 (25)
Beef semen in dairy cows	12.9 (9)
Estrus-detection aids (accelerometers or pedometers)	11.4 (8)
Semen company artificial insemination technicians	8.6 (6)
Sexed semen in dairy cows	7.1 (5)
Beef semen in dairy heifers	4.3 (3)