IMPROVING CATTLE FERTILITY:
THE INFLUENCES OF ASPIRIN, NUTRITION, AND PROSTAGLANDIN ON THE REPRODUCTIVE PERFORMANCE OF CATTLE

A Dissertation

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AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Jennifer Ann Spencer, submitted for the degree of Doctor of Philosophy with a Major in Animal Physiology and titled "Improving Cattle Fertility: The influences of aspirin, nutrition, and prostaglandin on the reproductive performance of cattle," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

High protein diets support milk production in lactating dairy cows, however, high protein diets have been linked to lowered reproductive performance in cattle. The objective of the first study was to determine the effects of high urea and low pH on bovine endometrial (BEND) cells response to interferon-tau (IFNτ) with protein expression of Mx1 and ISG-15. In cattle, early embryonic loss may be due to the premature secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) during the time of maternal recognition of pregnancy and luteolysis between days 14 and 16 after fertilization. Therefore, the objective of the second study was to determine the effect of aspirin administered during the time of maternal recognition of pregnancy on conception rates for lactating dairy cows that failed to conceive to a previous AI during the summer. Synchronization protocols aid in the synchrony of estrus and ovulation to maximize cattle fertility, however, many protocols often require multiple injections and animal handlings. The objective of the third study was to examine the effectiveness of one or two conventional $PGF_{2\alpha}$ (12 hours apart), or one high-concentration $PGF_{2\alpha}$ in a 5-day controlled internal drug release (CIDR)-Cosynch on progesterone (P_4) profiles from the time of CIDR removal until AI and complete luteolysis ($P_4 > 0.5$ ng/mL by AI) for suckling beef cows and lactating dairy cows.

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1.) Be impeccable with your word.

2.) Don't take anything personally.

3.) Don't make assumptions.

4.) Always do your best!

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DEDICATION

It is with my deepest and warmest gratitude that I dedicate this dissertation to my mentor Dr. Amin Ahmadzadeh. You have inspired and influenced who I have become more than you will ever know!

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LIST OF ABBREVIATIONS

305ME	305 mature milk equivalent
ABAM	Antibiotic-Antimycotic
AI	Artificial Insemination
ARMA	Autoregressive Moving Average
BCS	Body Condition Score
BEND	Bovine Endometrial
BS	Blood Sample
BUN	Blood Urea Nitrogen
BW	Body Weight
CIDR	Controlled Internal Drug Release Insert
CL	Corpus Luteum
COX	Cyclooxygenase
CP	Crude Protein
CV	Coefficients of Variance
DIM	Days in Milk
DMD	Dimethadione
DMI	Dry Matter Intake
DMSO	Dimethyl Sulfoxide
DO	Days Open
E_2	Estrogen
FM	Flunixin Meglumine
FSH	Follicle Stimulating Hormone
GLM	General Linear Model
GnRH	Gonadotropin-Releasing Hormone
i.m.	Intramuscular
i.v.	Intravenous
IFNτ	Interferon-tau
ISG-15	Interferon-Stimulated Gene 15
LH	Luteinizing Hormone

MUN	Milk Urea Nitrogen
Mx	Myovirus
NSAID	Non-Steroidal Anti-Inflammatory Drug
OTR	Oxytocin receptor
OXY	Oxytocin
P_4	Progesterone
P/AI	Pregnancy per Artificial Insemination
PBSα	Phosphate Buffered Saline
PGE_2	Prostaglandin E_2
PGES	Prostaglandin E Synthase
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGFM	Prostaglandin metabolite
PGFS	Prostaglandin F Synthase
PGH_2	Prostaglandin H_2
PGHS	Prostaglandin H endoperoxide synthase
PSPB	Pregnancy Specific Protein B
PTGS2	Prostaglandin Endoperoxide-Synthase 2
RDP	Rumen Degradable Protein
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation Assay Buffer
RUP	Rumen Undegradable Protein
S/C	Services per Conception
SE	Standard Error
TAI	Timed Artificial Insemination
TBRD	Number of Times Bred
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween 20
THI	Temperature-Humidity Index
TMR	Total Mixed Ration
ULT	Ultrasonography

CHAPTER ONE

"The influences of an acidic pH and high urea on bovine endometrial cell expression of Mx1 and ISG-15 in response to interferon-tau in the absence and presence of progesterone for maternal recognition of pregnancy"

ABSTRACT

High protein diets increase blood and uterine urea and reduce fertility in dairy cows. Elevated uterine urea may have direct effects on fertility, or indirect effects by disrupting uterine ion concentrations that lower uterine pH, resulting in reduced fertility. The objectives of these experiments were to investigate the effects of high urea and low pH on bovine endometrial (BEND) cell expression of myovirus 1 (Mx1) and interferon-stimulated gene 15 (ISG-15) in response to the ruminant maternal recognition protein interferon-tau (IFNτ) in the absence (Experiment 1) and presence (Experiment 2) of progesterone (P_4). Bovine endometrial cells were grown to 80% confluency, and cultured for an additional 24 hours in complete media with or without P_4 (10^{-7} *M*). Cells (90% confluent) were then treated with urea at final concentrations of 0, 5, 7.5, or 10 m*M* urea, or 0, 10, 15, or 20 m*M* dimethadione (DMD) to lower the media pH. Subsequently, cells were challenged with 0 or 10,000 antiviral units of recombinant IFNτ and incubated for an additional 24 hours. Once harvested, BEND cells were lysed and the cell supernatant was analyzed and quantified for Mx1 and ISG-15, using SDS-PAGE and Western Immunoblotting procedures. Adjusted volume percent of the band density were analyzed using a 2 × 4 factorial arrangement with two levels of IFNτ, and four levels of DMD or urea treatments. The model included the fixed effects of IFNτ, DMD or urea treatments, and the interaction between IFNτ and urea or DMD treatment. Each experiment was ran with two duplicates of IFNτ and DMD or urea treatments and repeated three times. Based on the adjusted volume percent of the band density, IFNτ increased ($P < 0.01$) Mx1 and ISG-15 in both experiments regardless of urea or DMD treatment. In experiment 1, urea had no effect on Mx1 ($P = 0.94$) or ISG-15 ($P = 0.99$) expression after 24 hours of culture. In addition, there was no urea by IFNτ interaction

on Mx1 ($P = 0.88$) or ISG-15 ($P = 0.99$) protein expression. Treatment of DMD effectively reduced media pH ($P < 0.01$) to 7.3, 7.2, 7.0, and 6.8 for 0, 10, 15, and 20 m*M* DMD, respectively. Additionally, there were effects of DMD ($P < 0.01$) and DMD by IFNτ ($P < 0.01$) on Mx1 and ISG-15 expressions. As DMD concentrations increased and media pH decreased ($P < 0.01$), expression of Mx1 ($P < 0.01$) and ISG-15 ($P < 0.01$) were reduced in the absence of P_4. In experiment 2, urea also had no effect on Mx1 ($P = 0.57$) or ISG-15 ($P = 0.72$) protein expressions. In addition, there were no interactions between urea and IFNτ on expression of Mx1 ($P = 0.34$) or ISG-15 ($P = 0.75$) expression. In the presence of P_4, treatment of DMD significantly reduced media pH ($P < 0.01$). Media pH was 7.4, 7.1, 6.9, and 6.7 for 0, 10, 15, and 20 m*M* DMD, respectively. Similar to experiment 1, in the presence of P_4, DMD significantly reduced protein expression of Mx1 ($P < 0.01$), and there was also a DMD by IFNτ interaction ($P < 0.01$). Interestingly, in the presence of P_4, there were no effects of DMD treatment ($P = 0.28$) or DMD by IFNτ interaction ($P = 0.41$) on ISG-15 expression. These results provide evidence that urea does not have a direct effect on IFNτ-stimulated proteins Mx1 and ISG-15 in the absence or presence of P_4. However, a lower pH can reduce BEND cell response to IFNτ with expression of Mx1 and ISG-15 in the absence of P_4 and Mx1 in the presence of P_4. It appears that ISG-15, an IFNτ-stimulated protein may be more resistant to the negative effects of a low pH in the presence of P_4.

Key Words: high urea, low pH, interferon-tau, bovine endometrial cells

INTRODUCTION

Reproductive efficiency is an important contributor to dairy farm profitability. Unfortunately, the reproductive performance of dairy cattle has steadily declined over the last few decades as evidenced by decreased estrous detection rates, decreased conception rates, and increased number of services per conception (Lucy, 2001; Washburn et al., 2002; de Vries and Risco, 2005). It is known that nutrition contributes to reproductive performance and thus, the profitability of dairy herds. Most notably, high protein diets have been shown to have detrimental effects on the reproductive performance of lactating dairy cows (Dietz and Flipse, 1969; Turner and Howards, 1978; Jordan et al., 1983; Kaim et al., 1983;

Ferguson et al., 1988; Ferguson and Chalupa, 1989; Blanchard et al., 1990; Canfield et al., 1990; Elrod and Butler, 1993; Elrod et al., 1993; Ferguson et al., 1993; Garcia-Bojalil et al., 1994; Bishonga et al., 1996; Butler et al., 1996; Larson et al., 1997; Butler, 1998; Westwood et al., 1998; Wittwer et al., 1999; Hammon et al., 2000b; Bode et al., 2001; De Wit et al., 2001; Rajala-Shultz et al., 2001; Dawuda et al., 2002; Ocon and Hansen, 2003; Iwata et al., 2006; Rhoads et al., 2006; Law et al., 2009).

Lactating dairy cow rations contain high levels of protein to support milk production and to increase the palatability of feed to stimulate intake (Butler, 1998). Protein is the most expensive feed component, however, overfeeding may lead to inefficient utilization thereby increasing diet cost and reducing producer profitability. In addition, it is well known that high protein diets are linked to reduced fertility in lactating dairy cows (Jordan et al., 1983; Kaim et al., 1983; Ferguson et al., 1988; Ferguson and Chalupa, 1989; Canfield et al., 1990; Elrod and Butler, 1993; Elrod et al., 1993; Ferguson et al., 1993; Butler et al., 1996; Larson et al., 1997; Butler, 1998; Westwood et al., 1998; Wittwer et al., 1999; Rajala-Shultz et al., 2001; Law et al., 2009). With growing evidence that as pregnancy rates increase so does dairy producer profitability, it is financially important to the U.S. dairy industry to further investigate the effect of high dietary protein intake and fertility. In fact for every 1% increase in pregnancy rate there is a return of approximately $20 to 25 per cow per year regardless of herd size (Overton and Cabrera, 2017).

REVIEW OF LITERATURE

HIGH DIETARY PROTEIN

Dairy producers have been successful in maximizing dairy cattle milk production in part through nutritional management including increased dietary protein during lactation. In fact, dietary protein increases feed intake by increasing palatability and thus, helps to increase dry matter intake (DMI). Total protein, or crude protein (CP) is composed of rumen degradable (RDP) and rumen undegradable protein (RUP) (Butler, 1998). An imbalance of protein utilization of the nitrogen and amino acid contents of protein, or lack of energy availability can lead to elevated ruminal ammonia and higher urea concentrations, which are often measured in the blood or milk as blood urea nitrogen (BUN) or milk urea nitrogen

(MUN) (Roffler and Thacker, 1983; Ferguson and Chalupa, 1993; Ferguson et al., 1993; Staples et al., 1993; Butler, 1998).

Protein Metabolism

Rumen degradable protein is digested in the rumen by microorganisms, by which, ammonia is produced and utilized for protein synthesis (Butler, 1998; Butler, 2000). However, excess RDP may produce additional ammonia that exceeds rumen microbial requirements. The resulting ammonia diffuses through the rumen wall, entering into circulation via portal blood (Butler, 1998). In the blood, ammonia is toxic, and must be detoxified into urea, a non-toxic substance, by the liver through a process called ureogenesis (Butler, 1998). Upon formation of urea, it enters circulation where it can be recycled back to the rumen for microbial protein synthesis, otherwise it is excreted in the urine (Van Soest, 1994). Elevated rumen ammonia and liver urea synthesis can occur if there is excessive dietary CP, an imbalance of RDP to RUP, lack of appropriate amino acid profiles, and/or lack of fermentable carbohydrate for microbial energy to utilize CP (Butler, 1998).

The quantity of ammonia produced and amount that escapes reflects dietary RDP and availability of fermentable carbohydrates to support microbial growth and protein synthesis (Nocek and Russell, 1988; Clark et al., 1992). The protein metabolite urea is measured by MUN or BUN, which can be used to assess the status of dietary protein fed to dairy cattle.

HIGH UREA & FERTILITY

It is well documented that elevated concentrations of BUN or MUN negatively affect fertility in lactating dairy cows (Kaim et al., 1983; Ferguson et al., 1988; Ferguson and Chalupa, 1989; Canfield et al., 1990; Ferguson et al., 1993; Butler et al., 1996; Larson et al., 1997; Butler, 1998; Westwood et al., 1998; Wittwer et al., 1999; Rajala-Schultz et al., 2001; Law et al., 2009). Studies have shown that BUN or MUN > 19 mg/dL (> 6.7 m*M*), which occurs when excess dietary protein is fed, results in approximately 20% decrease in pregnancy per AI (P/AI) (Ferguson et al., 1988; Ferguson et al., 1993; Butler et al., 1996; Elrod and Butler, 1993; Rajala-Schultz et al., 2001; Law et al., 2009). Moreover, a study by Rajala-Shultz et al. (2001) demonstrated that cows with MUN concentrations >15.4 mg/dL were 2.5 times less likely to become pregnant compared with cows that had <10 mg/dL.

Urea is a small water-soluble molecule that is capable of permeating cells (Gustafsson and Palmquist, 1993). Therefore, excess dietary protein will increase ammonia and urea concentrations in body fluids and tissues including the reproductive tract (Jordan et al., 1983; Elrod and Butler, 1993; Butler, 1998; Hammon et al., 2000a, 2000b; Rajala-Schultz et al., 2001; Rhoads et al., 2006; Gunaretnam et al., 2013). In fact, BUN and MUN are strongly correlated with uterine and oviductal urea concentrations (Butler et al., 1996). The mechanisms by which excessive dietary protein resulting in a high BUN negatively affect fertility have been investigated and it has been shown that high urea influences oocyte health (De Wit et al., 2001; Ocon and Hansen, 2003), sperm viability and motility (Dietz and Flipse, 1969; Turner and Howards, 1978), fertilization (Iwata et al., 2006), and embryo quality and development (Blanchard et al., 1990; Garcia-Bojalil et al., 1994; Bishonga et al., 1996; Hammon et al., 2000b; Bode et al., 2001; Dawuda et al., 2002; Ocon and Hansen 2003; Rhoads et al., 2006).

Studies have shown elevated BUN concentrations affect oocyte maturation and development, as embryos transferred from high BUN donors to moderate BUN recipients were less likely to survive (Ocon and Hansen, 2003; Rhoads et al., 2006) compared with moderate BUN donor cows. Additionally, Blanchard et al. (1990) demonstrated that a greater percentage of fertilized ova and transferable embryos are recovered from cows fed low RDP compared to those fed high RDP. This emphasizes the impact of elevated BUN concentrations and high RDP effects on oocyte viability. In addition, high BUN concentrations also negatively affect embryo viability and development. For example, super-ovulated lactating dairy cows with high BUN (8.7 m*M*) that were similar to those observed by Dawada et al. (2002) when cows were fed 19% CP, had reduced pregnancy rates and compromised embryo viability (Rhoads et al., 2006). The authors concluded that the negative effects observed on embryo viability were a result of high urea affecting the oocyte or embryo prior to recovery on day 7 after fertilization.

LOW pH & FERTILITY

It has been shown that excess dietary protein intake during the luteal phase of the estrous cycle significantly increases BUN and specifically decreases uterine pH (Elrod and Butler, 1993; Elrod et al., 1993). These authors (Elrod et al., 1993) suggested that changes in uterine pH (from 7.13 to 6.85) observed in cows fed 25% excess RDP or RUP. Similarly, it

has been shown that increasing BUN (16.6 to 22.6 mg/dL), as a result of intravenous infusion of urea, reduces uterine luminal pH from 7.1 to 6.8 in lactating cows (Rhoads et al., 2004). Rhoads et al. (2004) hypothesized that high urea could alter uterine pH by changing carbonic anhydrase which is involved in the selective transport of hydrogen and bicarbonate ions. Carbonic anhydrase is an enzyme present in secretory epithelium that transports hydrogen and bicarbonate ions out of epithelial cells and sodium, potassium or chloride into cells (Rodriguez-Martinez, 1991). These alterations in ion concentrations disrupt pH and can create a hostile environment not conducive for embryo development. Jordan et al. (1983) demonstrated that when cows are fed high dietary protein (23%), uterine secretions of magnesium, potassium, and phosphorus were altered, further supporting the hypothesis that high dietary protein alters carbonic anhydrase activity and lowers uterine pH.

Lower uterine pH (< 7) during the luteal phase of the estrous cycle has been shown to have detrimental effects on oocyte health, fertilization, implantation, embryo quality, and early embryo development (Elrod et al., 1993; Garcia-Bojalil et al., 1994; Bishonga et al., 1996; Butler, 1998; Hammon et al., 2000a, 200b; Ocon and Hansen, 2003). In fact, high dietary protein intake (≥ 20%) during the luteal phase of the estrous cycle increases BUN and decreases uterine pH in both cows and heifers and decreases fertility by affecting oocyte development and embryo survival (Jordan et al., 1983; Elrod and Butler, 1993; Elrod et al., 1993; Butler, 1998; Dawuda et al., 2002).

There is evidence that to maintain pregnancy, the uterus must secrete various proteins (Brooks and Spencer, 2015), and any change in uterine secretions may negatively affect pregnancy. *In vitro* studies have shown that acidic pH has a negative effect on embryo development and quality (Butler, 1998; Hammon et al., 2000a, 2000b; Ocon and Hansen, 2003). Therefore, it appears that high uterine urea and/or low uterine pH may negatively affect oocyte development and embryo quality. However, changes in uterine secretions and environment through high dietary protein is not well understood and there has been no direct evidence indicating how high urea and acidic pH affect uterine secretions in response to embryo signaling.

INTERFERON-TAU STIMULATED PROTEINS

Interferon-tau stimulated proteins are produced when the endometrium-trophectoderm complex forms during implantation and placentation. During this time, IFNτ

causes the stimulation of immune cells within the endometrium and release of IFNτ proteins, which aid in many physiological functions (e.g. maternal recognition of pregnancy). During maternal recognition of pregnancy, the uterine endometrium responds to IFNτ signals from the embryo by becoming highly secretory, producing a variety of proteins (histotroph or "uterine milk"), which are essential for embryo survival and attachment (Hansen et al., 1999; Emond et al., 2000; Brooks et al., 2014). In cattle, uterine glands secrete approximately 27 proteins in response to IFNτ, two of which include Myovirus 1 (Mx1) and Interferon-stimulated gene 15 (ISG-15) (Hansen et al., 1999; Johnson et al., 1999; Ott, 2000). Previous studies have shown Mx1 (Hicks et al., 2003; Spencer et al., 2014) and ISG-15 (Johnson et al., 1999; Austin et al., 2004) are upregulated over time in response to IFNτ *in vivo* and in BEND cells *in vitro*.

Myovirus

Myovirus proteins (1 and 2) were identified in an inbred strain of laboratory mice that were resistant to infection by several orthomyxoviruses, hence the name Mx (Lindenmann, 1962). Mx is a ~70 kDa intracellular protein identified in nearly all species (Horisberger and Gunst, 1991). Myovirus is induced by type I IFN's, possesses antiviral activity, and is structurally similar to monomeric GTPases (Horisberger, 1992). Myovirus proteins share structural homology with a class of proteins called mechanochemical enzymes that function in intracellular protein and vesicle trafficking (Horisberger and Gunst, 1991). This suggests that Mx may have alternative functions outside the immune response to viral infection. In ovine glandular epithelial cells, Racicot et al. (2008) demonstrated that reduced concentrations of Mx *in vitro* increased cell proliferation, and therefore, Mx may be involved in implantation and endometrial functions during early pregnancy in ruminants.

Highest concentrations of Mx proteins were found in the pregnant uterine horn of unilaterally pregnant ewes during early gestation (Charleston and Stewart, 1993; Ott et al., 1998). This suggests there is a local effect of the conceptus on uterine Mx expression during early pregnancy. In the ewe, Mx expression was greatest when IFNτ concentration were the highest (day 12-17 in the ewe) during the time of maternal recognition, and continued to be expressed until day 25 of pregnancy in ewes (Ott et al., 1998).

In cattle, Mx was measured by isolating ribonucleic acid (RNA) from leukocytes in plasma (Green et al., 2010). This study demonstrated that Mx is upregulated in pregnant

cows and can be detected as early as day 18 of pregnancy. However, these authors observed a parity effect where primiparous cows were less likely to have false positives compared with multiparous cows. The specific function of the IFNτ stimulated protein Mx is still unknown, but is involved in maternal recognition of pregnancy.

Interferon-Stimulated Gene-15

Interferon-stimulated gene-15 is an ubiquitin cross-reactive protein that is expressed during early pregnancy in response to secretion of IFNτ by the embryo (Austin et al., 1996; Hansen et al., 1997; Johnson et al., 1998; Austin et al., 1999; Hansen et al., 1999; Johnson et al., 2002). This IFNτ stimulated protein has both intracellular (Johnson et al., 1998; Liu et al., 2003) and extracellular functions (D'Cunha et al., 1996).

The extracellular functions of ISG-15 occur through involvement in the cytokine cascade and manipulating immune cell function (D'Cunha et al., 1996). This would be important during early pregnancy as establishment and maintenance of pregnancy rely on a supportive immune system (Hansen et al., 1999; Emond et al., 2000; Chełmońska-Soyta, 2002; Brooks and Spencer, 2015). The intracellular functions of this protein include involvement in cytoskeleton protein fraction (Liu et al., 2003) and conjugating to cytosolic proteins (Johnson et al., 1998) that may help during establishment of early pregnancy.

It has been shown that ISG-15 can be induced as early as day 15 and last until almost 50 days of gestation, well beyond synthesis and secretion of IFNτ (Han et al., 2006). Sustained ISG-15 until day 50 of pregnancy might be required for stabilization of conjugated proteins that could be involved in implantation and development of the placenta (Han et al., 2006). However, this has not been verified. Nevertheless, more recently researchers have been able to identify ISG-15 in the blood and have been able to use its presence along with P_4 to diagnose pregnancy, and even allude to the occurrence of embryonic loss (Han et al., 2006; Haq et al., 2016; Carvalho et al., 2017).

CELL MODELS

Cell models are useful as they can provide researchers with the ability to investigate complex physiological mechanisms that represent an *in vivo* model. Cell culture has been used for many years to examine cellular mechanisms and interactions and is a useful tool as researchers are able to control the *in vitro* environment eliminating the risk of any confounding effects. In addition, cell culture is a rapid way to test a hypothesis while

reducing the impact on live animals. However, the limitation of a cell culture experiment is that it does not represent the whole animal and the various physiological influences. Therefore, results from a cell culture experiment must be validated in a whole animal system. One method of cell culture is known as primary cell culture and involves the isolation of cells, tissues, or organs that are obtained directly from the species of interest (Cano and Colome, 1988). This method of developing cell culture lines, however, is more resource and time consuming when compared with other methods (Binelli et al., 2000) such as immortalized cells.

Immortalized BEND cells from day 14 cyclic cows were developed at the University of Wyoming, and are widely used as a model to examine dietary and conceptus regulation on endometrial secretions (Austin et al., 1996; Staggs et al., 1998; Austin et al., 1999; Johnson et al., 1999; Binelli et al., 2000; Badinga et al., 2002; Mattos et al., 2003). Bovine endometrial cells possess the characteristics typical of uterine epithelial cells (Rodriguez-Sallaberry et al., 2006), and BEND cells from day 14 of the estrous cycle provide P_4 primed cells which serve as an excellent model for examining the regulation of IFNτ-stimulated proteins (Binelli et al., 2000). In fact, it has been shown that bovine Mx1 (Hicks et al., 2003; Spencer et al., 2014) and bovine ISG-15 protein (Austin et al., 1999; Johnson et al., 1999) are induced by recombinant IFNτ in BEND cells, allowing for the investigation on the response of uterine cells to IFNτ.

RATIONALE

While researchers recognize the problem of low fertilization rates and/or abnormal embryo development associated with high dietary protein (Hammon et al., 1997; Bode et al., 2001; De Wit et al., 2001; Dawuda et al., 2002; Ocon and Hansen, 2003), there are still questions that need to be addressed about potential mechanisms and factors involved in the interaction between high dietary protein and fertility. A large body of evidence appears to focus on high BUN and low pH changes in uterine secretion. However, there is no direct evidence as to how the altered uterine environment may respond to embryo signaling. If uterine gland secretion is reduced or abrogated in cows experiencing high BUN and decreased uterine pH, then it is plausible to hypothesize that a lack of uterine response to

IFNτ, may also be attributed to low fertility in cows fed high dietary protein (≥ 20%).

HYPOTHESIS

We hypothesized that BEND cells reduce expression of Mx1 and ISG-15 in response to IFNτ when cultured in low pH media or media containing high urea in the absence (Experiment 1) or presence (Experiment 2) of P_4.

OBJECTIVES

1. To determine the direct effect of high urea on protein expression and secretion of Mx1 and ISG-15 of BEND cells in response to IFNτ in the absence (Experiment 1) and presence (Experiment 2) of P_4.
2. To determine the direct effect of low pH on protein expression and secretion of Mx1 and ISG-15 of BEND cells in response to IFNτ in the absence (Experiment 1) and presence (Experiment 2) of P_4.

MATERIALS & METHODS

Details about the preparation and contents of all cell culture solutions are described in Appendix 1.

CELL THAWING & PLATING

Aliquot tubes containing BEND cells suspended in dimethyl sulfoxide (DMSO) were removed from liquid nitrogen and placed in a 37ºC water bath and inverted every 30 seconds until thawed. Cells were then poured into a 50 mL conical tube with 10 mL of complete culture media (40% Hams F-12 w/ D-valine, 40% valine-modified MEM, 1% antibiotic-antimycotic (ABAM) solution, insulin [0.2 U/ml], 10% fetal bovine serum, 10% horse serum, pH 7.3; Appendix 1.1). Conical tubes were centrifuged at 1,500 × *g* for 5 minutes and then cells were re-suspended in complete media.

Propagation of BEND cells was achieved by plating ~1×10^6 cells initially in a sterile, polystyrene, tissue culture flask (T_{75}) using complete culture medium and grown to 90% confluency at 37°C under a humidified atmosphere containing 5% CO_2. Cell morphology was observed by light microscopy every 24 hours to ensure plating and determine confluency. After 48 hours, media was removed from the flask and cells were

rinsed with phosphate buffered saline (PBSα; pH 7.4; Appendix 1.1) and placed in fresh complete culture media.

CELL COUNTING & SPLITTING

Once T_{75} flasks (approximately 5 days) reached 80% confluency, cells were counted for splitting into petri dishes. Cells were rinsed twice with PBSα and then 2 mL of Trypsin (Sigma-Aldrich; St. Louis, MO) was added and flasks were incubated at 37°C for 15 minutes. To aid in detaching the cells, the flasks were removed from the incubator every five minutes and agitated gently. Subsequently, culture media (10 mL) was added to the flask and cells were transferred to a 50 mL conical tube. Cells were then centrifuged at 1,500 × *g* for 5 minutes. The media was carefully removed from the pelleted cells and additional media was added (20 mL) to break up the pellet of cells. A volume of 50 µl of cells and media were removed from the conical tube and placed into a 1.5 mL aliquot tube containing 950 µl of PBSα. The solution was mixed using the pipette, and 25 µl of the solution was added to each side of a clean hemacytometer slide (Bright-Line; Hausser Scientific, Horsham, PA). The number of cells contained in each side and within each box (8 values) were averaged and then used to calculate the number of cells and desired volume to achieve 150,000 cells/mL. Accordingly, cells were then added to 16 petri dishes (10 mm) and cultured for an additional week until 80% confluency was reached before subjected to urea or DMD treatments.

TREATMENT

At 80% confluency, cells were washed 3 times with PBSα and placed in complete media containing no P_4 (Experiment 1), or complete media containing 10^{-7} m*M* P_4 (Experiment 2; Appendix 1.1). The purpose of experiment 2 was to examine the effects of high urea and low pH in a P_4 environment, as the protein expression IFNτ stimulated proteins are known to be altered in the presence of P_4 (Ott et al., 1998). At the time of P_4 (0 or 10^{-7} m*M*), urea treatments (0, 5, 7.5, or 10 m*M*; Sigma-Aldrich; St. Louis, MO; Appendix 1.1) were also added to their respective plates. After 24 hours incubation, in media containing 0 or 10^{-7} m*M* P_4, cells were approximately 90% confluent (Figure 1.1). At this time, individual plates were washed 3 times with PBSα and fresh complete media without P_4 (Experiment 1) or with P_4 (Experiment 2) were added back to the plates (Figure 1.1). Subsequently, the 16 plates (two per concentration) were treated with again with urea (0, 5,

7.5, or 10 m*M*; Appendix 1.1) or treated with DMD (0, 10, 15, or 20 m*M*; 5, 5-dimethyloxazolidine-2, 4-dione; Acros Organics, New Jersey, USA; Appendix 1.1) (Figure 1.1). The DMD is a non-metabolizable weak acid that lowers the pH of the culture medium and has been used to study the effects of pH on embryo development *in vitro* (Bavister et al., 1983; Carney and Bavister, 1987; Edwards et al., 1998; Ocon and Hansen, 2003). Concentrations of DMD and urea treatments were determined based on a preliminary experiment (Appendix 1.2). After, half of the plates (one per concentration) were treated with IFNτ. Plates were incubated for an additional 24 hours and harvested to isolate protein (Figure 1.1). The pH of DMD treated plates was measured every 6 hours after DMD treatment during the 24-hour incubation using a pH meter probe (Appendix 1.1).

PROTEIN ISOLATION

Protein was extracted using 400 µL/plate of a cell lysis reagent, radioimmunoprecipitation assay buffer (RIPA; Pierce RIPA Lysis and Extraction Buffer; Thermo Fisher Scientific Inc., Waltham, MA). Cells were scraped gently from petri dishes and placed in aliquot tubes which were placed in the refrigerator at 4°C and agitated every couple of minutes for 30 minutes to collect cell lysates. Aliquot tubes were centrifuged at 1,500 × *g* for 10 minutes. Cell lysates were extracted and placed in a fresh aliquot tube and used to quantify total protein using a Bradford's Concentration Assay (BCA; Pierce, Rockford, IL) kit and bovine serum albumin was used as a standard.

SDS-PAGE & WESTERN IMMUNOBLOTING

Isolated protein was extracted (40 µg/sample) and placed into aliquot tubes and samples were brought to equal volumes using RIPA buffer. In addition, 4 µl of SDS-PAGE was added to each tube, which were then placed on a heating plate for 10 minutes at 95°C to denature proteins. Samples were removed from heating plate and loaded into separate wells within a 10 well, 4 to 15% gradient resolving gel (Mini-PROTEAN TGX Precast Gels; Bio-Rad Laboratories, Hercules, CA). Samples were separated out using an electrophoresis chamber at 90 volts for 1.5 hours, and filled with separating buffer solution (Appendix 1.1). Proteins were transferred from the resolving gel to a nitrocellulose membrane (BA83, Schleicher & Schuell, Keene, NH) in an electrophoresis chamber for 2 hours at 90 volts with a stirring bar in transfer buffer (Appendix 1.1) and placed on ice. Additional ice was added at one hour to maintain a cooler temperature throughout the 2 hours.

After transferring proteins to a nitrocellulose membrane, non-specific binding was blocked by incubating the membrane in a 5% non-fat dried milk solution with tris-buffered saline with tween 20 (TBST; Appendix 1.1) for Mx1 and 5% bovine serum albumin with TBST for ISG-15 for one hour at room temperature on a shaker plate. Membranes were rinsed with TBST 3 times for 5 minutes at room temperature on a shaker plate. Subsequently, the primary antibody for Mx1 (1:1000 dilution for polyclonal rabbit ovine Mx1; Multiple Peptide Systems, San Diego, CA) and ISG-15 (1:30,000 dilution polyclonal 5F10 anti-ISG-15; Hansen Laboratory, Laramie, WY) were added to the membranes, which were incubated overnight at 4ºC on a shaker plate. The membranes were removed from the solution containing the primary antibody and were washed with TBST 3 times for 10 minutes each. Subsequently, the secondary antibody (Mx1 = 1:2500 dilution goat anti-rabbit IgG-HRP conjugate; ISG-15 = 1:30,000 dilution anti-mouse IgG-HRP conjugate) was added in 5% non-fat dried milk with TBST for Mx1 or 5% bovine serum albumin with TBST for ISG-15 for 2 hours at room temperature on a shaker plate. The membranes were removed from solution containing the secondary antibody and were washed 3 times for 5 minutes in TBST and then once for 5 minutes in a tris-buffered saline (TBS; Appendix 1.1) solution without tween-20. A chemiluminescent signal was developed from the membranes onto a film using the protocol in the West Femto Maximum Sensitivity Substrate kit (Pierce, Rockford, IL). Band density and adjusted volume percent were calculated using the Fluor-S Multi-Imager system and Quantity One software (BioRad, Hercules, CA).

STATISTICAL ANALYSIS

Each experiment (Experiment 1 = no P_4; Experiment 2 = P_4) was set up as a 2 × 4 factorial arrangement with two levels of IFNτ (0 or 10,000 IU) and four levels of treatments either urea (0, 5, 7.5, and 10 m*M*) or DMD (0, 10, 15, and 20 m*M*). Each treatment was run in duplicate and replicated three times. The results of each experiment were analyzed using the analysis of variance procedure. The model included the main effects of IFNτ (2 levels), treatment (urea at 4 levels or DMD at 4 levels), and IFNτ levels by treatment interactions. Significance was declared at a $P < 0.05$ and a tendency at $P \leq 0.1$. Statistical analyses were carried out using SAS (v. 9.4; SAS Institute Inc., 2015).

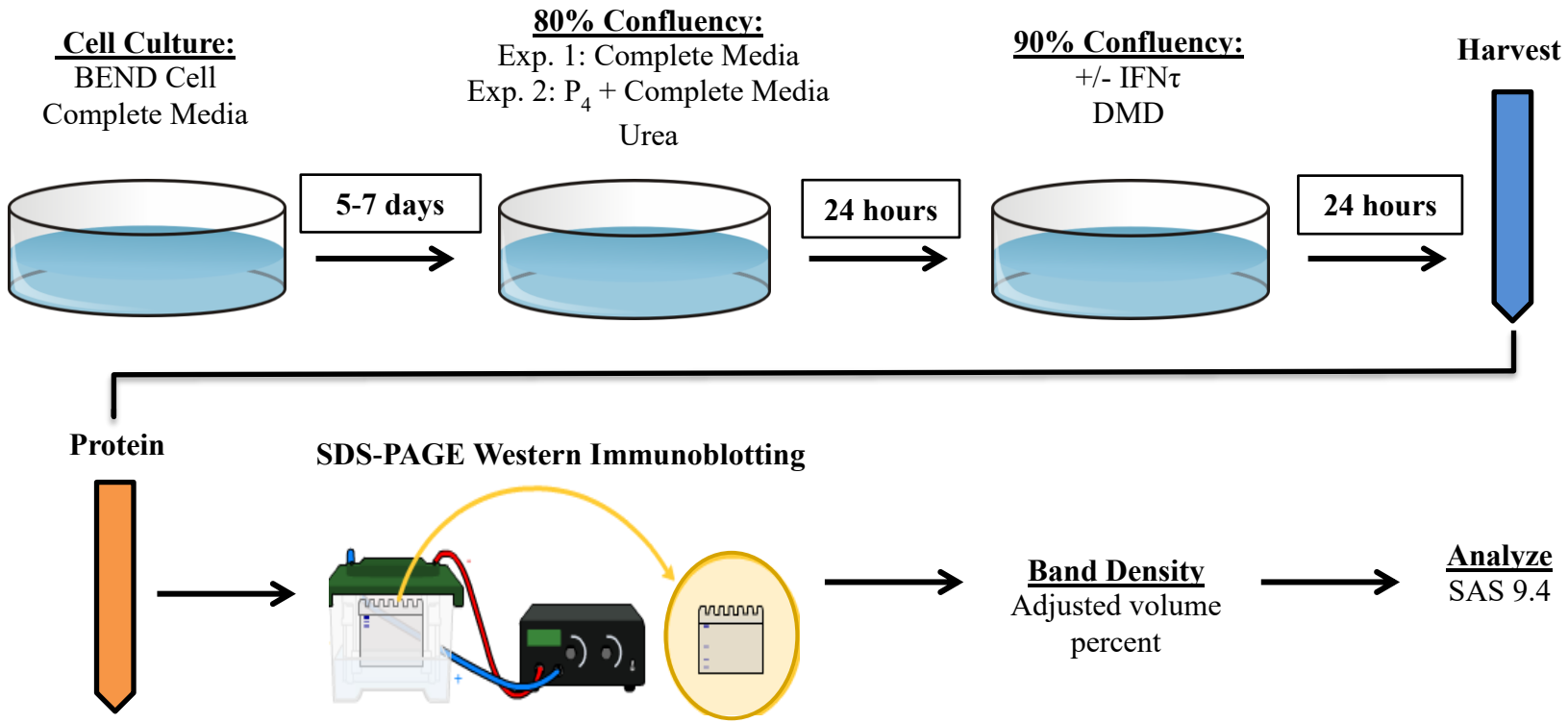

Figure 1.1 Experimental design for culturing bovine endometrial (BEND) cells and analyzing protein concentrations of Mx1 and ISG-15 after treatment with high urea or low pH. Bovine endometrial cells were cultured in complete media for 5 to 7 days until cell confluency was approximately 80%. Then media was changed and replaced with complete media without progesterone (P_4) (Experiment 1) or with P_4 (Experiment 2), and urea treatments (0, 5, 7.5, or 10 m*M*) were added at this time. After 24 hours, media was replaced with respective P_4 (0 or 10^{-7} m*M*) and urea treatments and cells were treated with or without interferon-tau (IFNτ). Simultaneously, dimethadione (DMD) treatments (0, 10, 15, or 20 m*M*) were added to respective plates in order to lower the pH of culture media. Cells were harvested 24 hours after cells were treated with urea or DMD and analyzed for protein concentration. Each sample was analyzed for Mx1 and ISG-15 protein expression using Western Immunoblotting technique. The band density was analyzed using the Fluor-S Multi-Imager system and Quantity One software.

RESULTS

EXPERIMENT 1 – ABSENCE OF PROGESTERONE

High Urea and Mx1

Protein expression of Mx1 by the BEND cells was increased in the presence of IFNτ at all urea concentrations ($P < 0.01$; Figure 1.2). This indicated that the cell culture system was functional, as cells were viable and able to respond to IFNτ. Increasing concentrations of urea had no effect on Mx1 protein expression in the absence of P_4 ($P = 0.34$). There were also no interactions between treatment and urea on Mx1 protein expression ($P = 0.16$). Mean adjusted volume percent ± SE for cells treated with IFNτ and urea were 19.32 ± 3.25 for 0 m*M*, 31.31 ± 2.66 for 5 m*M*, 29.06 ± 2.66 for 7.5 m*M*, and 28.26 ± 2.66 for 10 m*M* urea (Figure 1.2).

High Urea and ISG-15

Protein expression of ISG-15 increased when BEND cells were treated with IFNτ regardless of urea treatments ($P < 0.01$; Figure 1.3). There were no effects of urea treatment ($P = 0.99$), nor any IFNτ by urea treatment interaction ($P = 0.99$) on ISG-15 protein expression. The mean adjusted volume percent ± SE were 13.63, 12.95, 12.98, and 13.23 (SE ± 1.99) for 0, 5, 7.5, and 10 m*M* urea and IFNτ treated, respectively (Figure 1.3).

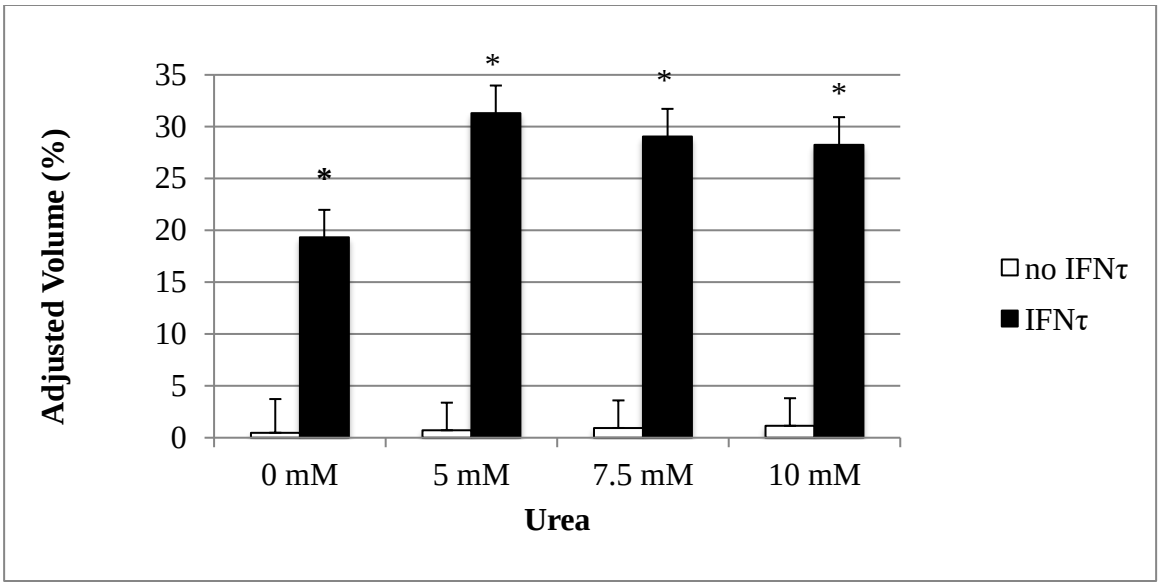

Figure 1.2 Percent adjusted volume for bovine endometrial (BEND) cell expression of Mx1 in response to interferon-tau (IFNτ) cultured with increasing concentrations of urea in the absence of progesterone (P_4). There were no effects of urea treatment ($P > 0.05$) on IFNτ stimulated Mx1 protein expression.
* Different from no IFNτ ($P < 0.01$).

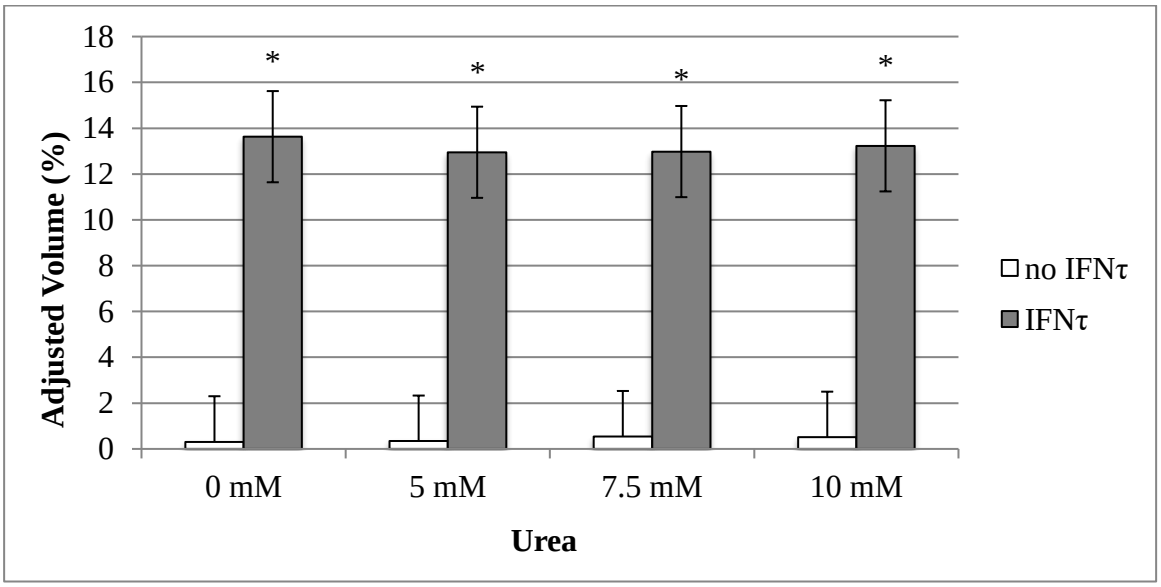

Figure 1.3 Percent adjusted volume for bovine endometrial (BEND) cell expression of ISG-15 in response to interferon-tau (IFNτ) cultured with increasing concentrations of urea in the absence of progesterone (P_4). There were no effects of urea treatment ($P > 0.05$) on IFNτ stimulated ISG-15 protein expression.
* Different from no IFNτ ($P < 0.01$).

Low pH and Mx1

In the absence of P_4 in the culture media, treatment of DMD had an effect on culture media, and as DMD concentrations increased, culture media pH decreased ($P < 0.01$; Table 1.1). Regardless of DMD treatment, protein expression of Mx1 by BEND cells was increased in response to IFNτ ($P < 0.01$; Figure 1.4). Once again, this indicates that the cell culture system was functional as cells were viable and responded to IFNτ. However, there was an effect of DMD treatment ($P < 0.01$), and DMD treatment by IFNτ interaction ($P = 0.01$) on Mx1 protein expression (Figure 1.4). As DMD concentrations increased, and culture media pH decreased, Mx1 protein expression significantly decreased ($P < 0.01$). Mean adjusted volume percent ± SE for cells treated with IFNτ and DMD were 34.36, 20.25, 8.48, 1.23 (± 3.50) for 0 (pH = 7.3), 10 (pH = 7.2), 15 (pH = 7.0), and 20 (pH = 6.8), m*M* DMD, respectively (Figure 1.4).

Low pH and ISG-15

In the absence of P_4 in culture media, DMD reduced media pH ($P < 0.01$; Table 1.1). Regardless of DMD treatment, IFNτ increased protein expression of ISG-15 ($P < 0.01$; Figure 1.5). Treatment of DMD had an effect on ISG-15 expression ($P < 0.01$). There was also an effect of DMD by IFNτ interaction ($P < 0.01$) on ISG-15 protein expression. As DMD concentrations increased, and culture media pH decreased, ISG-15 protein expression decreased (Figure 1.5). However, reduced ISG-15 protein expression was not observed until DMD concentrations reached 15 m*M* and culture media pH was 7.0 (Figure 1.7). Mean adjusted volume percent ± SE for cells treated with IFNτ and DMD were 18.76, 18.71, 11.58, 1.58 (± 1.72) for 0 (pH = 7.3), 10 (pH = 7.2), 15 (pH = 7.0), and 20 (pH = 6.8), m*M* DMD, respectively (Figure 1.5).

Table 1.1 Means ± SE pH values in bovine endometrial (BEND) cell culture media containing increasing concentrations of dimethadione acid (DMD) in the absence of progesterone (P_4). Means were pooled between cells stimulated with or without interferon-τ (IFNτ).

DMD	0 m*M*	10 m*M*	15 m*M*	20 m*M*
pH	7.3 ± 0.05	7.2 ± 0.05 °	7.0 ± 0.05 *	6.8 ± 0.05 *

° Tend to differ from 0 m*M* DMD treatment ($P = 0.1$).
* Means differ from 0 m*M* DMD treatment ($P < 0.05$).

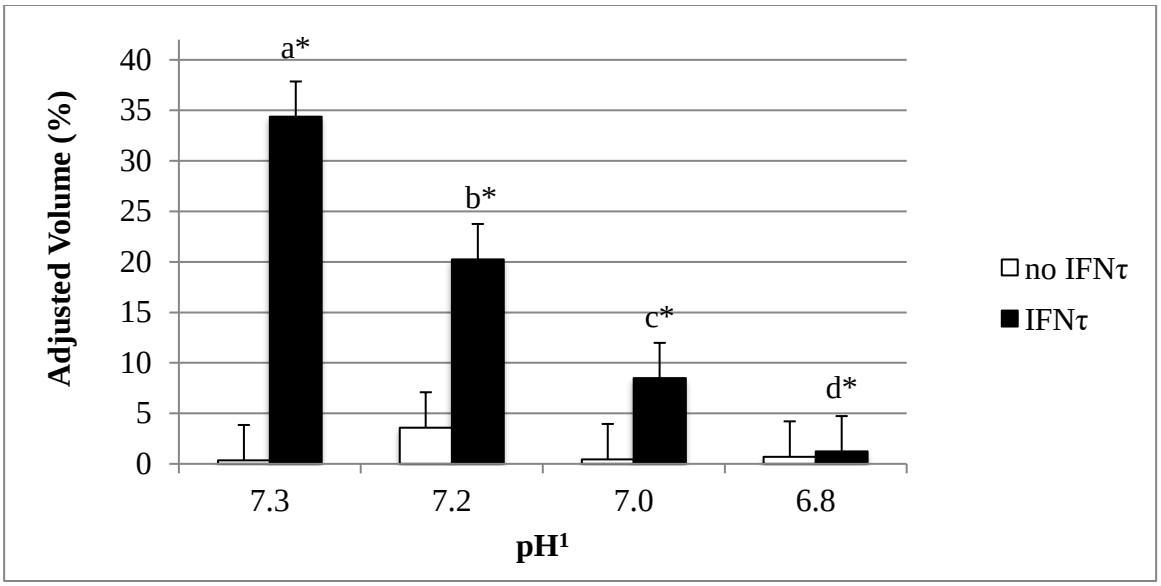

Figure 1.4 Percent adjusted volume for bovine endometrial (BEND) cell expression of Mx1 in response to interferon-tau (IFNτ) and cultured in media containing dimethadione (DMD) acid to reduce the pH in the absence of progesterone (P_4).
[1] pH of 7.3, 7.2, 7.0, and 6.8 correspond to DMD concentrations of 0, 10, 15, and 20 m*M*.
* Different from no IFNτ ($P < 0.01$).
[a,b,c,d] Bars designated with different letters differ ($P < 0.05$).

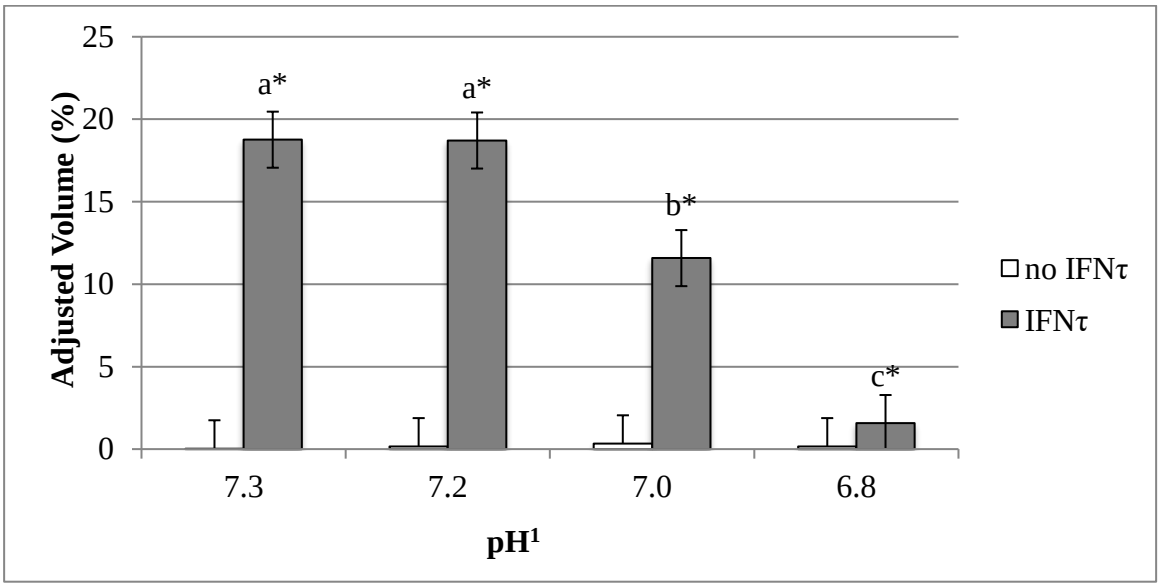

Figure 1.5 Percent adjusted volume for bovine endometrial (BEND) cell expression of ISG-15 in response to interferon-tau (IFNτ) and cultured in media containing dimethadione (DMD) acid to reduce the pH in the absence of progesterone (P_4).
[1] pH of 7.3, 7.2, 7.0, and 6.8 correspond to DMD concentrations of 0, 10, 15, and 20 m*M*.
* Different from no IFNτ ($P < 0.01$).
[a,b,c] Bars designated with different letters differ ($P < 0.05$).

EXPERIMENT 2 – PRESENCE OF PROGESTERONE

High Urea and Mx1

Protein expression of Mx1 increased when BEND cells were treated with IFNτ, across all urea concentrations ($P < 0.01$; Figure 1.6). This indicated that the cell culture system was functional, and cells in the presence of P_4 were viable and responsive to IFNτ. There were no effects of urea treatment ($P = 0.57$), nor any IFNτ by urea treatment interaction ($P = 0.34$) on Mx1 protein expression in the presence of P_4 in the culture media (Figure 1.6). The mean adjusted volume percent ± SE for cells treated with IFNτ and 0, 5, 7.5, and 10 m*M* urea were 9.78, 16.14, 9.98, 11.36 (SE ± 2.21) respectively (Figure 1.6).

High Urea and ISG-15

Protein expression of ISG-15 increased when BEND cells were treated with IFNτ, regardless of urea treatments ($P < 0.01$; Figure 1.7). Once again, this demonstrates that the cell culture system was functional, and cells were viable and responsive to IFNτ in the presence of P_4 and low pH. There were no effects of urea treatment ($P = 0.72$), nor any urea by IFNτ treatment interaction ($P = 0.75$) on ISG-15 protein expression in the presence of P_4 (Figure 1.7). The mean adjusted volume percent ± SE were 3.79, 4.75, 4.60, and 3.87 (SE ± 0.53) for 0, 5, 7.5, and 10 m*M* urea stimulated with IFNτ (Figure 1.7).

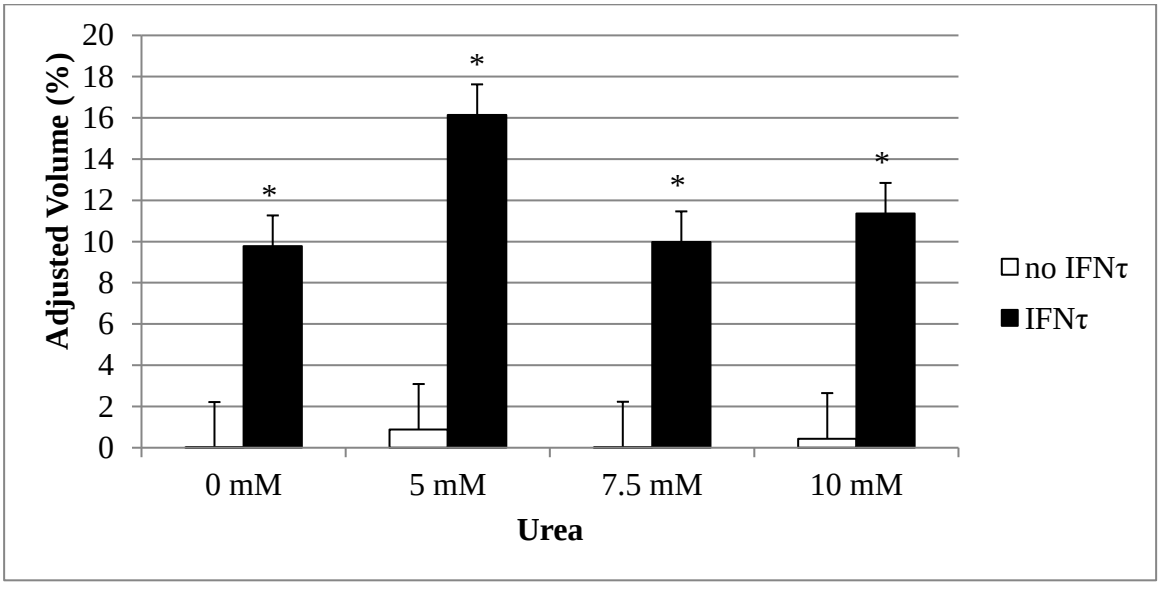

Figure 1.6 Percent adjusted volume for bovine endometrial (BEND) cell expression of Mx1 in response to interferon-tau (IFNτ) cultured with increasing concentrations of urea in the presence of progesterone (P_4). There were no effects of urea treatment ($P > 0.05$) on IFNτ stimulated Mx1 protein expression.
* Different from No IFNτ ($P < 0.01$).

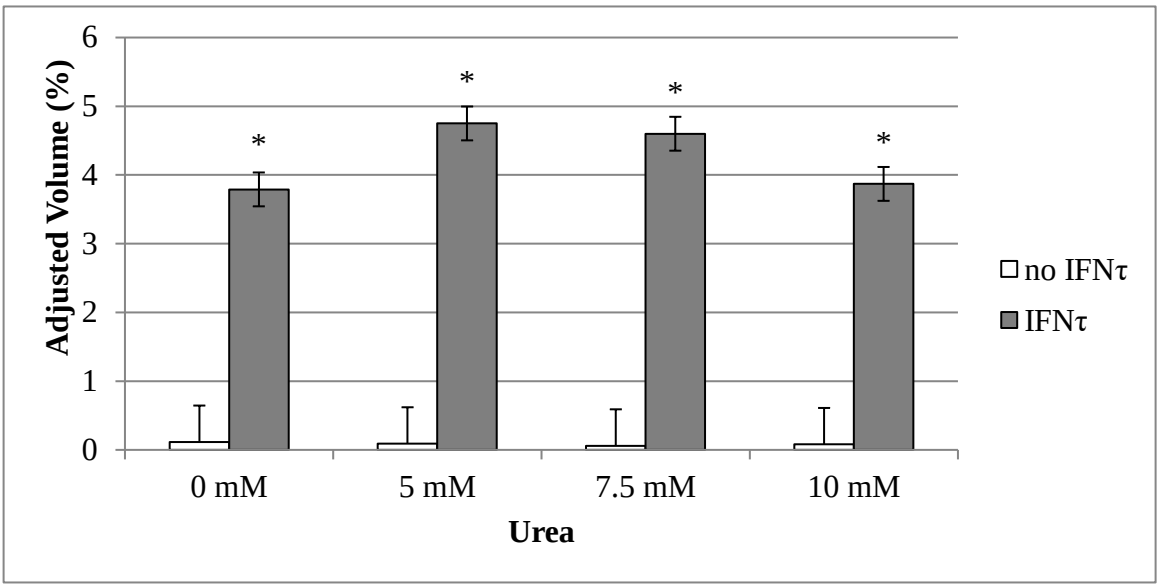

Figure 1.7 Percent adjusted volume for bovine endometrial (BEND) cell expression of ISG-15 in response to interferon-tau (IFNτ) cultured with increasing concentrations of urea in the presence of progesterone (P_4). There were no effects of urea treatment ($P > 0.05$) on IFNτ stimulated ISG-15 protein expression.
* Different from No IFNτ ($P < 0.01$).

Low pH and Mx1

In the presence of P_4 in culture media, DMD treatment decreased culture media pH ($P < 0.01$; Table 1.2). Regardless of DMD treatment, IFNτ increased protein expression of Mx1 ($P < 0.01$; Figure 1.8). Treatment with DMD, lowered media pH and reduced Mx1 expression ($P < 0.01$). There was also an effect of DMD by IFNτ interaction ($P < 0.01$) on Mx1 protein expression. In the presence of P_4, increasing DMD concentrations reduced Mx1 protein expression, however, the difference was not observed until DMD concentrations reached 15 m*M* (pH of 6.9) (Figure 1.8). Mean adjusted volume percent ± SE for cells treated with IFNτ and DMD were 18.79, 23.28, 10.07, 1.49 (± 1.71) for 0 (pH = 7.4), 10 (pH = 7.1), 15 (pH = 6.9), and 20 (pH = 6.7) m*M* DMD, respectively (Figure 1.8).

Low pH and ISG-15

In the presence of P_4, DMD treatment reduced media pH ($P < 0.01$; Table 1.2). Regardless of DMD treatment, the protein expression of ISG-15 by BEND cells increased in the presence of IFNτ ($P < 0.01$; Figure 1.9). There were no effects of increasing DMD concentrations, resulting in lower media pH ($P = 0.28$), nor any IFNτ by DMD treatment interactions ($P = 0.41$) on BEND cell protein expression of ISG-15 (Figure 1.9). The mean

adjusted volume percent ± SE were 12.54, 19.09, 17.99, 14.63 (SE ± 2.10) for 0 (pH = 7.4), 10 (pH = 7.1), 15 (pH = 6.9), and 20 (pH = 6.7) m*M* DMD and IFNτ treated, respectively (Figure 1.9).

Table 1.2 Means ± SE pH values in bovine endometrial (BEND) cell culture media containing increasing concentrations of dimethadione acid (DMD) in the presence of progesterone (P_4). Means were pooled between cells stimulated with or without interferon-τ (IFNτ).

DMD	0 mM	10 mM	15 mM	20 mM
pH	7.4 ± 0.01	7.1 ± 0.01 *****	6.9 ± 0.01 *	6.7 ± 0.01 *

* Means differ from 0 m*M* DMD treatment ($P < 0.05$).

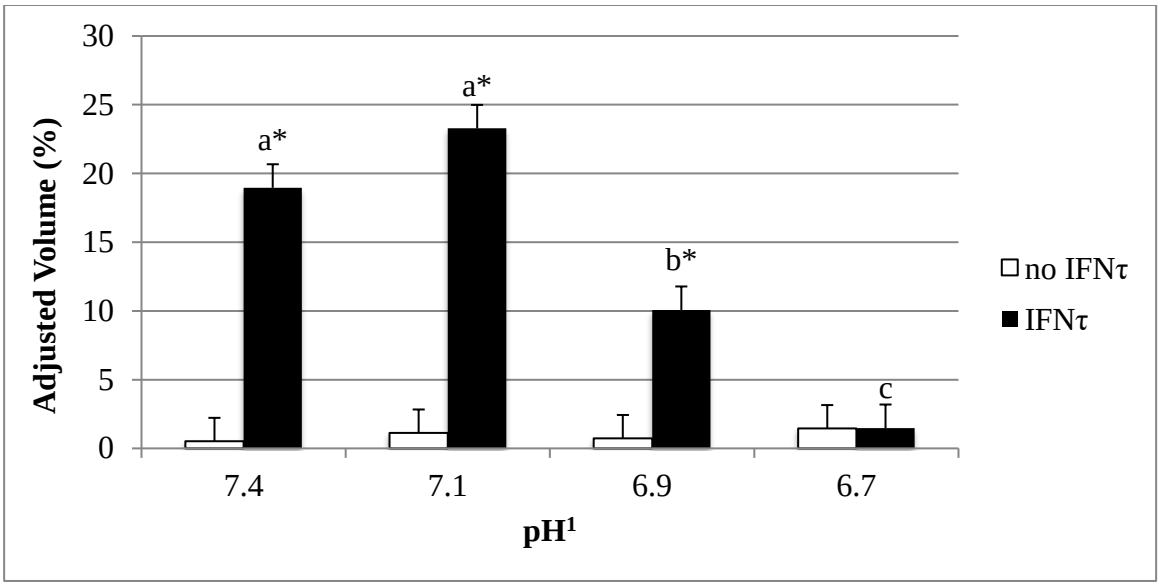

Figure 1.8 Percent adjusted volume for bovine endometrial (BEND) cell expression of Mx1 in response to interferon-tau (IFNτ) and cultured in media containing dimethadione (DMD) acid to reduce the pH in the presence of progesterone (P_4).
[1] pH of 7.4, 7.1, 6.9, and 6.7 correspond to DMD concentrations of 0, 10, 15, and 20 m*M*.
* Different from no IFNτ ($P < 0.01$).
[a,b,c] Bars designated with different letters indicate statistical significance ($P < 0.05$).

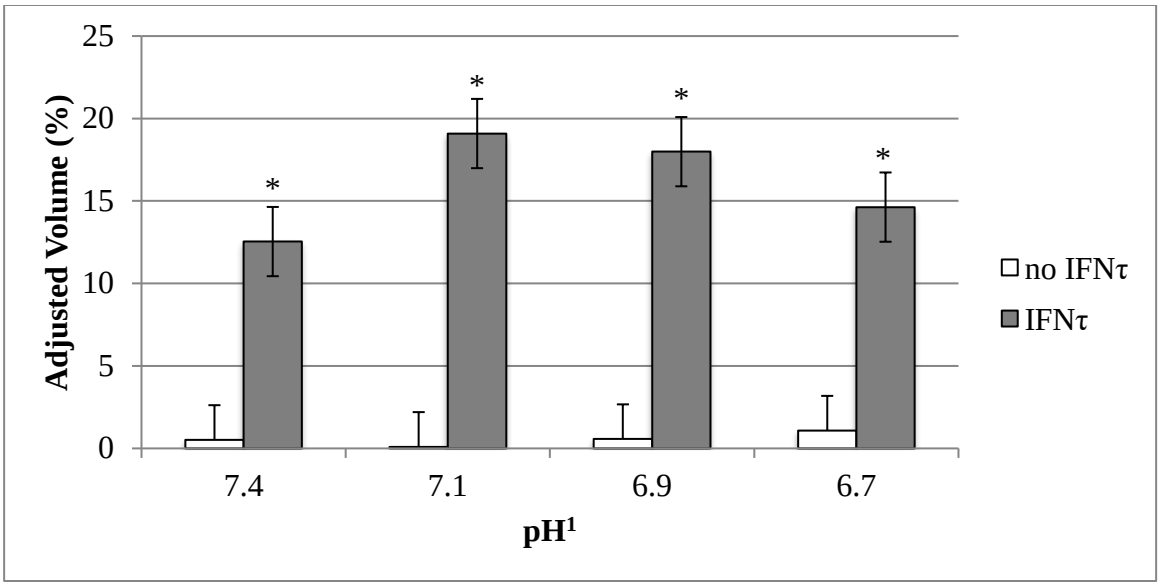

Figure 1.9 Percent adjusted volume for bovine endometrial (BEND) cell expression of ISG-15 in response to interferon-tau (IFNτ) and cultured in media containing dimethadione (DMD) acid to reduce the pH in the presence of progesterone (P_4). There were no effects of DMD treatment ($P > 0.05$) on IFNτ stimulated ISG-15 protein expression.
[1] pH of 7.4, 7.1, 6.9, and 6.7 correspond to DMD concentrations of 0, 10, 15, and 20 m*M*.
* Different from no IFNτ ($P < 0.01$).

DISCUSSION

High BUN or MUN, associated with high dietary protein diets (≥ 20% CP) have been linked with low fertility in lactating dairy cows (Jordan et al., 1983; Kaim et al., 1983; Ferguson et al., 1988; Ferguson and Chalupa, 1989; Canfield et al., 1990; Elrod and Butler, 1993; Elrod et al., 1993; Ferguson et al., 1993; Butler et al., 1996; Larson et al., 1997; Butler, 1998; Westwood et al., 1998; Wittwer et al., 1999; Rajala-Schultz et al., 2001; Law et al., 2009). Research has shown that high dietary protein (≥ 20% CP) that elevate BUN or MUN to greater than 19 mg/dL (> 6.7 m*M*), reduces P/AI by approximately 20% compared with BUN or MUN concentrations < 10 mg/dL (Ferguson et al., 1988; Ferguson et al., 1993; Butler et al., 1996; Elrod and Butler, 1993; Rajala-Schultz et al., 2001; Law et al., 2009). Besides the research that has focused on high dietary protein effects on pregnancy rates of cattle, most other research has focused on the effects of high dietary protein diets on sperm viability (Dietz and Flipse, 1969), oocyte maturation (De Wit et al., 2001; Ocon and Hansen, 2003), and embryonic development (Blanchard et al., 1990; Garcia-Bojalil et al., 1994;

Bishonga et al., 1996; Hammon et al., 2000b; Bode et al., 2001; Dawuda et al., 2002; Ocon and Hansen, 2003; Rhoads et al., 2006). However, there have been no studies to investigate the effects of high uterine urea or low uterine pH, as a result of high dietary protein diets, on uterine cell response to IFNτ. Therefore, the purpose of these experiments was to evaluate the direct effect of low pH media or media containing high urea concentrations on BEND cell response to IFNτ with protein expression of Mx1 and ISG-15 in the absence (Experiment 1) or presence of P_4 (Experiment 2). In the experiment 2, BEND cells were co-cultured with P_4 to mimic the uterine environment at the time of maternal recognition, when P_4 is high and the uterus is exposed to this hormone. The BEND cell line possess characteristics of uterine epithelial cells, with a cobblestone appearance and expression of cytokeratin, and therefore offers a flexible and useful model to investigate the regulation of IFNτ stimulated proteins in various environments.

This study provides the first evidence that high urea has no direct effect on protein expression of two IFNτ stimulated proteins, Mx1 and ISG-15, in the presence or absence of P_4. As a result of high dietary protein, circulating urea concentrations increase, and concentrations begin to equilibrate proportionally in tissues of the reproductive tract and blood (Wathes et al., 2007). It has been shown that high concentrations of urea negatively affect the oocyte and embryo (De Wit et al., 2001; Ocon and Hansen, 2003; Leroy et al., 2004; Hammon et al., 2005), however, we observed no effects of high urea concentrations on uterine response to IFNτ in the presence or absence of P_4. Although it has been suggested that higher urea concentrations may have detrimental effects on endometrial epithelial cell migration, differentiation, and proliferation through reduced gene expression (Gunaretnam et al., 2013), this study showed no difference in protein expression of ISG-15 or Mx1 *in vitro* suggesting IFNτ stimulated gene expression may not be affected by elevated urea in cell cultured uterine cells.

It has been hypothesized that high urea disrupts carbonic anhydrase, an enzyme that controls cellular pH (Rhoads et al., 2004). Carbonic anhydrase functions as a selective transporter of hydrogen and bicarbonate ions apically or basally across secretory epithelia for sodium, potassium, and chloride ions in order to regulate uterine lumen pH (Rodriguez-Martinez et al., 1991). Therefore, urea may indirectly affect uterine pH, and alter uterine

responsiveness to IFNτ-stimulated proteins during the time of maternal recognition of pregnancy.

A study investigating the effects of 12% vs. 23% CP on uterine secretions demonstrated that high CP disrupted uterine ion concentrations (Jordan et al., 1983). The uterine urea concentrations in lactating dairy cows fed 23% CP were 2.7 times greater than those fed a 12% CP diet. In addition, Jordan et al. (1983) also observed a disruption of potassium, phosphorous, magnesium and zinc ion concentrations within the plasma and uterine secretions, thus creating a hostile environment for developing embryos. In fact, these researchers found that 23% CP diet lower uterine secretion concentrations of magnesium when compared with the 12% CP diet. Magnesium has been shown to be important for cellular adhesion and deficiencies of magnesium are associated with abortions (Hurley et al., 1976).

Another interesting observation Jordan et al. (1983) found was related to zinc. During the first estrous cycle after 50 days postpartum, zinc concentrations decreased for the 12% CP diet, and increased in the 23% CP diet (Jordan et al., 1983). Excess zinc has been shown to reduce P_4 binding to its receptor (Habib et al., 1980), and therefore it could be hypothesized that uterine cells would be non-responsive to increasing P_4 concentrations and or IFNτ. In other words, if high protein diets increase zinc concentrations during the estrous cycle as observed by Jordan et al. (1983), then uterine cells may not be primed with P_4. Hypothetically a low pH, caused by high dietary protein, may change zinc ion concentrations reducing uterine cell response to IFNτ with secretion of proteins such as Mx1 even in the presence of P_4. However, further research is still needed in order to examine these theories *in vitro* and *in vivo*.

Provided that change in uterine urea and pH may cause a hostile uterine environment, it is conceivable that the uterus does not respond properly to embryonic signaling during the time of maternal recognition. In this study, low pH reduced IFNτ-stimulated protein expression of Mx1 and ISG-15 in the absence of P_4, and Mx1 in the presence of P_4. This may indicate a possible mechanism by which fertility is negatively affected in cows receiving high dietary protein and having a low uterine pH. In other words, it is possible that lower fertility in this case may in part be attributable to abrogated uterine response to embryo signaling with protein secretion of IFNτ-stimulated proteins.

The mechanism of action by which a low pH suppressed IFNτ-induced Mx1 secretion cannot be determined from this *in vitro* study. However, it is possible that low pH affects intra- and intercellular ion concentrations of uterine epithelial cells, hence affecting the cell function. It has been hypothesized that a shift in uterine pH may be related to a change in carbonic anhydrase, which is an enzyme present in secretory epithelium that transports hydrogen and bicarbonate ions out of epithelial cells and sodium, potassium or chloride into cells (Rhoads et al., 2004; Rodriguez-Martinez, 1991). These alterations in ion concentrations may affect uterine epithelial function such that they do not respond to IFNτ with Mx1 secretion.

Although a low pH reduced protein expression of Mx1 regardless of P_4 presence, and ISG-15 only in the absence of P_4, it is not clear how a low pH disrupted protein synthesis. For example, a low pH could have reduced the presence of IFNτ receptors, however both IFNτ-stimulated proteins (Mx1 and ISG-15) would have had reduced expression regardless of P_4. Previous studies have shown that when cells are exposed to an acidic environment, there is a disruption in normal cellular function (Dautry-Varsat et al., 1983; Gerdes et al., 1989; Chanat and Huttner, 1991; Lodish et al., 1995; Colomer et al., 1996; Wu et al., 2001). For example, when the cytosolic pH is low, some cells have altered cellular metabolism, DNA synthesis stops, amino acid content within cells is altered, and a reduced rate of RNA and protein synthesis (Lodish et al., 1995). Therefore it is possible that the reduced protein expression observed in this study could be due to disruption of DNA synthesis, post-transcriptional disruption of mRNA, or even ribosomal translation alterations for synthesis of proteins. In addition, post-translational events such as packaging of proteins into secretory vesicles within the Golgi could have been altered. Nevertheless, we are unable to determine these possible causes of reduced protein expression of Mx1 and ISG-15 in response to IFNτ, and further research is needed.

Studies have investigated the effects of cytosolic pH and examined the endoplasmic reticulum, Golgi, and secretory vesicles (Gerdes et al., 1989; Chanat and Huttner, 1991; Colomer et al., 1996; Wu et al., 2001). These studies have shown that a low pH allows for the sorting of constantly secreted proteins from those stimulated in order to be secreted (Gerdes et al., 1989; Chanat and Huttner, 1991; Colomer et al., 1996; Wu et al., 2001).

However, these packaged vesicles of sorted proteins may not be secreted and end up being degraded by lysosomes when the cytosolic pH is low.

Unlike what we observed with Mx1 secretion in the presence of P_4 in culture media, in this study, ISG-15 expression in response to IFNτ was not negatively affected by low pH in the presence of P_4 in the culture media. This suggests that not all IFNτ-stimulated proteins secreted by BEND cells are suppressed when cells are exposed and primed with P_4. How P_4 inhibited the suppressive effect of low pH on ISG-15 secretion is not clear. More studies, using other IFNτ stimulated protein markers, should be conducted to find out if the effect of low pH on BEND cell secretion in response to IFNτ is more universal or is exclusive to a few selective proteins.

Bovine endometrial cells possess typical characteristics of uterine epithelial cells and therefore are adequate models for investigating uterine responsiveness to IFNτ *in vitro* (Rodriguez-Sallaberry et al., 2006). This study provided evidence that BEND cells secrete measurable amounts of bovine Mx1 and ISG-15 in response to recombinant IFNτ in the media with or without P_4. However, there are a few limitations in regards to cell culture models. For example, in this study, BEND cells were a mixture of granular epithelial cells and stromal cells. In this study the number of passages from the time of propagation until treatments were minimized. By maintaining a low passage number, we were able to maintain a cell population of granular epithelial and stromal cells more similar to uterine tissue in the live animal. Nonetheless, cell culture models are not able to mimic the entire animal system. The overall physiological status of an animal can influence the uterine environment and cellular mechanisms of uterine cells. Therefore, investigating different types and amounts of dietary protein for lactating dairy cows may allow for maximizing the uterine response to IFNτ for maternal recognition of pregnancy.

REFERENCES

Austin, K. J., S. K. Ward, M. G. Teixeira, V. C. Dean, D. W. Moore, and T. R. Hansen . 1996. Ubiquitin cross-reactive protein is released by the bovine uterus in response to interferon during early pregnancy. Biol. Reprod. 54:600–606.

Austin, K. J., A. L. Carr, J. K. Pru, C. E. Hearne, E. L. George, E. L. Belden, and T. R. Hansen. 2004. Localization of ISG-15 and conjugated proteins in bovine endometrium using immunohistochemistry and electron microscopy. Endocrinology 145:967–975.

Austin, K. J., C. P. King, J. E. Vierk., R. G. Sasser, and T. R. Hansen. 1999. Pregnancy-specific protein B induces release of an alpha chemikine in bovine endometrium. Endocrinology 140: 542-545.

Badinga, L., A. Guzeloglu, and W. W. Thatcher. 2002. Bovine somatotropin attenuates phorbol ester-induced prostaglandin F2α production in bovine endometrial cells. J. Dairy Sci. 85:537–543.

Bavister, B. D., M. L. Leibfried, and G. Lieberman. 1983. Development of preimplantation embryos of the golden hamster in a defined culture medium. Biol. Reprod. 28:235-247.

Binelli, M., A. Guzeloglu, L. Badinga, D. R. Arnold, J. Sirois, T. R. Hansen, and W. W. Thatcher. 2000. Interferon-tau modulates phorbol ester-induced production of prostaglandin and expression of cyclooxygenase-2 and phospholipase-A(2) from bovine endometrial cells. Biol. Reprod. 63:415-424.

Bishonga, C., J. J. Robinson, T. G. McEvoy, P. Findlay, R. P. Aiten, and I. Robertson. 1996. Excess dietary urea intake in ewes and its effect on ovulation rate and embryo development. Jpn. J. Bet. Res. 44:139-151.

Blanchard, T., J. D. Ferguson, L. Love, T. Takeda, B. Henderson, J. Hasler, and W. Chalupa. 1990. Effect of dietary crude-protein type on fertilization and embryo quality in dairy cattle. Am. J. Vet. Res. 51:905–908.

Bode, M. L., R. O. Gilbert, and W. R. Butler. 2001. Effects of high plasma urea nitrogen levels on bovine embryo quality and development. J. Dairy Sci. 84(Suppl.):116.(Abstr.).

Brooks, K., G. Burns, and T. E. Spencer. 2014. Conceptus elongation in ruminants: roles of progesterone, prostaglandin, interferon tau and cortisol. J. Anim. Sci. Biotechnol. 5(1):53.

Brooks, K., and T. E. Spencer. 2015. Biological roles of interferon tau (IFNT) and type I IFN receptors in elongation of the ovine conceptus. Biol. Reprod. 92(2):47-1.

Butler, W. R., J. J. Calaman, and S. W. Beam. 1996. Plasma and milk urea nitrogen in relation to pregnancy rate in dairy cattle. J. Anim. Sci. 74:858–865.

Butler, W. R. 1998. Review: Effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J. Dairy Sci. 81:2533–2539.

Butler, W. R. 2000. Nutritional interactions with reproductive performance in dairy cattle. Anim. Reprod. Sci. 60:449-457.

Canfield, R. W., C. J. Sniffen, and W. R. Butler. 1990. Effects of excess degradable protein on postpartum reproduction and energy balance in dairy cattle. J. Dairy Sci. 73:2342–2349.

Cano, R. J., and J. S. Colome. 1988. Essentials of Microbiology. West Publishing Co. St. Paul, Minnesota p. 273.

Carney, E. W., and B. D. Bavister. 1987. Regulation of hamster embryo development in vitro by carbon dioxide. Biol. Reprod. 36:1155-1163.

Carvalho, P. D., C. C. Consentini, S. R. Weaver, R. V. Barleta, L. L. Hernandez, and P. M. Fricke. 2017. Temporarily decreasing progesterone after timed artificial insemination decreased expression of interferon-tau stimulated gene 15 (ISG15) in blood leukocytes, serum pregnancy-specific protein B concentrations, and embryo size in lactating Holstein cows. J. Dairy Sci. 100:3233-3242.

Chanat, E., and W. B. Huttner. 1991. Milieu-induced, selective aggregation of regulated secretory proteins in the trans-Golgi network. The Journal of Cell Biology, 115(6), pp.1505-1519.

Charleston B., and H. J. Stewart. 1993. An interferon-induced Mx protein: cDNA sequence and high-level expression in the endometrium of pregnant sheep. Gene 137:327–331.

Chełmonska-Soyta, A. 2002. Interferon tau and its immunobiological role in ruminant reproduction. Archivum immunologiae et therapiae experimentalis 50(1):47-52.

Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Symposium: Nitrogen metabolism and amino acid nutrition in dairy cattle. Microbial protein synthesis and flows of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75: 2304-2323.

Colomer, V., G. A. Kicska, and M. J. Rindler. 1996. Secretory granule content proteins and the luminal domains of granule membrane proteins aggregate in vitro at mildly acidic pH. J. Biol. Chem. 271(1):48-55.

D'Cunha, J., E. Knight, A. L. Haas, R. L. Truitt, and E. C. Borden. 1996. Immunoregulatory properties of ISG15, an interferon-induced cytokine. Proceed. National Academy Sci. 93(1):211-215.

Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. Proc. Natl. Acad. Sci. 80:2258-2262.

Dawuda, P. M., R. J. Scaramuzzi, H. J. Leese, C. J. Hall, A. R. Peters, S. B. Drew, and D. C. Wathes. 2002. Effect of timing of urea feeding on the yield and quality of embryos in lactating dairy cows. Theriogenology. 58:1443-1455.

De Wit., A. A., M. L. Cesar, and T. A. Kruip. 2001. Effect of urea during in vitro maturation on nuclear maturation and embryo development of bovine cumulus-oocyte-complexes. J. Dairy Sci. 84:1800-1804.

de Vries, A., and C. A. Risco. 2005. Trends and seasonality of reproductive performance in Florida and Georgia dairy herds from 1976 to 2002. J. Dairy Sci. 88:3155-3165.

Dietz, R. W., and R. J. Flipse. 1969. Metabolism of bovine semen. XX. Role of ammonia in interactions between the citric acid and urea cycles. Biol. Reprod. 1(2):200-206.

Elrod, C. C., and W. R. Butler. 1993. Reduction of fertility and alteration of uterine pH in heifers fed excess ruminally degradable protein. J. Anim. Sci. 71:694–701.

Elrod, C. C., M. Van Amburgh, and W. R. Butler. 1993. Alterations of pH in response to increased dietary protein in cattle are unique to the uterus. J. Anim. Sci. 71:702–706.

Edwards, L. J., D. A. Williams, and D. K. Gardner. 1998. Intracellular pH of the preimplantation mouse embryo: effects of extracellular pH and weak acids. Mol. Reprod. Dev. 50:434-442.

Emond, V., É. Asselin, M. A. Fortier, B. D. Murphy, and R. D. Lambert. 2000. Interferon-tau stimulates granulocyte-macrophage colony-stimulating factor gene expression in bovine lymphocytes and endometrial stromal cells. Biol. Reprod. 62(6):1728-1737.

Ferguson, J.D., T. Blanchard, D. T. Galligan, D. C. Hoshall, and W. Chalupa. 1988. Infertility in dairy cattle fed a high percentage of protein degradable in the rumen. J. Am. Vet. Med. Assoc. 192:659-662.

Ferguson, J. D., and W. Chalupa. 1989. Impact of protein nutrition on reproduction in dairy cows. J. Dairy Sci. 72:746-766.

Ferguson, J. D., D. T. Galligan, T. Blanchard, and M. Reeves. 1993. Serum urea nitrogen and conception rate: The usefulness of test information. J. Dairy Sci. 76:3742–3746.

Folman, Y., M. Rosenberg, I. Asscarrelli, M. Kaim, and Z. Herz. 1983. The effect of dietary and climatic factors on fertility and on plasma progesterone and oestradiol-15β levels in dairy cows. J. Steroid Biochem. 19: 863–868.

Garcia-Bojalil, C. M., C. R. Staples, W. W. Thatcher, and M. Drost. 1994. Protein intake and development of ovarian follicles and embryos of superovulated nonlactating dairy cows. J. Dairy Sci. 77:2537–2548.

Gerdes, H. H., P. Rosa, E. Phillips, P. A. Baeuerle, R. Frank, P. Argos, and W. B. Huttner. 1989. The primary structure of human secretogranin II, a widespread tyrosine-sulfated secretory granule protein that exhibits low pH-and calcium-induced aggregation. J. Biol. Chem. 264(20):12009-12015.

Green, J. C., C. S. Okamura, S. E. Poock, and M. C. Lucy. 2010. Measurement of interferon-tau (IFN-τ) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18- 20 d after insemination in dairy cattle. Anim. Reprod. Sci. 121:24-33.

Gunaretnam, I., T. Pretheeban, and R. Rajamahendran. 2013. Effects of ammonia and urea in vitro on mRNA of candidate bovine endometrial genes. Anim. Reprod. Sci.141:42-51.

Gustafsson, A. H., and D. L. Palmquist. 1993. Diurnal variation of rumen ammonia, serum urea, and milk urea in dairy cows at high and low yields. J. Dairy Sci. 76:475-484.

Habib, F.K., S. Q. Maddy, and S. R. Stitch. 1980. Zinc induced changes in the progesterone binding properties of the human endometrium. Acta Endocrinol. 94:99.

Hammon, D. S., G. R. Holyoak, and T. R. Dhiman. 2005. Association between blood plasma urea nitrogen levels and reproductive fluid urea nitrogen and ammonia concentrations in early lactation dairy cows. Anim. Reprod. Sci. 86:195-204.

Hammon, D. S., S. Wang, and G. R. Holyoak. 2000a. Ammonia concentration in bovine follicular fluid and its effect during in vitro maturation on subsequent embryo development. Anim. Reprod. Sci. 58:1-8.

Hammon, D. S., S. Wang, and G. R. Holyoak. 2000b. Effects of ammonia during different stages of culture on development of in vitro produced bovine embryos. Anim. Reprod. Sci. 59:23-30.

Hammon, D. S., S. Wang, G. Liu, R. D. Wiedmeier, and R. G. Holyoak. 1997. Effects of ammonia on in vitro development of bovine embryos. Theriogenology. 47: 321.

Han, H. K., J. Austin, L. A. Rempel, and T. R. Hansen. 2006. Low blood ISG-15 mRNA and progesterone levels are predictive of non-pregnant dairy cows. J Endocrinol. 191:505-512.

Hansen, T. R., K. J. Austin, and G. A. Johnson. 1997. Transient ubiquitin cross-reactive protein gene expression in the bovine endometrium. Endocrinology 138(11):5079-5082.

Hansen, T. R., K .J. Austin, D. J. Perry, J. K. Pru, M. G. Teixeira, and G. A. Johnson. 1999. Mechanism of action of interferon-tau in the uterus during early pregnancy. J. Reprod. Fertil. Suppl. 54:329-339.

Haq, I. U., Y. Han, T. Ali, Y. Wang, H. Gao, L. Lin, Y. Wu, S. Wu, and S. Zeng. 2016. Expression of interferon-stimulated gene ISG15 and ubiquitination enzymes is upregulated in peripheral blood monocyte during early pregnancy in dairy cattle. Reprod. Biol. 16(4):255-260.

Hicks, B. A., S. J. Etter, K. G. Carnahan, M. M. Joyce, A. A. Assiri, S. J. Carling, K. Kodali, G. A. Johnson, T. R. Hansen, M. A. Mirando., G. L. Woods, D. Vanderwall, and T. L. Ott. 2003. Expression of the uterine Mx protein in cyclic and pregnant cows, gilts, and mares. J. Anim. Sci. 81:1552-1561.

Horisberger, M. 1992. Interferon-induced human protein MxA is a GTPase which binds transiently to cellular proteins. J. Biol. 66:4705-4709.

Horisberger, M. A., and M. C. Gunst. 1991. Interferon induced proteins: Identification of Mx proteins in various mammalian species. Virology 180:185-190.

Hurley, L. S., G. Cosens, and L. L. Theriault. 1976. Teratogenic effects of magnesium deficiency in rats. J. Nutrition 106(9):1254-1260.

Iwata, H., J. Inoue, K. Kimura, T. Kuge, T. Kuwayama, and Y. Monji. 2006. Comparison between the characteristics of follicular fluid and the developmental competence of bovine oocytes. Anim. Reprod. Sci. 91: 215-223.

Johnson, G. A., K. J. Austin, E. A. Van Kirk, and T. R. Hansen. 1998. Pregnancy and interferonτ- induce conjugation of bovine ubiquitin cross-reactive protein to cytosolic uterine proteins. Biol. Reprod. 58:898–904.

Johnson, G. A., K. J. Austin, A. M. Collins, W. J. Murdoch, and T. R. Hansen. 1999. Endometrial ISG-15 mRNA and a related mRNA are induced by interferon-tau and localized to glandular epithelial and stromal cells from pregnant cows. Endocrine. 10:243–252.

Johnson, G.A., M. M. Joyce, S. J. Yankey, T. R. Hansen, and T.L. Ott. 2002. The interferon stimulated gene (ISG) 15 and Mx have different temporal and spatial expression in the ovine uterus suggesting more complex regulation of the Mx gene. J. Endocrinol. 154:R7-R11.

Jordan, E.R., T.E. Chapman, D.W. Holtan, and L.V. Swanson. 1983. Relationship of dietary crude protein to composition of uterine secretions and blood in high-producing postpartum dairy cows. J. Dairy Sci. 66:1854-1862.

Jordan E. R., and L. V. Swanson. 1979a. Effect of crude protein on reproductive efficiency, serum total protein, and albumin in the high-producing dairy cow. J. Dairy Sci. 62:58-63.

Jordan E. R., and L. V. Swanson. 1979b. Serum progesterone and luteinizing hormone in dairy cattle fed varying levels of crude protein. J. Anim. Sci. 48:1154-1158.

Kaim, M., Y. Folman, and H. Neumark. 1983. The effect of protein intake and lactation number on post-partum body weight loss and reproductive performance of dairy cows. Anim. Prod. 37:229-235.

Katagiri, S., and Y. Takahashi. 2004. Changes in EGF concentrations during estrous cycle in bovine endometrium and their alterations in repeat breeder cows. Theriogenology 62: 103–112.

Kenny, D. A., P. G. Humpherson, H. J. Leese, D. G. Morris, A. D. Tomos, M. G. Diskin, and J.M. Sreenan. 2002. Effect of elevated systemic concentrations of ammonia and urea on the metabolite and ionic composition of oviductal fluid in cattle. Biol. Reprod. 66:1797-1804.

Larson, S. F., W. R. Butler, W. B. and Currie. 1997. Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. J. Dairy Sci. 80(7):1288-1295.

Law, R. A., F. J. Young, D. C. Patterson, D. J. Kilpatrick, A. R. G. Wylie, and C. S. Mayne. 2009. Effect of dietary protein content on the fertility of dairy cows during early and mid lactation. J. Dairy Sci. 92:2737-2746.

Leroy, J. L. M. R., T. Vanholder, J. R. Delanghe, G. Opsomer, A. Van Soom, P. E. J. Bols, and A. de Kruif. 2004. Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. Anim. Reprod. Sci. 80:201-211.

Lindenmann J. 1962. Resistance of mice to mouse-adapted influenza A virus. Virology 16:203–204.

Liu, M., X. L. Li, and B. A. Hassel. 2003. Proteasomes modulate conjugation to the ubiquitin-like protein, ISG15. J. Biol. Chem. 278(3):1594-1602.

Lodish, H., A. Berk, S. L. Zipursky, P. Matsudaira, D. Baltimore, and J. Darnell. 1995. Molecular cell biology. Vol. 3 New York: WH Freeman.

Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: Where will it end? J. Dairy Sci. 84:1277–1293.

Mattos, R., A. Guzeloglu, L. Badinga, C. R. Staples, and W. W. Thatcher. 2003. Polyunsaturated fatty acids and bovine interferon- modify phorbol ester-induced secretion of prostaglandin F2α and expression of prostaglandin endoperoxide synthase-2 and phospholipase-A2 in bovine endometrial cells. Biol. Reprod. 69:780–787.

Nocek, J. E., and J. B. Russell. 1988. Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production. J. Dairy Sci. 71: 2070-2107.

Ocon, O. M., and P. J. Hansen. 2003. Disruption of bovine oocytes and preimplantation embryos by urea and acidic pH. J. Dairy Sci. 86:1194–1200.

Ott, T. L., J. Yin, A. A. Wiley, H. T. Kim, B. Gerami-Naini, T. E. Spencer, F. F. Bartol, R. C. Burghardt, and F. W. Bazer. 1998. Effects of the estrous cycle and early pregnancy on uterine expression of Mx protein in sheep (Ovis aries). Biol. Reprod. 59:784-794.

Ott, T.L. 2000. Interferon-tau: paracrine mediator of conceptus-maternal dialogue in ruminants. Pages 1-5 in Sectio DD, Medicina Veterinaria. Vol LV/B. Annales, Universitatis Mariae Curie-Sklodowoska. Poland.

Overton, M. W. and V. E. Cabrera. 2017. Monitoring and quantifying value of change in reproductive performance. In: Large Dairy Herd Management. 3rd ed. D. K. Beede, ed. American Dairy Science Association, Champaign, IL. 2017.

Racicot K. E., K. Toyokawa, and T. L. Ott. 2008. The interferon stimulated gene, Mx1, interacts with tubulin beta in ovine grandular cells and regulates cell proliferation. Biol. Reprod. 78:(Suppl. 1)172.

Rajala-Schultz, P. J., W. J. A. Saville, G. S. Frazer, and T. E. Wittum. 2001. Association between milk urea nitrogen and fertility in Ohio dairy cows. J. Dairy Sci. 84:482–489.

Rhoads M. L., T. R. Bilby, R. P. Rhoads, and L. H. Baumgard LH. 2008. Effects of nutrient metabolism and excess protein catabolism on dairy cow fertility. Proceed. 24th Southwest Nutrition and Management Conference. Arizona: Department of Animal Sciences-The University of Arizona.

Rhoads, M. L., R. O. Gilbert, M. C. Lucy, and W. R. Butler. 2004. Effects of urea infusion on the uterine luminal environment of dairy cows. J. Dairy Sci. 87:2896-2901.

Rhoads, M. L., R. P. Rhoads, R. O. Gilbert, R. Toole, and W. R. Butler. 2006. Detrimental effects of high plasma urea nitrogen on viability of embryos from lactating dairy cows. Anim. Reprod. Sci. 91:1-10.

Rodriguez-Martinez, H., E. Ekstedt, and Y. Ridderstrale. 1991. Histochemical localization of carbonic anhydrase in the female genitalia of pigs during the oestrous cycle. Acta Anat. 140:41-47.

Rodriguez-Sallaberry, C., C. Caldari-Torres, E. S. Greene, and L. Badinga. 2006. Conjugated linoleic acid reduces phorbol ester-induced prostaglandin $F_{2\alpha}$ production by bovine endometrial cells. J. Dairy Sci. 89:3826-3832.

Roffler, R. E., and D. L. Thacker. 1983. Early lactational response to supplemental protein by dairy cows fed grass-legume forage. J. Dairy Sci. 66(10):2100-2108.

Rowlett, R. S., N. J. Gargiulo, F. A. Santoli, J. M. Jackson, and A. H. Corbett. 1991. Activation and inhibition of bovine carbonic anhydrase III by dianions. J. Biol. Chem. 266:933-941.

SAS Institute. 2015. SAS 9.4, Version 4. SAS Institute Inc. Cary, NC.

Spencer, J. A., K. Austin, K. Carnahan, and A. Ahmadzadeh. 2014. The role of pH and progesterone on bovine uterine protein secretion in response to maternal recognition, interferon-tau. J. Dairy. Sci. 97(Abstract, E-Suppl. 1): 242.

Staggs, K. L., K. J. Austin, G. A. Johnson, M. G. Tiexeira, C. T. Talbot, V. A. Dooly, and T. R. Hansen. 1998. Complex induction to bovine uterine proteins by interferon-tau. Biol. Reprod. 59: 293-297.

Staples, C. R., C. Garcia-Bojalil, B. S. Oldick, W. W. Thatcher, and C. A. Risco. 1993. Protein intake and reproductive performance of dairy cows: a review, a suggested mechanism, and blood and milk urea measurements. In Proceedings of the 4th Annual Florida Ruminant Nutrition Symp. Gainesville, FL, Univ. of FL.

Turner, T. T., and S. S. Howards. 1978. Factors involved in the initiation of sperm motility. Biol. Reprod. 18(4):571-578.

Vallet, J. L., P. J. Barker, G. E. Lamming, N. Skinner, and N .S. Huskisson. 1991. A low molecular weight endometrial secretory protein which is increased by ovine trophoblast protein-1 is a beta 2-microglobulin-like protein. J. Endocrinol. 130:R1-4.

Van Soest, P. J. 1994. Nutritional ecology of the ruminant. 2 ed. Comstock Publishing Associates.

Washburn, S. P., W. J. Silvia, C. H. Brown, B. T. McDaniel, and A. J. McAllister. 2002. Trends in reproductive performance in Southeastern Holstein and Jersey DHI herds. J. Dairy Sci. 85:244–251.

Wathes, D. C., Z. Cheng, N. Bourne, V. J. Taylor, M. P. Coffey, and S. Brotherstone. 2007. Differences between primiparous and multiparous dairy cows in the inter-relationships between metabolic traits, milk yield and body condition score in the periparturient period. Domest. Anim. Endocrinol. 33(2):203-225.

Westwood, C. T., I. J. Lean, and R.C. Kellaway. 1998. Indications and implications for testing of milk urea in dairy cattle: A quantitative review. Part 2. Effect of dietary protein on reproductive performance. New Zealand Vet. J. 46:123-130.

Wittwer, F. G., P. Gallardo, J. Reyes, and H. Optiz. 1999. Bulk milk urea concentrations and their relationship with cow fertility in grazing dairy herds in southern Chile. Prev. Vet. Med. 38:159-166.

Wright, T. C., S. Moscardini, P. H. Luimes, P. Susmel, and B. W. McBride. 1998. Effects of rumen-undegradable protein and feed intake on nitrogen balance and milk protein production in dairy cows. J. Dairy Sci. 81:784-793.

Wu, M. M., M. Grabe, S. Adams, R. Y. Tsien, H. P. H. Moore, and T. E. Machen. 2001. Mechanisms of pH regulation in the regulated secretory pathway. J. Biol. Chem. 276(35):33027-33035.

CHAPTER TWO

"Using aspirin during the summer to enhance conception and reduce the risk of embryonic loss in lactating dairy cows"

ABSTRACT

The occurrence of embryonic loss in cattle may be related to a hormonal imbalance resulting in untimely secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) around the time of maternal recognition, between day 14 and 16 after fertilization. The objective of this study was to examine the effect of aspirin, a non-steroidal anti-inflammatory drug (NSAID), on pregnancy per AI (P/AI), blood progesterone (P_4), and blood pregnancy specific protein B (PSPB) in lactating dairy cows inseminated more than once. On day 14 after the second or greater AI, 556 cows were assigned randomly to aspirin (187.2 g total; n = 277) or control (n = 279) treatments. Aspirin (187.2 g total) was administered orally using a balling gun 24 hours apart on day 14 and 15 after AI, and the control group was subjected to sham bolus administration. On day 25 following AI, blood samples were collected from a subset of cows (n = 194) and measured for P_4 and PSPB concentrations. Pregnancy status was determined by transrectal palpation of uterine contents 35 to 42 days after AI. Logistic regression analysis was used to examine the effects of treatment, number of inseminations 14 days before initiation of the experiment (TBRD; 2^{nd} or 3^{rd} vs. $\geq 4^{th}$), parity, and all two-way interactions with treatment on P/AI. Progesterone and PSPB concentrations data on day 25 after AI were analyzed using analysis of variance procedures for the main effects of treatment, pregnancy status (pregnant vs. non-pregnant) after treatment, parity, TBRD, and all two-way interactions with treatment. The maximum daily ambient temperature on day 14 and 15 ranged from 38 to 41°C. There were no differences in P/AI between aspirin (21.6%) and control (27.5%). Additionally, there were no effects of parity (primiparous 26.2% vs. multiparous 23.8%), or TBRD (2^{nd} or 3^{rd} 26.2% vs. $\geq 4^{th}$ 21.5%) on P/AI, nor any two-way interactions with treatment. As expected, P_4 and PSPB concentrations on day 25 were greater for cows diagnosed pregnant vs. non-pregnant ($P < 0.01$). There were no effects of treatment ($P = 0.54$), parity ($P = 0.12$), TBRD ($P = 0.33$) or any two-way interactions of

treatment by pregnancy status (P = 0.27), treatment by parity (P = 0.81), or treatment by TBRD (P = 0.73) on P_4 concentrations. Additionally, there were no effects of treatment or treatment by pregnancy status on PSPB concentrations (P = 0.40). However, multiparous cows tended (P = 0.07) to have greater PSPB concentrations when compared with primiparous cows (0.58 ± 0.04 ng/mL vs. 0.49 ± 0.05 ng/mL). Also, cows inseminated for the 2nd or 3rd time before treatment tended (P = 0.07) to have greater PSPB concentrations compared to cows inseminated four or more times (0.59 ± 0.04 ng/mL vs. 0.48 ± 0.05 ng/mL). Nevertheless, there were no parity by treatment (P = 0.47), TBRD by treatment (P = 0.19), or pregnancy status by treatment (P = 0.39) interactions. These results provide evidence that aspirin does not improve P/AI or alter PSPB or P_4 concentrations in lactating dairy cows subjected to second or greater AI during the summer.

Keywords: aspirin, conception, pregnancy specific protein B, dairy cow

INTRODUCTION

Reproductive efficiency is an important contributor to dairy farm profitability. Many factors cause reproductive inefficiencies and decrease pregnancy rates. Embryonic loss is one factor that contributes to the reproductive inefficiencies of dairy cattle and negatively impacts pregnancy rates (Lamming et al., 1989; Chebel et al., 2004). In dairy cattle, fertilization rates are approximately 85 to 95%, however, conception rates at day 27 to 31 after AI are approximately 35 to 4%, indicating the majority of cows that conceive do not maintain pregnancy following fertilization (Diskin and Sreenan, 1980; Thatcher et al., 1994; Sreenan et al., 2001; Whitlock and Maxwell, 2008). The majority of embryonic mortality (70 to 80% of the total loss) occurs shortly after fertilization between days 8 and 16 (Sreenan et al., 2001).

A specific sequence of events must occur in order for embryos to survive and for pregnancy to be maintained. In cattle, these events are in part dependent upon sufficient progesterone (P_4) secretion from the corpus luteum (CL), and timely secretion of interferon-τ (IFNτ) from the embryo. Interferon-tau directly and indirectly inhibits synthesis and secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) preventing luteolysis (Anthony et al., 1988; Farin et

al., 1990; Dunne et al., 2000). Any disruption in the previously mentioned events may lead to early embryonic death. Therefore, it is critical to investigate methods to regulate luteolysis and assist in the maintenance of early pregnancy in dairy cattle.

High concentrations of P_4 prior to fertilization are necessary to prime the uterus for embryo development and inhibit secretion of $PGF_{2\alpha}$ (Thatcher et al., 2001). However, if P_4 concentrations are insufficient, then embryonic mortality increases because the uterine environment is not suitable for embryonic development (Spencer and Bazer, 2004). Another possible scenario that may lead to early embryonic loss is the inability of the embryo to in produce sufficient concentrations of IFNτ, or delayed secretion of IFNτ. This may result in untimely secretion of $PGF_{2\alpha}$, luteolysis, and embryonic loss (Thatcher et al., 1991).

Several *in vivo* studies have shown the negative effects of premature secretion of $PGF_{2\alpha}$ on embryonic survival in beef cows (Bazer, 1992; Schrick et al., 1993; Seals et al., 1998). Given that the majority of embryonic loss occurs between days 8 and 16, and maternal recognition occurs between days 14 and 16, embryonic loss may occur due to the inability of the embryo to prevent $PGF_{2\alpha}$ secretion. Any strategy to inhibit or reduce $PGF_{2\alpha}$ secretion near the time of maternal recognition may help to reduce embryonic loss and improve reproductive performance in lactating dairy cows.

REVIEW OF LITERATURE

LUTEOLYSIS

The process of luteolysis is two-fold; structural luteolysis caused by apoptosis, and functional luteolysis caused by the loss of steroidogenic capacity to produce P_4, which is caused by pulsatile secretion of endometrial $PGF_{2\alpha}$ (McCracken et al., 1999). Prostaglandin $F_{2\alpha}$ is transported through the uterine vein and diffuses into the ovarian artery that leads to the hilus of the ovary. Prostaglandin $F_{2\alpha}$ will bind to receptors primarily on large luteal cells, which have a greater affinity for $PGF_{2\alpha}$ than small luteal cells, and ultimately elicits a series of endocrine, paracrine, and autocrine effects. Specifically, the binding of $PGF_{2\alpha}$ causes auto-synthesis of $PGF_{2\alpha}$ by luteal cells that elicit autocrine and paracrine effects to cause luteolysis. In addition, CL derived $PGF_{2\alpha}$ creates a positive feedback loop with the uterus for the continuation and completion of luteolysis (Arosh et al., 2016).

Synthesis of Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ is a 20-carbon fatty acid that is synthesized and secreted by the endometrial cells of the uterus. During the luteal phase of the estrous cycle, P_4 produced by the CL primes the uterus by causing the accumulation of lipids for $PGF_{2\alpha}$ synthesis (McCracken et al., 1999). However, P_4 also reduces the presence of estradiol (E_2) and oxytocin receptors present on endometrial cells, the essential components for $PGF_{2\alpha}$ synthesis. Thus, a reduction of P_4 concentrations must happen for synthesis of E_2 and ultimately $PGF_{2\alpha}$. Initially, in the absence of an embryo, P_4 begins to down-regulate its own receptors on the endometrium consequently removing the inhibitory effect of E_2 receptors on the endometrium. Concurrently, E_2 from the second follicular wave in dairy cows begins to increase as P_4 begins to down-regulate its own receptors. Estradiol, a steroid hormone that is synthesized and secreted from the follicular granulosa cells, is transported through circulation either freely or bound to albumin to extend its half-life. In circulation, during late diestrus, E_2 targets endometrial cells where it enters through a carrier protein to bind cytosolic or nuclear receptors. Upon binding, especially to nuclear receptors, transcription of mRNA for oxytocin receptor synthesis occurs. Oxytocin receptors are translated in the endoplasmic reticulum, and the Golgi apparatus packages them into vesicles, which are transported to the plasma membrane for secretion (McCracken et al., 1999).

Oxytocin is synthesized and secreted from two sources. First, oxytocin is released upon stimulation of afferent neurons located in the dendritic area of the paraventricular nuclei within the hypothalamus. This stimulus causes oxytocin to be released into the capillary circulatory system within the posterior pituitary gland from the terminal axons of hypothalamic neurons that extend into the posterior pituitary. The second source of oxytocin is from secretory granules within the large luteal cells of the CL (McCracken et al., 1999). After secretion, oxytocin binds to endometrial cells and causes the activation of cyclooxygenase (COX) enzymes (COX-1 and COX-2), which convert arachidonic acid into prostaglandin H_2 (PGH_2), the substrate for $PGF_{2\alpha}$ and prostaglandin E_2 (PGE_2) (McCracken et al., 1999; Arosh et al., 2004). In the absence of an embryo, PGH_2 is converted to $PGF_{2\alpha}$ by prostaglandin F synthase (PGFS) for luteolysis. In contrast, if an embryo is present, it secretes IFNτ for maternal recognition of pregnancy, and causes the shift in production of

$PGF_{2\alpha}$ to PGE_2 by increasing the enzyme prostaglandin E synthase (PGES) in the CL contributing to CL maintenance and establishment of pregnancy (Arosh et al., 2004).

MATERNAL RECOGNITION OF PREGNANCY

Upon fertilization in the ampulla of the oviduct, the male and female gametes undergo syngamy and develop into a zygote. The zygote is a single blastomere cell that begins cleavage (mitotic division) within the oviduct to become a 2-celled, 4-celled, and then 8-celled embryo (Senger, 2012). Approximately 4 days after fertilization (16-cell stage), the bovine embryo enters the uterus and forms into a morula followed by a blastocyst. The blastocyst contains a fluid field cavity, which expands and eventually causes the hatching of embryonic cells from the zona pellucida forming the conceptus (embryo and extra-embryonic membranes) by day 9 and 10 (Senger, 2012). As the embryo continues to grow, the morphology differentiates from a sphere to ovoid by day 13 (Roberts et al., 1992). Between day 14 and 19 after fertilization, the conceptus continues to grow and elongate, the embryo becomes filamentous in form and begins to attach to the uterine wall and initiate the process of placentation (Güillomot et al., 1981; King and Thatcher, 1993; Sreenan et al., 2001; Roberts et al., 2008). The growth and development of the conceptus is enhanced by P_4 concentrations, and initiates secretion of IFNτ for maternal recognition of pregnancy (Mann and Lamming, 2001). Between days 10 and 25 the bovine embryo secretes IFNτ from the embryonic trophectoderm with increasing concentrations as the conceptus continues to grow. Larger embryos produce more IFNτ allowing for establishment of pregnancy (Mann and Lamming, 2001; Amiridis et al., 2009). The greatest IFNτ concentrations are secreted between days 14 and 16 after fertilization, which also coincides with the usual time of luteolysis (Farin, 1990; Bazer, 1992; Thatcher et al., 1995; Roberts et al., 2008). If the conceptus does not grow at an appropriate rate, it will not produce a sufficient amount IFNτ to inhibit $PGF_{2\alpha}$ and prevent luteolysis (Demmers et al., 2001; Mann and Lamming 2001). Therefore, proper embryonic development, adequate secretion of IFNτ by the embryo, and synchrony between the uterus and embryo are essential to inhibit luteolysis, establish pregnancy, and prevent early embryonic loss.

Interferon-tau

Interferon-tau is comprised of 172 amino acids. Since the 1990's the functions of IFNτ have been more clearly defined (Thatcher et al., 1995). Bazer (1992) first hypothesized

that IFNτ elicited four effects to prevent luteolysis, which have since been verified by other researchers (Mirando et al., 1993; Hansen et al., 1999; Arosh et al., 2004; Roberts et al., 2008). First, IFNτ maintains P_4 concentrations from the endometrium and CL, and inhibits $PGF_{2\alpha}$ synthesis (Arosh et al., 2004). Maintenance of P_4 concentrations is essential for embryonic growth. Second, IFNτ directly inhibits E_2 receptor synthesis, hence regulation of oxytocin receptors. It should be noted that oxytocin receptors are necessary to stimulate enzymes for $PGF_{2\alpha}$ synthesis (Mirando et al., 1993; Hansen et al., 1999). Inhibition of oxytocin receptor synthesis and expression, leads to deactivation of COX enzymes for $PGF_{2\alpha}$ synthesis. Lastly, IFNτ alters post-oxytocin mechanisms, which shift prostaglandin production from $PGF_{2\alpha}$ to PGE_2, a luteotrophic hormone (Arosh et al., 2004; Roberts et al., 2008).

Interferon-τ is known as antiluteolytic, luteotrophic, immunomodulatory, and maintains uterine quiescence during the time of embryo implantation in cattle (Thatcher et al., 1995; Arosh et al., 2004). It has been shown that low doses of IFNτ cause the down regulation of COX-2 and PGFS to inhibit $PGF_{2\alpha}$ synthesis (Xiao et al., 1999; Binelli et al., 2000). More specifically, IFNτ down regulates the expression of the prostaglandin-endoperoxide synthase 2 (PTGS2) gene; a gene that encodes the expression of COX-2 enzyme, the rate-limiting step for synthesis of $PGF_{2\alpha}$ (Thatcher et al., 1997; Roberts et al., 2008). Although some studies have indicated that IFNτ decreases COX-2 and $PGF_{2\alpha}$ (Xiao et al., 1999) others have indicated that IFNτ may actually increase COX-2 and PGE_2, a luteotrophic hormone (Asselin et al., 1997; Arosh et al., 2002). These differences may be explained by the fact that both $PGF_{2\alpha}$ and PGE_2 synthesis require COX-2; however, the specific enzymes PGFS and PGES regulate the synthesis of either $PGF_{2\alpha}$, or PGE_2 syntheses and secretions. In fact, studies have shown that expression of COX-2 is important during the peri-attachment period as early as day 17 after insemination (Charpigny et al., 1997; Emond et al., 2004; Guzeloglu et al., 2004), which may be due to the increased synthesis of PGE_2 for luteal maintenance. Xiao et al. (1999) demonstrated that bovine endometrial epithelial cells cultured in the presence of P_4 and treated with IFNτ resulted in a down-regulation of PGFS. Therefore, IFNτ alter the ratio of PGE_2 to $PGF_{2\alpha}$ in favor of higher concentrations of PGE_2 that would support luteal function, maintain P_4 concentrations, and aid in establishment of pregnancy.

Arosh et al. (2004) investigated the cellular effects of IFNτ on bovine endometrium, myometrium, and luteal cells. In this study, endometrial and myometrial cells were collected in three-day intervals throughout the estrous cycle (7 time points), which were verified by ovarian and uterine morphology. Corpora lutea were collected on day 16 of the estrous cycle from beef heifers following intrauterine infusion of saline or IFNτ (every 12 hours from day 14 to 16 of the estrous cycle). Tissues collected were cultured and proteins were measured using Western and Northern blot analysis. The results of this study indicated that IFNτ elicits selective and specific effects on the transition of luteolytic factors towards antiluteolytic factors, and these differ between the endometrium, myometrium, and luteal tissues (Arosh et al., 2004). This study showed that IFNτ influences the endometrium, myometrium, and CL in a time and tissue specific manner to shift the synthesis from $PGF_{2\alpha}$ to PGE_2. This transient shift allows for an increase in PGE_2 that will act as a polycrine signal (endocrine, exocrine, paracrine and autocrine) for endometrial receptivity, myometrial quiescence, and luteal maintenance (Arosh et al., 2004). Thus, these authors concluded that during maternal recognition of pregnancy IFNτ inhibits $PGF_{2\alpha}$ synthesis and increases PGE_2 synthesis, which appear to be essential requirements for establishment of pregnancy.

EARLY EMBRYONIC LOSS

Dairy cows and heifers have fertilization rates of 85 to 95%; however, between 35 to 40% of bred cows undergo embryonic loss (Diskin and Sreenan, 1980; Thatcher et al., 1994; Sreenan et al., 2001; Whitlock and Maxwell, 2008). Of the total embryonic loss, 70 to 80% occurs early between days 8 and 16 after fertilization and during which critical events such as maternal recognition of pregnancy and luteolysis occur (Dunne et al., 2000; Sreenan et al., 2001; Silke et al., 2002). Embryonic loss can occur for a variety of reasons such as lethal genes, milk production as it relates to metabolic rate and clearance of steroid hormones, heat stress, parity, nutrition, and disease.

Research has shown that abnormal P_4 and E_2 profiles are associated with early embryonic loss (Beard et al., 1994). In fact, low concentrations of P_4 have been shown to increase $PGF_{2\alpha}$ concentrations predisposing cows to embryonic loss due to premature luteolysis (Mann and Lamming, 1995; Sreenan et al., 2001; Seals et al., 1998). In addition, low concentrations of P_4 are incapable of supporting embryonic growth may delay secretion of IFNτ (Mann and Lamming, 2001; Thatcher et al., 2001). This could result in untimely

secretion of $PGF_{2\alpha}$ before embryonic signaling for maternal recognition of pregnancy, and may increase the risk of embryonic loss due to luteolysis. Moreover, a delay or low concentrations of IFNτ secreted by the developing embryo during the time of maternal recognition of pregnancy may also contribute to the incidence of embryonic loss.

Measuring Embryonic Loss

Researchers have been trying to identify cattle that are susceptible to embryonic loss by measuring hormone concentrations such as P_4 and pregnancy specific protein B (PSPB) concentrations (Humbolt, 2001; Gábor et al., 2007, Prvanović et al., 2009; Gábor et al., 2016; Pohler et al., 2016). It is known that adequate concentrations of P_4 are needed during early embryonic development and implantation (Spencer et al., 2004) as P_4 down regulates oxytocin receptors by suppressing the presence of E_2 receptors on the endometrium (Spencer and Bazer, 2004), thereby inhibiting subsequent $PGF_{2\alpha}$ synthesis (Skarzynski and Okuda 1999). Low concentrations of P_4 between days 5 to 7 after AI have been associated with lower pregnancy rates in dairy cows (Stronge et al., 2005). If P_4 concentrations are reduced, embryos will develop at a slower rate, and may not secrete IFNτ in a timely manner (Mann et al., 1999; Mann and Lamming, 2001). In addition, lower P_4 concentrations may not prevent synthesis of $PGF_{2\alpha}$ resulting in premature luteolysis. Moreover, further reduction in P_4 concentrations, because of premature secretion of $PGF_{2\alpha}$, creates a hostile uterine environment that is not conducive for embryonic development.

Serum PSPB concentrations can be used to determine pregnancy status in cattle (Sasser et al., 1986). Researchers have also investigated the use of PSPB concentrations to determine embryonic loss, as PSPB begins to decrease approximately 2.5 days after initiation of pregnancy loss (Giordano et al., 2012). In addition, an *in vitro* study by Del Vecchio et al. (1996) demonstrated that bovine luteal cells from mid (day 10 to 12) and late (day 17 and 18) estrous cycles respond to PSPB with increased P_4 and PGE_2 production. Therefore, the relationship between P_4 and PSPB during early pregnancy may allow for the prediction of early embryonic loss.

Recently Gábor et al. (2016) demonstrated that cows with low PSPB concentrations have a greater risk of pregnancy loss. During this study, blood samples were taken 29 to 35 days after AI to measure P_4 and PSPB concentrations. These authors noted that when PSPB concentrations were 0.6 to 0.8 ng/mL there was a 78.2% risk of pregnancy loss compared

with PSPB concentrations greater than 1.1 ng/mL (15% risk of pregnancy loss). In addition, the risk of pregnancy loss was 76.3% when PSPB concentrations were low (0.6 to 1.1 ng/mL) and P_4 concentrations were also low (< 2 ng/mL). Nevertheless, future research is needed to determine PSPB and P_4 concentration thresholds and timing of these hormones in order to adequately diagnose embryonic loss.

HEAT STRESS

The occurrence of embryonic loss increases during the summer as high temperatures and humidity results in heat stress that negatively affects normal physiological functions of reproduction (López-Gatius et al., 2004; Whitlock and Maxwell, 2008). Heat stress has been defined as ambient temperatures ≥ 29°C; however, the effects depend on the relative humidity, and the severity and duration of heat stress (Chebel et al., 2004). Heat stress is measured using the temperature humidity index (THI). The THI value is influenced by a variety of factors, including solar radiation, air movement, and precipitation, but the most important factors influencing this value are the ambient temperature and relative humidity (Bilby, 2014). A THI value > 68 constitutes heat stress in dairy cattle (Armstrong, 1994; Collier et al., 2011). Temperature humidity index is calculated according to the following formula (NRC, 1971):

$$THI = Temperature\ (F°) - [0.55 - (0.55 \times Humidity/100)] \times (Temperature - 58)$$

As temperatures increase, respiration rates and rectal temperatures also increase which affects metabolism and reproductive function (Kadzere et al., 2002). Heat stress negatively affects follicular and oocyte development, embryo development, the uterine environment, hormonal secretions, and overall fertility (Baumgartner and Chrisman, 1981; Badinga et al., 1985; Putney et al., 1989; Ray et al., 1992; Ealy et al., 1993; Badinga et al., 1993; Howell et al., 1994; Wolfenson et al., 1995; Hansen and Arechinga, 1999; Lucy, 2001; Roth et al., 2001a, 2001b; De Rensis et al., 2002).

During the summer, the conception rates of dairy cattle decrease by 20 to 30% (Badinga et al., 1985; Lucy, 2001; De Rensis et al., 2002). In fact, heat stress contributes, in part, to the increased number of inseminations required in dairy cows during the summer (Ray et al., 1992; Thompson et al., 1996; Al-Katanani et al., 1999). This is due to the decreased duration and intensity of estrus, increased pregnancy loss. All these are a result of the negative effects of heat stress on ovarian, uterine, and embryo function (Gwazdauskas et

al., 1981; Younas et al., 1993; Wilson et al., 1998a, 1998b; De Rensis and Scaramuzzi, 2003; López-Gatius et al., 2004; Whitlock and Maxwell, 2008; Gábor et al., 2016).

Effects on Follicular and Oocyte Development

If cattle are exposed to heat stress before ovulation, there is a disruption of oocyte meiotic maturation that delays selection of a dominant follicle and subsequent ovulation (Baumgartner and Chrisman, 1981; Badinga et al., 1993; Roth et al., 2001a, 2001b). Oocytes and follicles are sensitive to increased temperatures (Leibfried-Rutledge, 1999). Research has shown that as temperatures increased and follicular selection is delayed, there is a decrease in the degree of dominance by the dominant follicle. In turn, more medium subordinate follicles develop that may increase the risk of twinning (Ryan and Boland, 1991; Badinga et al., 1993; Wolfenson et al., 1995; Wilson et al., 1998b; Roth et al., 2000). The development of subordinate follicles, in part, is due to the increased degenerative theca and granulosa cells which compromises steroidogenesis, and subsequent development of a functional CL (Badinga et al., 1993; Howell et al., 1994; Wolfenson et al., 1995; Roth et al., 2001a, 2001b). In addition, heat stress negatively affects oocyte development and maturation reducing future embryo development and quality (Putney et al., 1989; Ray et al., 1992; Ealy et al., 1993; Hansen and Arechinga, 1999). Collectively these heat-induced issues can increase the risk of embryonic loss in cattle.

Effects on Embryo Development

In a study by Putney et al. (1989), 16 Holstein heifers were superovulated following exposure to either thermoneutral or heat stressed temperatures. Heifers were heat stressed for 10 hours at the onset of estrus behavior by exposure to an ambient temperature of 42°C and relative humidity of 75%. This time coincides with the early stages of nuclear and cytoplasmic maturation in the preovulatory oocyte (Hyttel et al., 1986). On day 7 after AI, treatment did not affect the number of embryos recovered, however, there was a decrease in the quality of embryos, the percentage of normal embryos, and the number of cells observed within the developing embryos in heat stress-treated heifers. In fact, 85% of embryos collected from heifers that were heat stressed did not develop beyond the 8 to 16-celled stages by day 7. At this time, embryos should have been at the early to late morula stage. These results provide evidence that heat stress on the preovulatory oocyte negatively affects subsequent embryo development. Decreased embryonic development may result in delayed

secretion of IFNτ resulting in synthesis and secretion of $PGF_{2\alpha}$ and embryonic loss. Embryos exposed to heat stress between days 1 and 3 after fertilization have reduced development and viability; however, by day 3 embryos appear to become more heat resistant (Ealy et al., 1993). Therefore, early embryonic loss may be due to a suboptimal uterine environment and hormonal milieu after embryonic development begins.

Effects on Endocrine Function and Uterine Environment

Cows experiencing heat stress exhibit prolonged luteal phases early postpartum (Wilson et al., 1998a, 1998b). Prolonged luteal phases and exposure to P_4 can increase the accumulation of lipid droplets that can be used to form arachidonic acid for $PGF_{2\alpha}$ synthesis (Jerome and Srivastava, 2012). During the luteal phase, growth and maturation of follicles results in increasing concentrations of E_2. If there is an abundance of arachidonic acid (the precursor for $PGF_{2\alpha}$ synthesis) due to prolonged exposure to P_4, perhaps lower concentrations of E_2 may result in subtle increases of $PGF_{2\alpha}$ prior to the time of maternal recognition. In an *in vitro* study, endometrial cells harvested from heat stressed cows at day 17 of pregnancy had increased secretion of $PGF_{2\alpha}$ compared to endometrial cells from cows that were not exposed to heat stress (Malayer et al., 1990). This is important as this time coincides with the timing of maternal recognition of pregnancy, and may indicate that heat stress causes premature secretion of $PGF_{2\alpha}$ and luteolysis before embryonic signaling. In this scenario, early luteolysis due heat stress would also reduce P_4 concentrations, delay embryonic growth, and further increase the risk of embryonic loss. Therefore, it may be possible to reduce embryonic loss by inhibiting $PGF_{2\alpha}$ synthesis and secretion thereby allowing time for the embryo to signal for maternal recognition of pregnancy.

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Non-steroidal anti-inflammatory drugs (NSAID) are known inhibitors of cyclooxygenase (COX) enzymes (COX-1 and COX-2), which are responsible for converting arachidonic acid to $PGF_{2\alpha}$ (Agrawal and Gupta, 2010). Non-steroidal anti-inflammatory drugs such as ketoprofen, meloxicam, flunixin meglumine (FM), and aspirin have been shown to affect reproductive performance in livestock (Aiumalamai et al., 1990; Breuhaus et al., 1999; Strahringer et al., 1999; Elli et al., 2001; Schrick et al., 2001; Purcell et al., 2005; Kafi et al., 2006; Guzeloglu et al., 2007; Amiridis et al., 2009; Erdam and Guzeloglu, 2010; Rabaglino et al., 2010; Sanchez-Rodriguez et al., 2011; Gramulia et al., 2015; Pfeifer et al.,

2016; Spencer et al., 2016a; Spencer et al., 2016b), rodents (Al-Janabi et al., 2005) and humans (Rubinstein et al., 1999).

Ketoprofen

Ketoprofen is a propionic NSAID; a non-selective inhibitor of COX-1 and 2 (Agrawal and Gupta, 2010). Ketoprofen inhibits prostaglandin production providing analgesic, antipyretic, and anti-inflammatory effects. Ketoprofen is administered orally to cattle for alleviating fever, pain, and inflammation associated with mastitis, and is practical for dairy producers because ketoprofen does not require a milk withdrawal period after administration (Agrawal and Gupta, 2010). When ketoprofen was administered to dairy cattle before and after ovulation, luteal regression was delayed and ketoprofen treated cattle had a greater daily increase in mean P_4 concentrations when compared to control cows from days 0 to 6 of estrous cycle (Kafi et al., 2006). Also, ketoprofen treated cows had greater E_2 concentrations compared with control cows at the time of estrus (Kafi et al., 2006). This delay in luteal regression provides evidence that ketoprofen may also suppress synthesis and secretion of $PGF_{2\alpha}$. Although luteal regression was delayed indicating prolonged CL function, the authors noted that that ketoprofen-treated cows had impaired follicular growth when compared to control cows (Kafi et al., 2006). The authors suggested that impaired follicular growth could disrupt normal processes of ovulation and luteolysis (Kafi et al., 2006); therefore not positively influence the reproductive efficiency of cattle.

Meloxicam

Meloxicam is a preferential, but not specific inhibitor of the COX-2 enzyme (Vane, 1971; Davies and Skjodt, 1999). Administration of meloxicam can be intramuscular (i.m.) or subcutaneous (s.c). Meloxicam is an effective anti-inflammatory and analgesic drug however, if administered, it requires a 96-hour milk withdrawal in dairy cows (Agrawal and Gupta, 2010). When compared with FM, meloxicam has a longer biological half-life (35 hours) and is effective for up to 72 hours after administration (Konigsson et al., 2002). The effectiveness of meloxicam on improving reproductive performance has been examined in buffalo (Rajkumar et al., 2010) and cattle (Amiridis et al., 2009; Erdem and Guzeloglu, 2010).

In dairy heifers, administration of meloxicam s.c. on day 15 post-AI reduced P/AI (24% meloxicam vs. 52% control) (Erdem and Guzeloglu, 2010). The authors postulated

that the decrease in pregnancy was due to the long half-life of meloxicam and explained that meloxicam may have interfered with embryonic implantation by inhibiting PGE_2 (Erdem and Guzeloglu, 2010). However, it should be noted that the number of animals used in the previously mention study were limited.

In lactating dairy cows, administration of meloxicam to cows (with four or more unsuccessful AI) on day 16, 17, and 18 post-AI, had no influence on P/AI (Amiridis et al., 2009). There were observed differences in estrous cycle length as they were longer for meloxicam-treated cows, but the authors suggested that meloxicam may have aided in the temporary survival of non-viable embryos.

Flunixin Meglumine

Flunixin meglumine is a potent COX inhibitor and commonly used for pyrexia and inflammation during bovine respiratory disease and endotoxemia, as it elicits analgesic, antipyretic, and anti-inflammatory effects (Anderson et al., 1990; Agrawal and Gupta, 2010). The half-life of FM in cattle ranges from 3 to 8 hours after administration (Odensvik and Johansson, 1995; Anderson et al., 1990; Odensvik, 1995). Flunixin meglumine is administered intravenously (i.v.) and has a milk withdrawal of 36 hours and a meat withdrawal of 4 days (Payne, 2001).

The use of FM to improve P/AI and pregnancy rates during embryo transfer has been extensively examined. Flunixin meglumine administration during embryo transfer has improved pregnancy rates in beef cows and lactating dairy cows (Schrick et al., 2001; Purcell et al., 2005; Scenna et al., 2005). In lactating dairy cows, FM at the time of embryo transfer increased pregnancy rates by 5% (Scenna et al., 2005). Additionally, Schrick et al. (2001) observed an increase in pregnancy rates when beef cows were treated with FM 2 to 5 minutes before embryo transfer of both fresh and glycerol-frozen embryos, which was associated with the stage and quality of embryos transferred (Schrick et al., 2001). Flunixin meglumine improved pregnancy rates when embryo quality grades were excellent, and when embryos were transferred at the morula stage compared with the blastocyst stage. In agreement, the administration of FM 2 to 12 min prior to embryo transfer along with a controlled internal drug release (CIDR) insert after embryo transfer improved pregnancy rates in beef cows (Purcell et al., 2005). It should be noted that the improvement in pregnancy rates could be a result of the CIDR insert, or the effects of FM on $PGF_{2\alpha}$

synthesis. Together these studies demonstrate the beneficial effects of FM at the time of embryo transfer and this improvement in pregnancy rates may be attributable to the ability of FM to reduce uterine manipulation induced release of $PGF_{2\alpha}$.

Some studies have also shown an improvement on P/AI when FM was administered during the time of luteolysis and maternal recognition of pregnancy. In dairy heifers, the use of 1.1 mg/kg BW of FM on day 15 and 16, improved P/AI on day 29 (77% FM vs. 50% control) and on day 65 (69% FM vs. 46% control) after AI (Guzeloglu et al., 2007). Likewise, P/AI were improved when lactating dairy cows were administered FM on day 15 and 16, 12 hours apart (Pfeifer et al., 2016). The P/AI 30 days after AI and 15 days after first FM administration were 37% for the FM-treated cows and 17% for the control cows. These previous researchers suggested that the observed differences in P/AI were attributable to the attenuation of $PGF_{2\alpha}$ secretion preventing early luteolysis of the CL (Guzeloglu et al., 2007; Pfeifer et al., 2016); however, PGFM concentrations were not measured in these studies.

The hypothesis by Guzeloglu et al. (2007) and Pfeifer et al. (2016) can be supported as other studies have shown that FM reduces PGFM concentrations. A study by Aiumlamai et al. (1990) noted a 30% reduction in PGFM concentrations during the luteal phase of the estrous cycle when dairy heifers were treated with FM. To determine the effects of FM on PGFM without the confounding effect of endogenous production of $PGF_{2\alpha}$ by the CL, oophorectomized cows were administered 2.2 mg/kg BW of FM. Within 30 minutes, PGFM concentrations were significantly reduced from basal concentrations and remained at a lower concentration until 6 hours after treatment (Aiumlamai et al., 1990). In agreement, when lactating dairy cows were synchronized to the luteal phase of the estrous cycle and administered 2.0 mg/kg BW FM on day 15 (day 0 = ovulation) of the estrous cycle (Spencer et al., 2016a), blood PGFM was decreased compared with saline treated cows. The decrease in PGFM concentrations observed by Spencer et al. (2016a) were similar to those observed by Aiumlamai et al. (1990). Within 60 minutes, PGFM concentrations for FM-treated cows were approximately 30% less than basal levels, whereas PGFM concentrations for the saline-treated cows remained unchanged (Spencer et al., 2016a). In addition, P_4 concentrations were measured daily from days 15 to 22 during the estrous cycle to examine the influences of FM on the CL function and (or) lifespan. There was no difference in the mean P_4 concentrations, however, the FM-treated cows tended to have a slower rate of P_4

decline between days 15 and 22 of the estrous cycle indicating that FM may help to extend luteal function of the CL (Spencer et al., 2016a). However, these previously mentioned studies did not investigate the effects of FM on P/AI.

Merrill et al. (2007) examined the effects of FM on both P/AI and PGFM concentrations in beef cows. Flunixin meglumine was administered on day 14 after AI and improved P/AI (71% FM vs. 61% control) compared to those that were not administered FM (Merrill et al., 2007). Also, mean PGFM concentrations were significantly reduced following the administration of FM (39.6 pg/mL FM vs. 60.6 pg/mL control; Merrill et al., 2007) and were similar to the observations seen in lactating (Spencer et al., 2016a) and oophorectomized dairy cows (Aiumlamai et al., 1990).

Although the above mentioned studies (Aiumlamai et al., 1990; Guzeloglu et al., 2007; Merrill et al., 2007; Pfeifer et al., 2016; Spencer et al., 2016a) in beef cows, dairy heifers, and lactating dairy cows have demonstrated a decrease in PGFM and (or) an increase in P/AI, other studies in dairy heifers have shown no effect of FM on overall P/AI. A study by Rabaglino et al. (2010), observed no differences in P/AI when FM was administered twice, 12 hours apart on day 15 and 16 after AI in dairy heifers synchronized using a 5-day CIDR-Cosynch timed-AI (TAI) protocol (59.4% FM vs. 59.5% control). Similarly, a study by von Krueger and Heuwieser (2010), showed no difference in P/AI (54.8% FM vs. 58.2% control) when FM was administered to dairy heifers 24 hours apart on either day 14 and 15 or 15 and 16 post-AI. Nevertheless, none of the previous studies in cows or heifers have examined the effects of FM on P/AI and (or) PGFM concentrations during the summer when embryonic loss is more prevalent.

Despite the evidence that FM may slow or prevent luteolysis and improve P/AI in lactating cows, the use of FM is not a practical reproductive management tool on dairy operations for a number of reasons. Flunixin meglumine requires a prescription from a veterinarian, is more expensive than other NSAID, must be administered i.v., and requires milk withdrawal of 36 hours resulting in milk profit loss (Payne, 2001). Thus, the use of another NSAID may be a better alternative and prove to be just as effective in inhibiting secretion of $PGF_{2\alpha}$ and potentially improve fertility.

Aspirin

Aspirin is carboxylic acid NSAID and a nonspecific inhibitor of COX enzymes (Campbell and Blikslager, 2000). It is mostly used in dairy cows for the treatment of pain, fever, and inflammation (Agrawal and Gupta, 2010). Aspirin is commonly administered orally in bolus form and contains lipophilic molecules, which allow for absorption through the gastrointestinal wall. However, only 50% of aspirin's metabolite (salicylate acid) binds to albumin for distribution, consequently within 24 hours following administration 90% of aspirin is eliminated through urine via the glycine conjunction pathway (Short et al., 1991; Agrawal and Gupta, 2010).

In cattle, an effective dose of orally administered aspirin is 100 mg/kg BW every 12 hours, which is equivalent to 1 ⅓ of 240-grain (20.68 g) bolus twice a day for a 1500 lb. cow (Gingerich, 1975; Payne, 2001). Aspirin has been shown to affect blood flow by shifting the local production of thromboxane and prostaglandins to prostacyclin (Elli et al., 2001). In sheep, 10 mg/kg BW aspirin i.v. decreased the basal thromboxane level by 95% (Nolan et al., 1990). Rubinstein et al. (1999) postulated that aspirin-induced reduction in thromboxane is the mechanism by which the smooth muscles of the uterine endometrial vessels relax, increasing blood flow to the uterus.

Administration of low dose aspirin in women, during embryo transfer following *in vitro* fertilization, has shown promising results because it improved embryo implantation and pregnancy rates (Rubinstein et al., 1999). Administration of aspirin improved ovarian responsiveness to human chorionic gonadotropin (hCG) with the number of oocytes ovulated, increased uterine and ovarian blood flow, and improved implantation rates and pregnancy rates (Rubinstein et al., 1999). In mice, administration of low dose aspirin during diestrus over long periods of time (5 to 20 days), significantly decreased uterine weight, and decreased blood gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Al-Janabi et al., 2005). Aspirin also increased P_4 levels, and the number and diameter of CL present on the ovary (Al-Janabi et al., 2005). In this study, however, the researchers did not determine any fertility aspects such as number of pups or pregnancy rates following aspirin treatment.

In Brahman cows (40 multiparous and 20 primiparous), 2.3 kg of aspirin was added to a concentrate diet (approximately 100 mg/kg BW), and given every 12 hours for 5 days

(day 7 to 13 postpartum) to observe its effects on PGFM and P_4 concentrations. In that study, multiparous cows showed a decrease in plasma PGFM concentrations, whereas primiparous cows exhibited an increase in plasma PGFM (Strahringer et al., 1999). Following aspirin treatment, both primiparous and multiparous cows were reported to have lower pregnancy rates, increased abnormal cycles, and a declined presence of a CL following ovulation (Strahringer et al., 1999). However, the length of the first estrous cycle, concentrations of P_4, and interval from calving until conception were all unaffected after aspirin administration (Stahringer et al., 1999). To our knowledge there was no evidence on the effects of oral administration of aspirin on PGFM and P_4 concentrations during the luteal phase of the estrous cycle in lactating dairy cows. Thus, in a previous study, our laboratory investigated the effects of aspirin on PGFM and P_4 concentrations during the luteal phase (day 14 and 15) of the estrous cycle in lactating dairy cows (Spencer et al., 2016b). In that study, 23 lactating Holstein dairy cows were synchronized to luteal phase (day 14 and 15) of the estrous cycle using a 5-day CIDR-Cosynch protocol. On day 14 (day 0 = ovulation), cows were stratified by parity and assigned randomly to aspirin (n = 11) or control (n = 12) treatments. For the aspirin treatment, cows received oral aspirin administration (46.65 g/dose) in the morning and evening of day 14 and in the morning of day 15, whereas the control cows received sham bolus administration. On day 14 prior to aspirin treatment, a blood sample was collected to measure pre-treatment PGFM concentrations. On day 15, six hours after the last aspirin treatment, hourly blood samples were collected for 6 hours to measure PGFM concentrations. Additionally, blood samples were collected every day from day 15 to 22 to measure P_4 concentrations and determine days to luteolysis, which was defined as two consecutive days of P_4 < 1ng/mL (Spencer et al., 2016b).

In that study, mean PGFM concentrations after treatment were lower ($P < 0.05$) for the aspirin-treated cows compared to the control cows (76.79 ± 14.83 pg/mL vs. 143.53 ± 26.52 pg/mL). In fact, administration of aspirin reduced PGFM concentrations by 1.8 fold and PGFM concentrations for the aspirin treatment remained lower for 30 hours after first administration, when compared with the control. There were seven cows that did not undergo luteolysis and their P_4 data was removed from the analysis of P_4 concentrations. In remaining cows, the mean P_4 concentrations were greater ($P < 0.05$) for the aspirin treatment when compared with the control (3.4 ± 0.5 ng/mL vs. 1.8 ± 0.5 ng/mL). In addition days to

luteolysis (two consecutive days of P_4 < 1 ng/mL) tended ($P < 0.1$) to be greater for the aspirin treatment compared with the control (20.6 ± 0.5 days vs. 19.2 ± 0.5 days; Spencer et al., 2016b).

RATIONALE

The results of our previous study (Spencer et al., 2016b) provided evidence that indicated that aspirin is effective in reducing circulating PGFM concentrations and may also delay days to luteolysis. Whether aspirin treatment after AI can improve P/AI in dairy cows, however, has not been investigated. Hypothetically, aspirin could reduce $PGF_{2\alpha}$ concentrations and delay premature luteolysis. This could potentially reduce the risk of embryonic loss that may be caused by premature secretion of $PGF_{2\alpha}$ or delayed secretion of IFNτ by the embryo in lactating dairy cows, especially during the summer when the occurrence of embryonic loss is exacerbated due to heat stress.

HYPOTHESIS

In lactating dairy cows with one or more failed AI conception, aspirin administration on day 14 and 15 after a subsequent AI will improve P/AI during the summer.

OBJECTIVES

The objectives of this study were to investigate the effects of aspirin on a) P/AI and b) concentrations of P_4 and PSPB in lactating dairy cows during the luteal phase of the estrous cycle (day 14 and 15 post-AI) after the second or greater insemination.

MATERIALS & METHODS

ANIMALS

All animal handling procedures and treatment protocols used in this experiment were in accordance with the protocols of the Animal Care and Use Committee at the University of Idaho and approved prior to experimentation. This project was conducted from June to August 2015 on a commercial dairy in Mesa, Washington. This dry-lot dairy milked approximately 6,000 lactating Holstein dairy cows with an average daily milk production of 37 kg and a 305-day mature milk equivalent (305ME) of 12,700 kg. All cows were synchronized to first AI using a modified 7-day CIDR-Cosynch protocol (CIDR insertion –

7 days – CIDR removal and $PGF_{2\alpha}$ – 2 days – GnRH and AI) with a voluntary waiting period of 50 days. The herd average days open (DO) was 131, with an average service per conception (S/C) of 2.8. The DO for cows that were inseminated more than once was 167. Annualized 21-day pregnancy rate was 23% for the calendar year 2014 to 2015. During June and July of 2014, the 21-day pregnancy rate was 17%. All cows were housed in dry-lot pens and milked twice daily. Cows were fed a total mixed ration (TMR) twice daily that was pushed up 4 to 6 times a day. The TMR was formulated to meet or exceed the nutritional requirements for high producing dairy cows (NRC, 2001). Cows had access to feed for more than 20 hours a day, and had ad libitum access to water.

CHARACTERISTICS OF RESEARCH COWS

During this experiment, 575 lactating Holstein dairy cows that failed to conceive to the previous AI were enrolled in this experiment. Nineteen cows were eliminated before data analysis. Of those eliminated, 15 were sold or died before the pregnancy results after treatment were determined. The additional 4 animals were eliminated as they were inseminated less than 18 days from the previous AI, indicating these cows were not during the luteal phase of the estrous cycle at the time of treatment administration. Therefore, a total of 556 primiparous and multiparous lactating dairy cows that failed to conceive to at least one AI were used in this study.

Treatments and Blood Sampling

All cows were synchronized to AI before treatment with either a 7-day Cosynch or a 7-day CIDR-Cosynch protocol. On day 14 following AI, cows were assigned randomly to treatment (aspirin) or control (no aspirin) groups. Treatment cows (n = 277) were administered aspirin (93.6 g/cow/dose; VetOne, Boise, ID) twice 24 hours apart (day 14 and 15 after AI) and control cows (n = 279) were subjected to sham bolus administration (Figure 2.1). After milking on Thursday (day 14) and Friday (day 15), cows were treated beginning at 0300 hours. Body condition scores were recorded by averaging scores from two individuals from a subset of cows (n = 262; 133 aspirin and 129 control) on day 25 of the experiment (Figure 2.1). Body condition scores were determined using a 1 to 5 point scale with 0.25 increments (Butler and Smith 1989). Additionally, on day 25 post-AI, coccygeal blood samples were collected from a sub-population (n = 194) of cows using 10 mL serum Covidien, Monoject Vacutainer® tubes (Covidien LLC, Mansfield, MA) and immediately

placed on ice. All samples were stored at 4ºC for 18 to 24 hours and then centrifuged for 25 minutes at 1,300 × *g* at 4ºC. Serum samples were immediately analyzed for PSPB, and the remaining serum was harvested and stored at -20ºC until assayed for P_4 concentrations. Pregnancy status was determined by transrectal palpation performed by the herd veterinarian between 35 and 42 days after AI (21 to 28 days after first treatment administration).

Hormone Assays

Blood samples were collected on day 25 following AI and analyzed for PSPB concentrations using BioPRYN (BioTracking, Inc., Moscow, Idaho). These blood samples were also used to measure P_4 concentrations using a double antibody RIA (MP Biomedicals, Costa Mesa, CA) under equilibrium conditions. The standard curve ranged from 0.05 to 25 ng/mL. All samples and standards were run in duplicate. The intra-assay CV was 7.6%, calculated using a known high and low reference samples.

STATISTICAL ANALYSIS

Descriptive data for the number of times bred (TBRD) before treatment, number of lactations (parity), somatic cell count (SCC), body condition score (BCS), and 305ME were analyzed between treatments using the analysis of variance procedure in SAS (v. 9.4; SAS Institute Inc. 2015).

The difference in P_4 and PSPB concentrations between aspirin and control cows on day 25 were also analyzed by analysis of variance using the GLM procedure. The model included the main effects of treatment (control vs. aspirin), parity (primiparous vs. multiparous), TBRD (2^{nd} and 3^{rd} vs. $\geq 4^{th}$), pregnancy status following treatment (pregnant vs. non-pregnant), and all two-way interactions with treatment. Cow within treatment was considered the random effect in this model.

Logistic regression was used to analyze the main effects of treatment (control vs. aspirin), parity (primiparous or multiparous), TBRD (2^{nd} and 3^{rd} vs. $\geq 4^{th}$), and all two-way interactions with treatment on P/AI. Significance was declared at $P \leq 0.05$ and a tendency at $P \leq 0.1$.

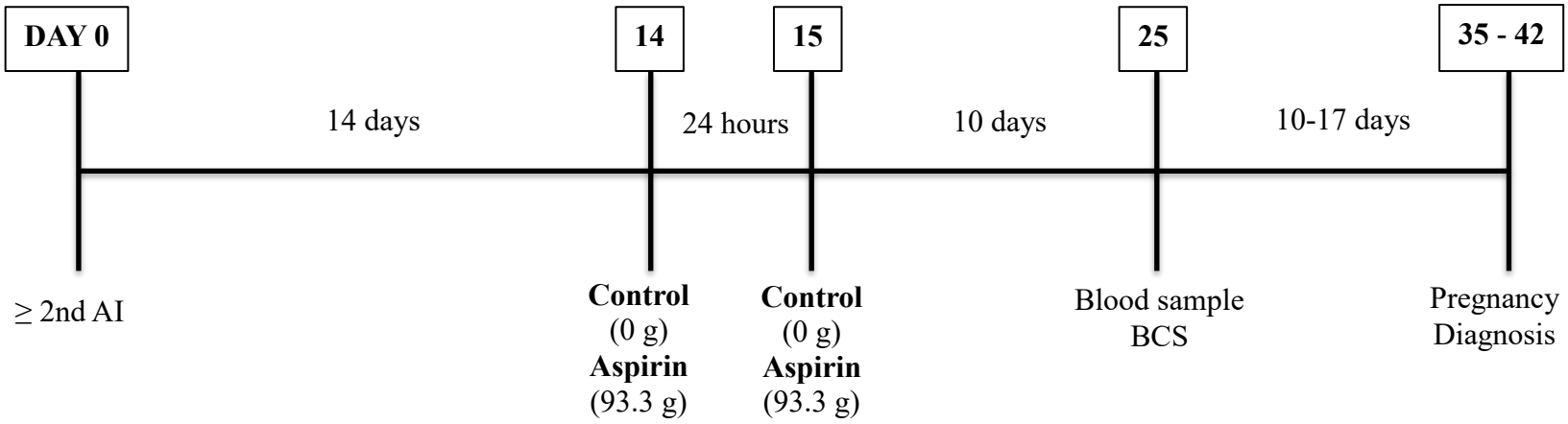

Figure 2.1 Schematic of experimental protocol for examining the effects of aspirin on percentage of pregnancies per AI (P/AI) in lactating dairy cows subjected to second or greater AI. All cows received AI, and were assigned randomly 14 days following AI to either the control group (n = 279) or aspirin group (n = 277). On day 14 and 15 of the experiment (24 hours apart) the control cows received sham bolus administration and the aspirin-treated cows received 93.3 g/dose/animal/day. Ten days later on day 25 of the experiment, coccygeal blood samples were collected from a subset of cows (n = 194) to measure progesterone (P_4) and pregnancy specific protein B (PSPB) concentrations. Body condition scores (BCS) on a subset of cows (n = 262) were recorded on day 25.

RESULTS

There were no differences ($P = 0.23$) in mean BCS between the subsample of 133 aspirin-treated cows and 129 control cows (Table 1). In addition, TBRD ($P = 0.49$), DIM ($P = 0.80$), parity ($P = 0.79$), 305ME ($P = 0.22$), and SCC ($P = 0.74$) did not differ between aspirin and control groups (Table 1).

There was no effect of treatment on P/AI ($P = 0.13$) and P/AI were 21.6% for the aspirin-treated cows and 27.6% for the control cows (Figure 2.2). There were also no differences in P/AI between 2nd or 3rd vs. ≥ 4th TBRD ($P = 0.14$). The P/AI for 2nd or 3rd TBRD were 26.2% and ≥ 4th were 21.5%, respectively. There were no P/AI differences between parities ($P = 0.55$; 23.8% multiparous vs. 26.2% primiparous; Figure 2.2), nor any treatment by parity ($P = 0.11$), or treatment by TBRD ($P = 0.26$) effects on P/AI.

Progesterone concentrations on day 25 after AI did not differ between treatment groups ($P = 0.54$). Mean P_4 concentrations were 5.15 ± 0.58 ng/mL for the aspirin group and 5.61 ± 0.45 ng/mL for the control group (Table 2). As expected, P_4 concentrations following treatment were greater ($P < 0.01$) for pregnant cows (8.97 ± 0.60 ng/mL) compared with non-pregnant cows (1.97 ± 0.58 ng/mL). There were no effects of parity ($P = 0.12$), TBRD ($P = 0.33$), nor any interactions between treatment and parity ($P = 0.81$), treatment and TBRD ($P = 0.73$), and treatment and pregnancy status ($P = 0.27$) on P_4 concentrations.

We observed no differences in PSPB concentrations on day 25 post-AI between treatment groups ($P = 0.63$). Mean PSPB concentrations were 0.52 ± 0.05 ng/mL for aspirin treated cows and 0.55 ± 0.04 ng/mL for control cows (Table 2). As expected, there was an effect of pregnancy status on PSPB concentrations ($P < 0.01$). Mean PSPB concentrations were greater for pregnant cows (0.97 ± 0.06 ng/mL) compared with non-pregnant cows (0.10 ± 0.03 ng/mL), however, there were no interactions with treatment by pregnancy status ($P = 0.39$). There was a tendency ($P = 0.07$) for cows inseminated for the 2nd or 3rd time to have greater PSPB concentrations (0.59 ± 0.04 ng/mL) compared with cows that were inseminated ≥ 4th time (0.48 ± 0.05 ng/mL). Nevertheless, there was no effect of treatment by TBRD interaction on PSPB concentrations ($P = 0.19$). Additionally, there was a tendency ($P = 0.07$) for multiparous cows to have greater PSPB concentrations on day 25 post-AI compared with primiparous cows (0.58 ± 0.04 ng/mL vs. 0.49 ± 0.05 ng/mL). To our knowledge this is the first study to report any differences in PSPB concentrations between

parities. Nevertheless, there was not an effect of treatment by parity on PSPB concentrations ($P = 0.47$).

Table 2.1 Least squares mean ± SEM for number of times bred before treatment, days in milk (DIM), parity, BCS, 305-day milk equivalent (305ME), and somatic cell count (SCC) in lactating Holstein cows treated with aspirin (187.2 g/cow total) or subjected to sham bolus administration (control) on day 14 and 15 following the second or greater insemination during the summer.

	Treatment	
Parameter	Aspirin (n = 277)	Control (n = 279)
Times bred[1]	3.4 ± 0.1	3.6 ± 0.1
DIM (d)	159 ± 5	161 ± 5
Parity	2.3 ± 0.1	2.3 ± 0.1
BCS[2,3]	2.73 ± 0.06	2.83 ± 0.06
305ME (kg)	13,945 ± 109	14,134 ± 109
SCC	245,491 ± 409	264,541 ± 408

[1] Number of times bred prior to treatment.
[2] BCS on scale of 1 to 5 in 0.25 increments (1 = emaciated; 5 = over conditioned).
[3] BCS on day 25 from subset of 133 aspirin-treated cows and 129 control cows.

Table 2.2 Least squares mean ± SEM of pregnancy specific protein B (PSPB) and progesterone (P_4) concentrations on day 25 post-AI for treatment groups[1], parities, number of times inseminated (TBRD) before experimental treatment, and pregnancy status in lactating Holstein cows.

	Treatment[1]		Parity		TBRD		Pregnancy Status[2]	
Hormone	Aspirin	Control	Primiparous	Multiparous	2 or 3	≥ 4	Non-Pregnant	Pregnant
PSPB (ng/mL)	0.52 ± 0.05	0.55 ± 0.04	0.49 ± 0.05[c]	0.59 ± 0.04[d]	0.59 ± 0.04[c]	0.48 ± 0.05[d]	0.10 ± 0.03[a]	0.97 ± 0.06[b]
P_4 (ng/mL)	5.15 ± 0.58	5.61 ± 0.45	4.94 ± 0.52	5.82 ± 0.40	5.08 ± 0.38	5.67 ± 0.56	1.97 ± 0.34[a]	8.79 ± 0.60[b]

[1] Treatments were either aspirin (186.2 g/cow total; n = 99) or sham bolus administration (control; n = 95) in lactating Holstein dairy cows on day 14 and 15 after the second or greater insemination during the summer.

[2] Status of pregnancy following treatments.

[a,b,] Means with different superscripts for each parameter within row differ ($P < 0.05$).

[c,d] Means with different superscripts for each parameter within row tend to differ ($P \leq 0.1$).

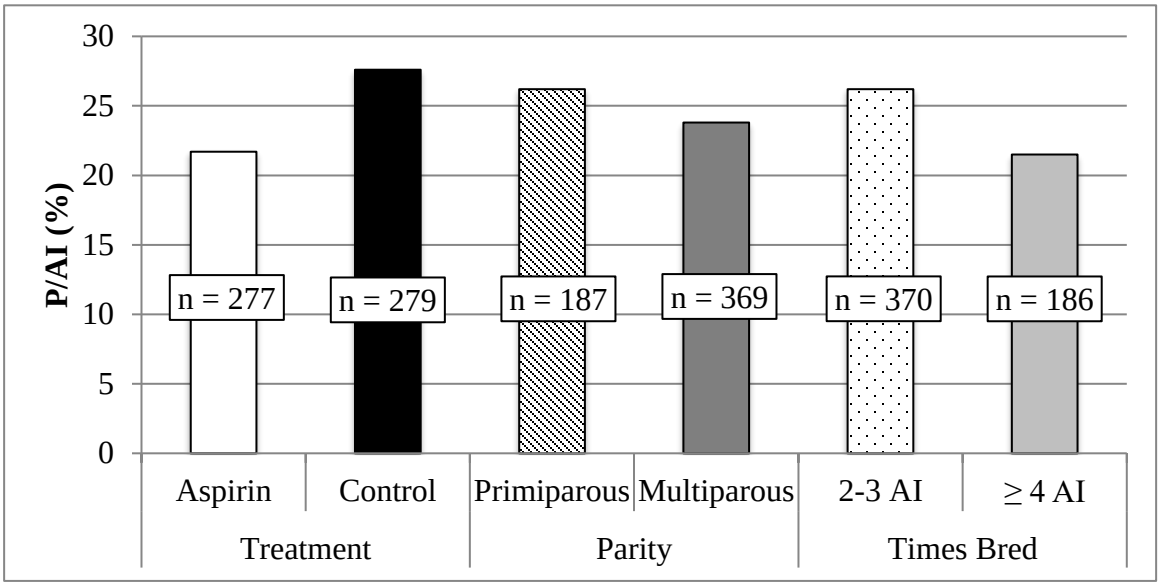

Figure 2.2 Pregnancy per AI (P/AI) for treatments (aspirin vs. control), parity (primiparous vs. multiparous), and number of inseminations (times bred) prior to treatment (2^{nd} or 3^{rd} AI vs. $\geq 4^{th}$ AI) in lactating Holstein cows.

DISCUSSION

The maximum ambient temperature on day 14 and day 15 ranged from 38 to 41°C, with an average relative humidity for both days of 46%. Using the previously described formula (NRC, 1971; Collier et al., 2011), the maximum THI on day 14 and 15 ranged from 86 to 88 indicating that the cows in our experiments were exposed to heat stress conditions. At a temperature humidity index greater than 68, dairy cows are heat stressed (Armstrong, 1994; Collier et al., 2011).

Our previous research showed that aspirin could significantly reduce PGFM concentrations for 30 hours after first aspirin administration (Spencer et al., 2016b). In addition, days to luteolysis (two consecutive days of P_4 < 1ng/mL) were 1.5 days longer for the aspirin-treated cows compared with the control cows. This delay in luteolysis may potentially allow more time for embryonic growth, hence embryonic signaling for maternal recognition (Spencer et al., 2016b). Based on these findings, we hypothesized that aspirin treatment may potentially influence pregnancy by reducing untimely secretion of $PGF_{2\alpha}$ and maintaining P_4 for a longer period of time. Thus, the current experiment was conducted to test the effect of aspirin on P/AI.

We observed no differences in P/AI between aspirin and control, and the P/AI were 21.6% for the aspirin-treated cows and 27.5% for the control cows. Although not significant, the P/AI for the aspirin-treated cows was 6-percentage points less than the control cows, and may indicate that aspirin has a detrimental effect on reproductive performance. However, in order to detect a 6-percentage point difference, approximately 400 cows per group would be required. Nevertheless, our results provide evidence that aspirin does not improve P/AI, and therefore would not be an effective reproductive management tool to reduce the risk of embryonic loss and improve P/AI.

In contrast to the current study, Sanchez-Rodriguez et al. (2011) observed a tendency for increased P/AI in beef cows administered low-dose aspirin during estrous synchronization and for 45 day following AI. It should be noted that the sample size in that study was limited (19 cows/treatment). In Brahman cows, aspirin treatment every 12 hours for (100 mg/kg BW) 13 days after calving reduced P/AI (Strahringer et al., 1999). Given the difference in timing and duration of aspirin administration between Strahringer et al. (1999), Sanchez-Rodriguez et al. (2011) and the current study, aspirin may have a broad impact on reproductive performance in cows. Perhaps the lack of differences in P/AI between aspirin and control cows in our study could be attributed to the fact that aspirin inhibits both COX-1 and 2 (Agrawal, 2010). Cyclooxygenase enzymes 1 and 2 are responsible for the synthesis of both $PGF_{2\alpha}$ and PGE_2. Conceivably, aspirin inhibited COX-1 and COX-2 enzymes, and although reducing the synthesis of $PGF_{2\alpha}$, aspirin may have also reduced the synthesis of PGE_2 a luteotrophic hormone important for establishment of pregnancy (Arosh et al., 2004). In fact, during maternal recognition of pregnancy (day 14 to 16), IFNτ causes the shift in preferential synthesis from $PGF_{2\alpha}$ to PGE_2 in the uterus (Asselin et al., 1997; Arosh et al., 2004). Therefore, aspirin treatment may have negated any potential benefits of inhibiting untimely secretion of $PGF_{2\alpha}$ by also inhibiting the synthesis of PGE_2 for establishment and maintenance of pregnancy.

There were also no differences in P/AI between cows inseminated for the 2^{nd} or 3^{rd} time compared with $\geq 4^{th}$ insemination. Additionally, there were no differences in P/AI between parities (23.8% multiparous vs. 26.2% primiparous). In contrast, Chebel et al. (2004) observed greater P/AI in primiparous cows compared with multiparous cows when cows were under heat stress. In fact, multiparous cows were 13% less likely to become

pregnant that primiparous when exposed to heat stress (Chebel et al., 2004). These authors postulated that the decreased P/AI in multiparous cows may have been due to the increased prevalence of postpartum diseases such as milk fever that are known to negatively influence fertility (Gröhn and Rajala-Schultz, 2000). Nevertheless, no effects were observed between treatment and parity, or treatment and TBRD on P/AI in the current study.

Progesterone concentrations on day 25 after AI did not differ between treatment groups, and averaged 5.15 ± 0.58 ng/mL for the aspirin-treated cows, and 5.61 ± 0.45 ng/mL for the control cows (Table 2). On day 25 after insemination (10 days after first treatment), pregnant cows had 4.5 times greater P_4 concentrations compared with non-pregnant cows (8.78 ± 0.60 ng/mL vs. 1.97 ± 0.34 ng/mL; Table 2). There were no differences between parities, or TBRD on P_4 concentrations 25 days after AI. Also, no interactions existed for treatment by parity, treatment by TBRD, or treatment by pregnancy status on P_4 concentrations. Our results are similar to previous studies in Brahman cows (Stahringer et al., 1999), beef heifers (Gramulia et al., 2015), and dairy cows (Spencer et al., 2016b), where it was shown that aspirin had no effect on P_4 concentrations following treatment. If aspirin decreased PGE_2 synthesis, we may have seen a decrease in P_4 concentrations for aspirin-treated cows as PGE_2 has been shown to stimulate P_4 synthesis in luteal cells (Shelton et al., 1990). On the other hand if aspirin inhibited $PGF_{2\alpha}$, P_4 concentrations could have been greater in aspirin-treated cows. As previously discussed, aspirin could have inhibited both COX-1 and COX-2 enzymes, thereby inhibiting synthesis and secretion of both PGE_2 and $PGF_{2\alpha}$, and not affecting P_4 concentrations.

It was hypothesized that if aspirin treatment reduced embryonic loss (by decreasing $PGF_{2\alpha}$ and maintaining P_4), then mean PSPB would be greater in aspirin-treated cows. We observed no difference in mean PSPB concentrations on day 25 post-AI between aspirin-treated and control cows (Table 2). As expected, PSPB concentrations were greater for pregnant cows compared to non-pregnant cows (0.96 ± 0.06 ng/mL vs. 0.10 ± 0.03 ng/mL) regardless of treatment (Table 2). There were no effects of treatment by pregnancy status interactions on day 25 PSPB concentrations. Surprisingly, there was a tendency for multiparous cows to have greater PSPB concentrations compared with primiparous cows (0.59 ± 0.04 ng/mL vs. 0.49 ± 0.05 ng/mL; Table 2). In addition, cows inseminated for the 2^{nd} or 3^{rd} time tended to have greater PSPB concentrations compared with cows inseminated

four or more times (0.59 ± 0.04 ng/mL vs. 0.48 ± 0.05 ng/mL; Table 2). There is no clear explanation as to why these differences exist in PSPB, however, it should be noted that the difference in PSPB for parity and TBRD were rather small (~0.1 ng/mL) and may not be physiologically significant.

We hypothesized that aspirin would reduce the occurrence of embryonic loss and improve P/AI. Our study however, did not show any difference in P/AI between cows receiving aspirin and cows receiving a sham bolus administration on day 14 and 15 after the second or greater insemination. We expected to observe differences in P_4 concentrations on day 25 after insemination, as we previously demonstrated that aspirin could reduce PGFM concentrations in lactating dairy cows during the luteal phase of the estrous cycle (Spencer et al., 2016b). Yet, the results of the current study do not support this theory. Although P_4 and PSPB have been used to measure embryonic loss (Gábor et al., 2007; Prvanović et al., 2009; Gábor et al., 2016), only one blood sample was collected in this study, making it difficult to assess the relationship between the changes in P_4 and PSPB concentrations and the occurrence of embryonic loss in this study.

This study provides evidence that high-dose, short-term aspirin administration in dairy cows on day 14 and 15 after AI does not improve P/AI in cows inseminated for the second or greater AI during the summer. Potentially, low-dose, long-term aspirin administration, or perhaps a more specific $PGF_{2\alpha}$ inhibitor may help to delay the secretion of $PGF_{2\alpha}$ and allow the embryo enough time to signal for maternal recognition. Further research on the use of practical reproductive management tools that can improve P/AI, reduce the occurrence of embryonic loss, and increase producer profitability are still imperative.

REFERENCES

Agrawal, P., and A. Gupta. 2010. NSAIDS. Retrieved from http://en.engormix.com/MA-dairy-cattle/dairy-industry/articles/nsaids-t1498/p0.htm.

Aiumlamai, S., K. Odensvik, G. Stabenfeldt, and H. Kindahl, H. 1990. Regulation of prostaglandin biosynthesis with flunixin meglumine in the bovine species. J. Vet. Med. 37(1):16-22.

Al-Janabi, A. S., A. M. A-lzohyri, and F. K. Al-Rubayai. 2005. Pharmacological effects of low-dose aspirin on corpus luteum functions in mature cycling female mice. Middle East Fertil. Soc. J. 10(2):150-162.

Al-Katanani, Y. M., D. W. Webb, and P. J. Hansen. 1999. Factors affecting seasonal variation in 90-day nonreturn rate to first service in lactating Holstein cows in a hot climate1. J. Dairy Sci. 82(12):2611-2616.

Amiridis, G. S., T. H. Tsiligianni, E. Dovolou, C. Rekkas, D. Vouzaras, and I. Menegatos. 2009. Combined administration of gonadotropin-releasing hormone, progesterone, and meloxicam is an effective treatment for the repeat-breeder cow. Theriogenology 72(4):542-548.

Anderson, K. L., C. A. Neff-Davis, L. E. Davis, and V. D. Bass. 1990. Pharmacokinetics of flunixin meglumine in lactating cattle after single and multiple intramuscular and intravaneous administrations. Am. J. Vet. Res. 51:1464-1467.

Anthony R. V., S. D. Helmer, S. F. Sharif, R. M. Roberts, P. J. Hansen, W. W. Thatcher, and F. W. Bazer. 1988. Synthesis and processing of ovine trophoblast protein-1 and bovine trophoblast protein-1, conceptus secretory proteins involved in the maternal recognition of pregnancy. Endocrinology 123:1224-1280.

Armstrong D. V. 1994. Heat stress interaction with shade and cooling. J. Dairy Sci. 77:2044-2050.

Arosh, J. A., S. K. Banu, S. Kimmins, P. Chapdelaine, L. A. Maclaren, and M. A. Fortier. 2004. Effect of interferon-τ on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E2. Endocrinology 145(11):5280-5293.

Arosh, J. A., S. K. Banu, and J. A. McCracken. 2016. Novel concepts on the role of prostaglandins on luteal maintenance and maternal recognition and establishment of pregnancy in ruminants1. J. Dairy Sci. 99(7):5926-5940.

Arosh, J. A., J. Parent, P. Chapdelaine, J. Sirois, and M. A. Fortier. 2002. Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle. Biol. Reprod. 67(1):161-169.

Asselin, E., F. W. Bazer, and M. A. Fortier. 1997. Recombinant ovine and bovine interferons tau regulate prostaglandin production and oxytocin response in cultured bovine endometrial cells. Biol. of Reprod. 56:402-408.

Badinga, L., R. J. Collier, W. W. Thatcher, and C. J. Wilcox. 1985. Effects of climatic and management factors on conception rate of dairy cattle in subtropical environment 1. J. Dairy Sci. 68(1):78-85.

Badinga, L., W. W. Thatcher, T. Diaz, M. Drost, and D. Wolfenson. 1993. Effect of environmental heat stress on follicular development and steroidogenesis in lactating Holstein cows. Theriogenology 39(4):797-810.

Baumgartner, A. P., and C. L. Chrisman. 1981. Cytogenetic analysis of ovulated oocytes following hyperthermic stress during meiotic maturation. Exp. Cell Res. 132:359-366.

Bazer F. W. 1992. Mediators of maternal recognition of pregnancy in mammals. Proc. Soc. Exp. Biol. Med. 199:373–84.

Beard, A. P., M. G. Hunter, and G. E. Lamming. 1994. Quantitative control of oxytocin-induced $PGF_{2\alpha}$ release by progesterone and oestradiol in ewes. J. Reprod. Fertil. 100:143–150.

Bilby, T. 2014. How Do I Determine?: How do I calculate Temperature-Humidity Index? Progressive Dairyman. https://www.progressivedairy.com/topics/herd-health/how-do-i-determine-how-do-i-calculate-temperature-humidity-index-thi.

Binelli, M., A. Guzeloglu, L. Badinga, D. R. Arnold, J. Sirois, T. R. Hansen, and W. W. Thatcher. 2000. Interferon-τ modulates phorbol ester-induced production of prostaglandin and expression of cyclooxygenase-2 and phospholipase-A2 from bovine endometrial cells. Biol. Reprod. 63(2):417-424.

Breuhaus, B. A., F. J. D. Graves, E. K. Honore, and M. G. Papich. 1999. Pharmacokinetics of ibuprofen after intravenous and oral administration and assessment of safety of administration to healthy foals. Am. J. Vet. Res. 60:1066-1073.

Butler, W. R., and R. D. Smith. 1989. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J. Dairy Sci. 72(3):767-783.

Campbell, N. B., and A. T. Blikslager. 2000. The role of cyclooxygenase inhibitors in repair of ischaemic-injured jejunal mucosa in the horse. Equine Vet. J. 32:59-64.

Charpigny, G., P. Reinaud, J. P. Tamby, C. Creminon, and M. Güillomot. 1997. Cyclooxygenase-2 unlike cyclooxygenase-1 is highly expressed in ovine embryos during the implantation period. Biol. Reprod. 57(5):1032-1040.

Chebel, R. C., J. E. P. Santos, J. P. Reynolds, R. L. A. Cerri, S. O. Juchem, M. Overton. 2004. Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. Anim. Reprod. Sci. 84:239-255.

Collier R. J., R. B. Zimbelman, R. P. Rhoads, M. L. Rhoads, and L. H. Baumgard. A re-evaluation of the impact of temperature humidity index (THI) and black globe humidity index (BGHI) on milk production in high producing dairy cows. Western Dairy Management Conf. March 9-11, 2011. Reno, NV 113-126.

Davies, N. M. and N. M. Skjodt. 1999. Clinical pharmacokinetics of meloxicam. Clin. Pharmacokinet. 36(2):115-126.

De Rensis F., and R. J. Scaramuzzi. 2003. Heat stress and seasonal effects on reproduction in the dairy cow – a review. Theriogenology 60:1139-1151.

De Rensis, F., P. Marconi, T. Capelli, F. Gatti, F. Facciolongo, S. Franzini, and R. J. Scaramuzzi. 2002. Fertility in postpartum dairy cows in winter or summer following estrus synchronization and fixed time AI after the induction of an LH surge with GnRH or hCG. Theriogenology 58(9):1675-1687.

Del Vecchio, R. P., W. D. Sutherland, and R. G. Sasser. 1996. Bovine luteal cell production in vitro of prostaglandin E2, oxytocin and progesterone in response to pregnancy-specific protein B and prostaglandin F2 alpha. J. Reprod. Fertil. 107:131-136.

Demmers, K. J., K. Derecka, and A. Flint. 2001. Trophoblast interferon and pregnancy. Reproduction 121(1):41-49.

Diskin M. G., and J. M. Sreenan. 1980. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J. Reprod. Fertil. 59:463-468.

Dunne, L. D., M. G. Diskin, and J. M. Sreenan. 2000. Embryo and fetal loss in beef heifers between day 14 of gestation and full term. Anim. Reprod. Sci. 58:39-44.

Ealy A. D., M. Drost, and P. J. Hansen. 1993. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. J. Dairy Sci. 76(10):2899-2905.

Elli, M., B. Gaffuri, A. Frigerio, M. Zanardelli, D. Covini, M. Candiani, and M. Vignali. 2001. Effect of a single dose of ibuprofen lysinate before embryo transfer on pregnancy rates in cows. J. Reprod. 121:151-154.

Emond, V., L. A. MacLaren, S. Kimmins, J. A. Arosh, M. A. Fortier, and R. D. Lambert. 2004. Expression of cyclooxygenase-2 and granulocyte-macrophage colony-stimulating factor in the endometrial epithelium of the cow is up-regulated during early pregnancy and in response to intrauterine infusions of interferon-τ. Biol. Reprod. 70(1):54-64.

Erdem, H., and A. Guzeloglu. 2010. Effect of meloxicam treatment during early pregnancy in Holstein heifers. Reprod. Domest. Anim. 45(4):625-628.

Farin, C. E., K. Imakawa, T. R. Hansen, J. J. McDonnell, C. N. Murphy, P. W. Farin, and R. M. Roberts. 1990. Expression of trophoblastic interferon genes in sheep and cattle. Bio. Repro. 43:210-218.

Gábor, G., J. P. Kastelic, Z. Abonyi-Tóth, P. Gábor, T. Endrődi, and O. G. Balogh. 2016. Pregnancy loss in dairy cattle: Relationship of ultrasound, blood pregnancy-specific protein B, progesterone and production variables. Reprod. Domest. Anim. 51(4):467-473.

Gábor, G., F. Tóth, L. Ozsvári, Z. Abonyi-Tóth, and R. G. Sasser. 2007. Early detection of pregnancy and embryonic loss in dairy cattle by ELISA tests. Reprod. Domest. Anim. 42(6):633-636.

Geisert, R. D., M. T. Zavy, and B. G. Biggers. 1988. Effect of heat stress on conceptus and uterine secretion in the bovine. Theriogenology 29(5):1075-1082.

Gingerich, D. A. 1975. Pharmacokinetics and dosage of aspirin in cattle. J. Anim. Vet. Med. Assoc. 167(10):945-948.

Giordano, J. O., J. N. Guenther, G. Lopes, and P. M. Fricke. 2012. Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein B, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. J. Dairy Sci. 95(2):683-697.

Gramulia, B. C., F. M. Walter, M. R. Crosswhite, D. N. Black, S.R. Underdahl, and C. R. Dahlen. 2015. Impact of aspirin feeding on ovulation, CL development and concentrations of progesterone in beef heifers. North Dakota Beef Report 49-51.

Gröhn, Y. T., and P. J. Rajala-Schultz. 2000. Epidemiology of reproductive performance in dairy cows. Anim. Reprod. Sci. 60-61, 605–614.

Güillomot, M., J. E. Flechon, and S. Wintenberger-Torres. 1981. Conceptus attachment in the ewe: an ultrastructural study. Placenta 2(2):69-182.

Guzeloglu, A., T. R. Bilby, A. Meikle, S. Kamimura, A. Kowalski, F. Michel, L. A. MacLaren, and W. W. Thatcher. 2004. Pregnancy and bovine somatotropin in nonlactating dairy cows: II. Endometrial gene expression related to maintenance of pregnancy. J. Dairy Sci. 87(10):3268-3279.

Guzeloglu, A., H. Erdem, M. K. Saribay, W. W. Thatcher, and T. Tekeli. 2007. Effect of the administration of Flunixin meglumine on pregnancy rates in Holstein heifers. Vet. Rec. 160:404-406.

Gwazdauskas, F. C., W. W. Thatcher, C. A. Kiddy, M. J. Paape, and C. J. Wilcox. 1981. Hormonal patterns during heat stress following PGF2α-tham salt induced luteal regression in heifers. Theriogenology 16(3):271-285.

Hansen, P. J., and C. F. Areéchiga. 1999. Strategies for managing reproduction in the heat-stressed dairy cow. J. Anim. Sci. 77(Suppl. 2):36-50.

Howell, J.L., J. W. Fuquay, and A. E. Smith, A.E., 1994. Corpus luteum growth and function in lactating Holstein cows during spring and summer. J. Dairy Sci. 77(3):735-739.

Humblot, P. 2001. Use of pregnancy specific proteins and progesterone assays to monitor the pregnancy, and determine the timing, frequencies and source of embryonic mortality in ruminant. Theriogenology 56:1417-1433.

Hyttel, P., H. Callesen, and T. Greve. 1986. Ultrastructural features of preovulatory oocyte maturation in superovulated cattle. J. Reprod. Fertil. 76:645-656.

Jerome, A., and N. Srivastava. 2012. Prostaglandins vis-à-vis bovine embryonic mortality: a review. Asian Pacific J. of Reprod. 1(3):238-246.

Kadzere, C. T., M. R. Murphy, N. Silanikove, and E. Maltz. 2002. Heat stress in lactating dairy cows: A review. Livestock Prod. Sci. 77, 59–91.

Kafi, M., S. Nazifi, R. Bagheri-Nejad, and M. Rahmani. 2006. The effects of ketoprofen on ovarian function in dairy cows. Comp. Clinic. Pathol. 15:70-75.

King, G. J., and W. W. Thatcher. 1993. Pregnancy. Reproduction in Domesticated Animals. Amsterdam, Elsevier 229-270.

Königsson, K., H. Gustafsson, and H. Kindahl. 2002. 15-ketodihydro-PGF2α, progesterone, and uterine involution in primiparous cows with induced retained placenta and post-partal endometritis treated with oxytetracycline and flunixin. Reprod. Domest. Anim. 37(1), pp.43-51.

Lamming, G. E., A. O. Darwash, and H. L. Back. 1989. Corpus luteum function in dairy cows and embryonic mortality. J. Reprod. Fertil. Suppl. 37:245-252.

Leibfried-Rutledge, M.L. 1999. Factors determining competence of in vitro produced cattle embryos. Theriogenology 51(2):473-485.

López-Gatius, F., P. Santolaria, J. L. Yániz, J. M. Garbayo, and R. H. F. Hunter. 2004. Timing of early foetal loss for single and twin pregnancy in dairy cattle. Reprod. Domest. Anim. 39(6):429-433.

Lucy M. C. 2001. Reproductive loss in high producing dairy cattle: where will it end? J. Dairy Sci. 86(6):1277-1293.

Malayer, J. R., P. J. Hansen, T. S. Gross, and W. W. Thatcher. 1990. Regulation of heat shock-induced alterations in the release of prostaglandins by the uterine endometrium of cows. Theriogenology 34(2):219-230.

Mann, G. E., and G. E. Lamming. 1995. Progesterone inhibition of the development of the luteolytic signal in cows. J. Reprod. Fertil. 104:1-5.

Mann, G. E., and G. E. Lamming. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction 121(1):175-180.

McCracken, J. A., E. E. Custer, and J. C. Lamsa. 1999. Luteolysis: A neuroendocrine-mediated event. Physiol. Rev. 79(2):263-323.

Merrill, M. L., R. P. Ansotegui, P. D. Burns, M. D. MacNeil, and T. W. Geary. 2007. Effects of flunixin meglumine and transportation on establishment of pregnancy in beef cows. J. Anim. Sci. 85:1547-1554.

Mirando, M. A., W. C. Becker, and S. S. Whiteaker. 1993. Relationships among endometrial oxytocin receptors, oxytocin-stimulated phosphoinositide hydrolysis and prostaglandin F2α secretion in vitro, and plasma concentrations of ovarian steroids before and during corpus luteum regression in cyclic heifers. Biol. Reprod. 48(4):874-882.

Nolan, A. M., B. A. Callingham, and R. J. Evans. 1990. Effects of aspirin on xylazine-induced hypoxaemia in sheep. Res. Vet. Sci. 48(3):386-388.

NRC. A Guide to Environmental Research on Animals. Natl. Acad. Sci. Washington, DC; 1971.

NRC. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington DC; 2001.

Odensvik, K., 1995. Pharmacokinetics of flunixin and its effect on prostaglandin F2α metabolite concentrations after oral and intravenous administration in heifers. J. Vet. Pharmacol. Ther. 18(4):254-259.

Odensvik, K. and I. M. Johansson. 1995. High-performance liquid chromatography method for determination of flunixin in bovine plasma and pharmacokinetics after single and repeated doses of the drug. Am. J. Vet. Res. 56(4):489-495.

Payne, M. 2001. Anti-inflammatory therapy in dairy cattle: therapeutic and regulatory considerations. California Veterinarian 55(2):10-12.

Pfeifer, L. F. M., N. Á. de Castro, P. de N. Duarte, L. Meneghello, and A. Schneider. 2016. Effect of flunixin meglumine on pregnancy rate of fixed-time inseminated dairy cows. PUBVET 10(2):168-172.

Pohler, K. G., M. H. C. Pereira, F. R. Lopes, J. C. Lawrence, D. H. Keisler, M. F. Smith, J. L. M. Vasconcelos, and J. A. Green. 2016. Circulating concentrations of bovine pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds. J. Dairy Sci. 99(2):1584-1594.

Purcell, S. H., W. E. Beal, and K. R. Gray. 2005. Effect of CIDR insert and Flunixin meglumine, administered at the time of embryo transfer, on pregnancy rate and resynchronization of estrus in beef cattle. Theriogenology 64:867-878.

Putney, D. J., S. Mullins, W. W. Thatcher, M. Drost, and T. S. Gross. 1989. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperatures between the onset of estrus and insemination. Anim. Reprod. Sci. 19:37-51.

Prvanović, N., A. Tomašković, J. Grizelj, and M. S. P. Kočila. 2009. Monitoring of early pregnancy and early embryonic mortality by ultrasound and determination of PAG and progesterone in cows. Veterinarski arhiv 79(3):259-267.

Rabaglino, M. B., C. A. Risco, M. J. Thatcher, F. Lima, J. E. P. Santos, and W. W. Thatcher. 2010. Use of a five-day progesterone-based timed AI protocol to determine if flunixin meglumine improves pregnancy per timed AI in dairy heifers. Theriogenology 73:1311-1318.

Rajkumar, R., S. K. Singh, S. K Agarwal, S. Mahmood, and U. Shankar. 2010. Effect of selective COX-2 inhibitor on conception rate, progesterone and PGFM profile in buffalo (Bubalus bubalis). J. Applied Anim. Res. 38(2):209-212.

Ray, D. E., T. J. Halbach, and D. V. Armstrong. 1992. Season and lactation number effects on milk production and reproduction of dairy cattle in Arizona1. J. Dairy Sci. 75(11):2976-2983.

Roberts, R. M., Y. Chen, T. Ezashi, and A. M. Walker. 2008. Interferons and the maternal-conceptus dialog in mammals. Sem. Cell Develop. Biol. 19:170-177.

Roberts, R. M., J. C. Cross, and D. W. Leaman. 1992. Interferons as hormones of pregnancy. Endocrin. Rev. 13(3):432-452.

Roth, Z., A. Arav, A. Bor, Y. Zeron, R. Braw-Tal, and D. Wolfenson. 2001b. Improvement of quality of oocytes collected in the autumn by enhanced removal of impaired follicles from previously heat-stressed cows. Reproduction 122(5):737-744.

Roth, Z., R. Meidan, R. Braw-Tal, and D. Wolfenson. 2000. Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. J. Reprod. Fertil. 120(1):83-90.

Roth, Z., R. Meidan, A. Shaham-Albalancy, R. Braw-Tal, and D. Wolfenson. 2001a. Delayed effect of heat stress on steroid production in medium-sized and preovulatory bovine follicles. Reproduction 121(5):745-751.

Rubinstein, M., A. Marazzi, and E. Polak de Fried. 1999. Low dose aspirin treatment improves ovarian responsiveness, uterine and ovarian responsiveness, uterine and ovarian blood flow velocity, implantation, and pregnancy rates in patients undergoing in vitro fertilization: a perspective, randomized, double-blind placebo controlled assay. Fertil. Steril. 71(5):825-829.

Ryan, D. P., and M. P. Boland. 1991. Frequency of twin births among Holstein-Friesian cows in a warm dry climate. Theriogenology 36(1):1-10.

Sanchez-Rodriguez, H. L., R. C. Vann, E. Baravik-Munsell, S. T. Willard and P. L. Ryan. 2011. Effects of acetylsalicylic acid on vasodilation or uterine arteries, right external iliac arterial blood flow, and pregnancy in beef cows. J. Anim. Sci. 89 (E-Suppl. 1):254 (Abstract).

SAS Institute. 2015. SAS 9.4, Version 2. SAS Institute Inc. Cary, NC.

Sasser, R. G., C. A. Ruder, K. A. Ivani, J. E. Butler, and W. C. Hamilton. 1986. Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. Biol. Reprod. 35(4):936-942.

Scenna, F. N., M. E. Hockett, T. M. Towns, A. M. Saxton, N. R. Rohrbach, M. E. Wehrman, and F. N. Schrick. 2005. Influence of a prostaglandin synthesis inhibitor administered at embryo transfer on pregnancy rates of recipient cows. Prostaglandins Other Lipid Mediat. 78:38-45.

Schrick, F. N., M. E. Hockett, T. M. Towns, A. M. Saxton, N. E. Wert, and M. E. Wehrman. 2001. Administration of a prostaglandin inhibitor immediately prior to embryo transfer improves pregnancy rates in cattle. Theriogenology 55(1):370.

Schrick F. N., E. K. Inskeep, and R. L. Butcher. 1993. Pregnancy rates for embryo transferred from early postpartum beef cows into recipients with normal estrous cycles. Biol. Reprod. 49:617-621.

Seals, R. C., J. W. Lemaster, F. M. Hopkins, and F. N. Schrick. 1998. Effects of elevated concentrations of prostaglandin $F_{2\alpha}$ on pregnancy rates in progestogen supplemented cattle. Prostaglandins Other Lipid Mediat. 56:377-389.

Senger, P. L. 2012. Pathways to Pregnancy and Parturition. (3rd Ed.). Redmond, OR: Current Conceptions, Inc.

Shelton, K., T. J. Parkinson, M. G. Hunter, R. W. Kelly, and G. E. Lamming. 1990. Prostaglandin E-2 as a potential luteotrophic agent during early pregnancy in cattle. J. Reprod. Fertil. 90(1):11-17.

Short, C. R., C. A. Neff-Davis, L. C. Hsieh, G. D. Koritz, M. S. Malbrough, S. A. Barker, and L. E. Davis. 1991. Pharmacokinetics & elimination of salicylic acid in rabbits. J. Vet. Pharmacol. Therio. 14(1):70-77.

Silke, V., M. G. Diskin, D. A. Kenny, M. P. Boland, P. Dillon, J. F. Mee, J. M. Sreenan. 2002. Extent, pattern and factors associated with late embryonic loss in dairy cows. Anim. Reprod. Sci. 71:1-12.

Skarzynski, D. J., and K. Okuda. 1999. Sensitivity of bovine corpora lutea to prostaglandin F2α is dependent on progesterone, oxytocin, and prostaglandins. Biol. Reprod. 60(6):1292-1298.

Spencer, T. E., and F. W. Bazer. 2004. Conceptus signals for establishment and maintenance of pregnancy. Reprod. Biol. Endocrin. 2(49):1-15.

Spencer, J. A., K. G. Carnahan, B. Shafii, S. Read, and A. Ahmadzadeh. 2016a. Effect of flunixin meglumine on prostaglandin metabolites and progesterone in lactating dairy cows. Clinic. Theriogenol. 8:41-48.

Spencer, J. A., K. Steinkamp, B. Shafii, and A. Ahmadzadeh. 2016b. The effect of aspirin on prostaglandin $F_{2\alpha}$ secretion in lactating dairy cows. Prof. Anim. Sci. 32:681-686.

Sreenan, J. M., M. G. Diskin, and D. G. Morris. 2001. Embryo survival in cattle: A major limitation to the achievement of high fertility. Fertility in the high producing dairy cow; proceedings of an international symposium, 26:93-104.

Stahringer, R. C., D. A. Neuendorff, R. D. Randel. 1999. The effect of aspirin administration and parity on plasma salicylate concentrations and postpartum reproductive parameters in Brahman cows. Prostaglandins Other Lipid Mediat. 58:125–138.

Stronge, A. J. H., J. M. Sreenan, M. G. Diskin, J. F. Mee, D. A. Kenny, and D. G. Morris. 2005. Post-insemination milk progesterone concentration and embryo survival in dairy cows. Theriogenology 64(5):1212-1224.

Thatcher, W. W., M. Binelli, J. Burke, C. R. Staples, J. D. Ambrose, and S. Coelho. 1997. Antiluteolytic signals between the conceptus and endometrium. Theriogenology 47:131-140.

Thatcher W. W., M. A. Driancourt, M. Terqui, L. Badinga. 1991. Dynamics of ovarian follicular development in cattle following hysterectomy and during early pregnancy. Domest. Anim. Endocrinol. 8:223-234.

Thatcher, W. W., A. Guzeloglu, R. Mattos, M. Binelli, T. R. Hansen, and J. K. Pru. 2001. Uterine-conceptus interactions and reproductive failure in cattle. Theriogenology 56:1435-1450.

Thatcher, W. W., M. D. Meyer, and G. Danet-Desnoyers. 1995. Maternal recognition of pregnancy. J. Reprod. Fertil. Suppl. 49:15-28.

Thatcher, W. W., C. R. Staples, G. Danet-Desnoyers, B. Oldick, and E. P. Schmitt. 1994. Embryo health and mortality in sheep and cattle. J. Anim. Sci. 72:16-30.

Thompson, J. A., D. D. Magee, M. A. Tomaszewski, D. L. Wilks, and R. H. Fourdraine. 1996. Management of summer infertility in Texas Holstein dairy cattle. Theriogenology 46(3):547-558.

Vane, J. R. 1971. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature (London) New Biol. 231:232-235.

von Krueger, X., and W. Heuwieser. 2010. Effect of flunixin meglumine and carprofen on pregnancy rates in dairy cattle. J. Dairy Sci. 93:5140-5146.

Wilson, S. J., C. J. Kirby, A. T. Koenigsfeld, D. H. Keisler, and M. C. Lucy. 1998a. Effects of controlled heat stress on ovarian function of dairy cattle. 2. Heifers. J. Dairy Sci. 81(8):2132-2138.

Wilson, S. J., R. S. Marion, J. N. Spain, D. E. Spiers, D. H. Keisler, and M. C. Lucy. 1998b. Effects of controlled heat stress on ovarian function of dairy cattle. 1. Lactating cows. J. Dairy Sci. 81(8):2124-2131.

Whitlock, B. K., and H. S. Maxwell. 2008. Pregnancy-associated glycoproteins and pregnancy wastage in cattle. Theriogenology 70(3):550-559.

Wolfenson, D., W. W. Thatcher, L. Badinga, J. D. Savio, R. Meidan, B. J. Lew, R. Braw-Tal, and A. Berman. 1995. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. Biol. Reprod. 52:1106-1113.

Xiao, C. W., B. D. Murphy, J. Sirois, and A. K. Goff. 1999. Down-regulation of oxytocin-induced cyclooxygenase-2 and prostaglandin F synthase expression by interferon-τ in bovine endometrial cells. Biol. Reprod. 60(3):656-663.

Younas, M., J. W. Fuquay, A. E. Smith, and A. B. Moore. 1993. Estrous and endocrine responses of lactating Holsteins to forced ventilation during summer. J. Dairy Sci. 76(2):430-436.

CHAPTER THREE

"Defining progesterone profiles and the occurrence of luteolysis after one or two prostaglandin $F_{2\alpha}$ injections in a 5-day CIDR-Cosynch protocol for lactating dairy cows and suckling beef cows"

ABSTRACT

The objective was to examine the effects of one or two doses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, dinoprost tromethamine), or one dose of high-concentration $PGF_{2\alpha}$ (HighCon) on progesterone (P_4) concentration profiles and luteolysis in lactating dairy cows (Experiment 1) and suckling beef cows (Experiment 2) subjected to a 5-day CIDR-Cosynch protocol. On day 0, 67 lactating Holstein dairy cows and 54 suckling Charolais beef cows received GnRH and a CIDR was inserted. On day 5, the CIDR was removed and cows were assigned randomly to receive one dose (5 mL) $PGF_{2\alpha}$ (1PG; 5 mg/mL; Exp. 1 n = 24; Exp. 2 n = 19), two doses (5 mL/dose) $PGF_{2\alpha}$ 12 hours apart (2PG; 5 mg/mL; Exp. 1 n = 22; Exp. 2 n =17), or one dose (2 mL) HighCon $PGF_{2\alpha}$ (12.5 mg/mL; Exp. 1 n = 21; Exp. 2 n = 17). One hour after CIDR removal and before treatment, a blood sample was collected to measure baseline P_4 concentrations (day 5). After treatments, blood samples were collected every 12 hours from days 5 to 8 to measure P_4. On day 8, all cows received a second GnRH and were inseminated. Ovaries were examined by ultrasonography on day 0, 5, and 8. A repeated measures analysis of variance assuming a lognormal distribution was used to assess treatment differences in P_4 concentrations. The model included terms for treatment, the repeated factor (time), and time by treatment interaction. Cow within treatment was the random effect within the model and day 5 P_4 concentrations were used as a covariate. All cows in both experiments had elevated P_4 concentrations and corpora lutea prior to treatment (day 5). For both experiments, blood P_4 concentrations decreased over time in all treatments ($P < 0.01$). In experiment 1, there was an effect of treatment by time interaction on P_4 concentrations ($P = 0.05$). By 24 hours after treatments, P_4 concentrations were lower, and remained at a lower concentration ($P < 0.01$) throughout the experiment for 2PG and

HighCon compared with 1PG. By 72 hours post-treatment, the average P_4 concentrations were 1.02, 0.05, and 0.22 ng/mL for 1PG, 2PG, and HighCon, respectively. The proportion of cows that went through luteolysis ($P_4 \leq 0.5$ ng/mL) were greater ($P < 0.01$) in 2PG and HighCon compared to 1PG. The results of experiment 1 provides evidence that 1PG was not as effective as 2PG and HighCon in causing luteolysis, and that one injection of HighCon was as effective as 2PG in decreasing blood P_4 and causing luteolysis by the time of AI. As expected, P_4 concentrations decreased over time in all treatments ($P < 0.01$). There were no significant treatment effects on P_4 ($P > 0.1$) in experiment 2, while there was a significant treatment by time interaction ($P = 0.02$). Serum P_4 concentrations at 72 hours after treatment tended ($P = 0.09$) to differ between treatments and averaged 0.50, 0.18, and 0.25 ng/mL for 1PG, 2PG, and HighCon, respectively. The proportion of cows that went through luteolysis tended to be greater ($P = 0.09$) in 2PG and HighCon compared to 1PG (89, 88, and 63%, respectively). In experiment 2, 1PG, 2PG, and HighCon were all effective in causing luteolysis by the time of AI in suckling beef cows.

Keywords: prostaglandin dose, progesterone, luteolysis, dairy cow, beef cow

INTRODUCTION

An effective systematic breeding program is a critical segment of an integrated management program in an efficient dairy and beef cow production system. Reproductive efficiency represents the future profitability and genetic improvement of the herd. The main objective is to manage cows during the early post-partum, identify eligible cows, and subject them to AI in an efficient and timely manner. Fixed-time AI (TAI) breeding protocols, such as Ovsynch or Ovsynch + controlled internal drug release (CIDR) insert, and their modified versions, are attractive reproductive management tools that enhance AI submission rate. However, AI pregnancy rates (P/AI) achieved through these programs vary significantly in dairy and beef. For example, P/AI for first insemination have been reported to range between 30 to 34% for dairy cattle (Norman et al., 2009), and between 60 to 70% in beef cattle (Johnson and Jones, 2004). Factors that may compromise reproductive performance in a breeding program include inadequate estrous detection, poor conception rates, delayed

ovulation, and improper timing of insemination (Hermas et al., 1987; Nebel and Jobst, 1998; Lucy, 2001; Washburn et al., 2002; de Vries and Risco, 2005). Inconsistent results and the above mentioned concerns provide opportunity for improvement in reproduction through the use of TAI breeding programs.

REVIEW OF LITERATURE

Synchronization protocols have become useful management tools to improve reproductive performance, decrease number of days open (DO), reduce calving season length, facilitate the use of AI, aid in genetic advancement, and improve producer profitability.

The purpose of synchronization protocols are designed to mimic the physiological process that occur during the bovine estrous cycle by using exogenous hormones such as gonadotropin-releasing hormone (GnRH), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and progesterone (P_4). Common synchronization protocols are Ovsynch (Pursley et al., 1997; Bisinotto et al., 2010; Figure 3.1) and Cosynch (Figure 3.2). A Presynch protocol (two $PGF_{2\alpha}$ injections 14 days apart) 10 to 14 days before the initiation of Ovsynch and Cosynch may also be used. Presynch protocols are often used to optimize the stage in the estrous cycle at which a TAI protocol is initiated, and to maximize the pregnancy outcomes of TAI protocols (Vasconcelos et al., 1999; Colazo et al., 2004; Bello et al., 2006; Galvão et al., 2007; Giordano et al., 2013; Wiltbank and Pursley, 2014). The addition of CIDR inserts between the initial GnRH and $PGF_{2\alpha}$ improve synchronization and P/AI by preventing premature estrus and ovulation before $PGF_{2\alpha}$ (Xu and Burton, 2000; Larson et al., 2006). Various synchronization protocols have been developed for use in cattle and ultimately provide producers with a systematic breeding program to maximize fertility and reproductive performance in their cows.

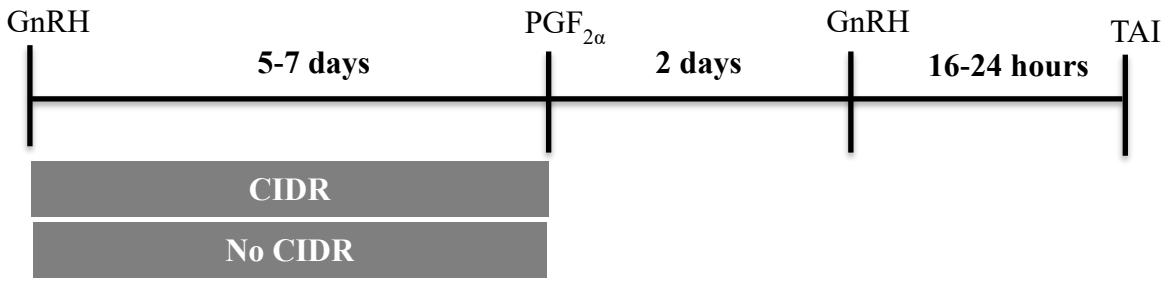

Figure 3.1 Ovsynch or Ovsynch with controlled internal drug release (CIDR) insert for systematic breeding. Gonadotropin-releasing hormone (GnRH) initially administered to cause ovulation of a dominant follicle if present. A CIDR insert is inserted vaginally to increase progesterone (P_4) concentrations for 5 to 7 days (only for Ovsynch+CIDR) between initial GnRH and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration. On day 5 or 7 CIDR inserts are removed and one or two $PGF_{2\alpha}$ injections are given to regress any corpus luteum (CL) that may have developed due to the initial GnRH-induced ovulation or regress a previously existing CL present at CIDR insertion. The second GnRH is administered 2 to 3 days later to cause ovulation and allow for timed artificial insemination (TAI) 16 to 24 hours after second GnRH.

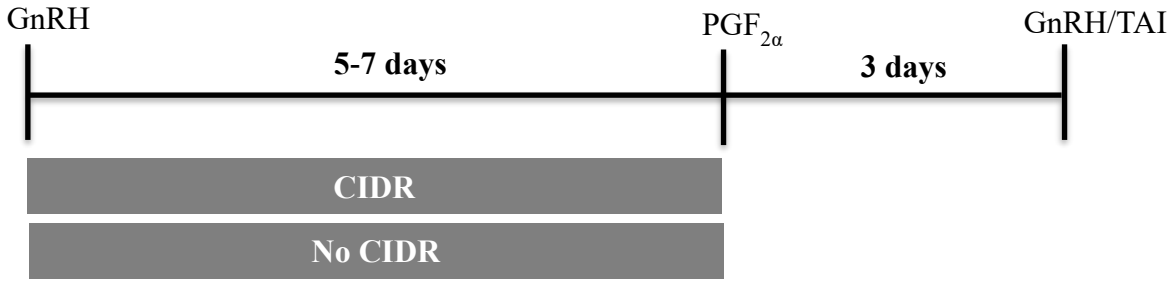

Figure 3.2 Cosynch or Cosynch with controlled internal drug release (CIDR) inserts for systematic breeding. Gonadotropin-releasing hormone (GnRH) is initially administered to cause ovulation of a dominant follicle if present. A CIDR insert is inserted vaginally to increase progesterone (P_4) concentrations for 5 to 7 days (only for Cosynch+CIDR) between initial GnRH and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration. On day 5 or 7 CIDR inserts are removed and one or two $PGF_{2\alpha}$ injections are given to regress any corpus luteum (CL) that may have developed due to the initial GnRH-induced ovulation or regress a previously existing CL present at CIDR insertion. The second GnRH is administered 3 days later and simultaneously with timed artificial insemination (TAI) to minimize animal handling.

ESTROUS AND (OR) OVULATION SYNCHRONIZATION HORMONES

Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone is synthesized and secreted from the hypothalamus and causes the release of two gonadotropins; luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary (Senger, 2012). Gonadotropins are

important for follicular growth, development and maturation, as well as synthesis and secretion of steroid hormones such as estradiol (E_2) and P_4 in the ovaries.

Gonadotropin-releasing hormone is commonly used at the initiation of TAI synchronization protocols to cause a surge of LH, which results in ovulation or luteinization of a dominant follicle, subsequently stimulating another follicular wave. If adominant follicle is not present at the time of GnRH administration, ovulation will not occur and may cause lutenization of a non-dominant follicle. Ovulation of a dominant follicle in response to GnRH relies on an increase in FSH for follicular growth, the acquisition of LH-dependency by the follicle, and the production of inhibin (Ginther et al., 1996). Gonadotropin-releasing hormone is also used at the end of TAI synchronization protocols to induce ovulation before or at the time of AI. Some examples of GnRH products include Factrel® (100 μg/dose; Zoetis Inc.), Cystorelin® (100 μg/dose; Merial), and Fertagyl® (86 μg/dose; Merck Animal Health).

Progesterone

Progesterone is synthesized and secreted by small and large luteal cells of the corpus luteum (CL) during the luteal phase of the estrous cycle. Prior to luteolysis, P_4 is required to prime the uterus for embryonic development. However, if P_4 is administered prior to the LH surge, ovulation can be prevented until P_4 concentrations decrease (Inskeep et al., 1973).

Controlled internal drug releasing devices are commonly used in synchronization programs. These intravaginal inserts release P_4 (1.38 mg) into the vaginal mucous, which aids in the absorption of P_4 into vaginal tissues and circulation. The amount of P_4 released into circulation is dependent on the surface area of the CIDR that comes in contact with the vaginal mucous (Rathbone et al., 2002). Progesterone from CIDR inserts act to suppress estrus, making it a useful tool for estrous synchronization. Specifically, CIDR inserts maintain high plasma P_4 concentrations, which cause a negative feedback on the hypothalamus preventing an LH surge and subsequent ovulation (Inskeep et al., 1973; Savio et al., 1993). Upon removal of CIDR inserts, there is a rapid decline in systemic P_4 concentrations. In the absence of CL, this rapid decline in P_4 allows for an increase in the pulsatile secretion of FSH and LH for follicular growth increased concentrations of E_2 and subsequent ovulation. If a CL, after CIDR removal is present, exogenous $PGF_{2\alpha}$ is required

to regress the existing CL and reduce P_4 for subsequent follicular growth estrus, and ovulation.

Progesterone Supplementation

The addition of a CIDR during Ovsynch or Cosynch following an initial GnRH injection helps to increase circulating P_4 and prevents ovulation of any follicle that did not ovulate to the GnRH. In fact, a 5 to 10% increase in P/AI have been observed when CIDR inserts were incorporated into TAI protocols (Chebel et al., 2006; Lamb et al., 2010). While some studies have shown an enhancement in P/AI (Xu et al., 1997; Lamb et al., 2001; Stevenson et al., 2003b; Chebel et al., 2006; Larson et al., 2006; Lamb et al., 2010; Chebel et al., 2010; Dewey et al., 2010; Rabaglino et al., 2010; Santos et al., 2010; Bilby et al., 2013; Bisinotto et al., 2013, 2015a, 2015b), others have shown no improvement with the inclusion of a CIDR insert (Chenault et al., 2003; El-Zarkouny et al., 2004; Galvão et al., 2007; Cerri et al., 2009a; Lima et al., 2009). Previous researchers suggested that any reduction in fertility associated with the use of CIDR inserts may be attributable to the development of a persistent dominant follicle and ovulation of an aged oocyte, thus decreasing embryo quality and embryo development (Savio et al., 1993; Cerri et al., 2004 & 2009b). Nevertheless, research has indicated no difference or an improvement in P/AI with the addition of a CIDR during Ovsynch or Cosynch protocols.

Progesterone supplementation ultimately prevents ovulation in the small percentage of cows that do not respond to the initial GnRH in an Ovsynch protocol, and also inhibits premature luteolysis of a CL (Vasconcelos et al., 1999; Xu and Burton, 2000; Lima et al., 2009). Progesterone supplementation may also inhibit the release of gonadotropins (LH/FSH) and follicle turnover or ovulation (Taylor and Rajamahendran, 1991). Using lactating dairy cows, Cerri et al. (2009a) showed that incorporation of a CIDR into the Ovsynch protocol for first AI helped induce resumption of cyclicity. A study in dairy heifers examining the effects of a 5-day Cosynch-TAI resynchronization protocol with and without a CIDR insert showed a 12.5% increase in P/AI when heifers received a CIDR for 5 days between the initial GnRH and $PGF_{2\alpha}$ (Rabaglino et al., 2010). Overall, it appears that the CIDR may prompt cyclicity in lactating cows, anovular cows, and heifers, as well as tighten the synchrony of estrus in non-pregnant lactating cows (Chenault et al., 2003; Cerri et al., 2009a; Rabaglino et al., 2010; Green et al., 2011).

Prostaglandin $F_{2\alpha}$

Prostaglandin $F_{2\alpha}$ is a lipid hormone that is synthesized and secreted by the uterine endothelium and causes the regression of the CL, therefore decreasing P_4 synthesis and secretion (Senger, 2012). In the absence of a conceptus, the endometrium secretes pulses of $PGF_{2\alpha}$, which stimulates oxytocin (OXY) from the hypothalamus and granules in the CL secretion in a similar pulsatile manner to initiate the process of CL regression known as luteolysis. Estrogen upregulates oxytocin receptor (OTR) in the uterus, thus increasing uterine responsiveness to OXY from the CL (Inskeep, 2004). This event stimulates further release of $PGF_{2\alpha}$ and completes luteolysis (Silvia et al., 1991). Definitively, luteolysis is the event by which $PGF_{2\alpha}$ downregulates LH receptors on luteal cells and causes necrosis and apoptosis of luteal tissue leading to a decrease in synthesis and secretion of P_4 (Senger, 2012). Exogenous administration of $PGF_{2\alpha}$ similarly causes luteolysis and a decline in systemic P_4 concentrations, promoting estrus and providing an adequate uterine environment for AI. However, administration of $PGF_{2\alpha}$ more effectively induces luteolysis if cows are in the diestrus phase between days 7 and 17 of the estrous cycle (Stevenson et al., 1989). Two common sources of $PGF_{2\alpha}$ that are commercially available in the U.S. are Estrumate® (Merck Animal Health, 500 μg cloprostenol sodium/dose) a synthetic analogue of $PGF_{2\alpha}$, and Lutalyse® (Zoetis, 25 mg dinoprost tromethamine/dose) an analogue of the naturally occurring $PGF_{2\alpha}$ compound. Both products have been found to be effective luteolytic products and shown to produce similar P/AI when used in 5-day CIDR-Cosynch protocol (Kasimanickam et al., 2009).

Recently, Zoetis investigated the luteolytic effect of a high-concentration $PGF_{2\alpha}$ analogue, Lutalyse HighCon® (Zoetis, 25 mg dinoprost tromethamine/dose), which is currently approved for either s.c. and i.m. administration in cattle. Following administration, Lutalyse Highcon reaches a higher maximum plasma concentration than conventional Lutalyse®, which may allow time for the CL to become responsive and undergo complete luteolysis by the time of AI. Lutalyse HighCon contains the same $PGF_{2\alpha}$ compound as the conventional Lutalyse product; however, Lutalyse HighCon is more concentrated (12.5 mg/mL dinoprost tromethamine) compared with conventional Lutalyse (5 mg/mL dinoprost tromethamine). Although current research does not show any difference in the biological half-life between conventional Lutalyse and Lutalyse HighCon, it is possible that Lutalyse

HighCon is stored and mobilized at a slower rate when compared to conventional Lutalyse. However, Bovaline (250 mcg/mL, cloprostenol), another prostaglandin product was shown to have a longer biological activity as it remained stored in lipid molecules (A. Tibary, personal communication, June 20, 2018). Therefore, it is possible that the more concentrated Lutalyse HighCon may have an extended period of biological activity by way of storage in lipid molecules, similar to Bovaline.

In addition, the cost of one Lutalyse HighCon dose is $2.48 to $2.56, whereas one conventional Lutalyse injection is more expensive and ranges from $2.89 to $3.42 (Zoetis, 2018). Nevertheless, information about the effectiveness of Lutalyse HighCon on improving or maintaining P/AI in a synchronization protocol is limited. To our knowledge, there are only two studies that have investigated the effects of Lutalyse HighCon in a synchronization protocol. Both of these studies were conducted in beef heifers and showed no difference in P/AI between Lutalyse HighCon and conventional Lutalyse (Oosthuizen et al., 2017; Lansford et al., 2018). It should be noted that in these studies, Lutalyse HighCon was administered s.c., whereas Lutalyse was administered i.m., and may have confounded the observed effects.

Given the reduced costs and luteolytic capability, it appears that Lutalyse Highcon may be a reasonable alternative for conventional Lutalyse. Nevertheless, the effects of Lutalyse HighCon on P/AI for lactating dairy cows and suckling beef cows requires further investigation.

TIMED-AI SYNCHRONIZATION PROTOCOLS

The length of proestrus and follicular dominance can be controlled through manipulation of the duration of the synchronization protocol and optimization of the interval between induction of ovulation and AI (Pursley et al., 1998; Bridges et al., 2008; Chebel et al., 2009). As such, synchronization protocols have been developed to cause ovulation using GnRH injections, with $PGF_{2\alpha}$ treatment to cause luteolysis and reduce P_4 concentrations by the time of AI. It is thought that the success of GnRH-based TAI protocols pivot, in part, around manipulation of the physiological processes involved in ovulation and/or lutienization of a dominant follicle as well as the recruitment of a new follicular waves with the administration of GnRH. However, a study by Stevenson (2016) demonstrated that there are three other main factors that may play important roles in the success of TAI

synchronization protocols. Stevenson (2016) argues that these three factors can explain 78% of the variation in pregnancy risk observed in TAI protocols: 1) ovulation to the second GnRH injection (near the time of AI), 2) low P_4 concentrations within 48 hours after $PGF_{2\alpha}$ administration, and 3) high P_4 concentrations before $PGF_{2\alpha}$ injection. In addition, the duration of follicular dominance, under the influence of P_4, especially in CIDR-based protocols may also affect the success of TAI protocols. For example, there is evidence that in the CIDR-Coysnch protocol, shortening the interval between initial GnRH and $PGF_{2\alpha}$ injections, and extending the time between the $PGF_{2\alpha}$ and until the time of the second GnRH and AI can optimize the length of proestrus, allow time for a follicle to reach dominance, and improve ovulation response to the second GnRH (Bridges et al., 2008; Lopes et al., 2013; Stevenson, 2016).

5-day vs. 7-day CIDR

The use of Cosynch, particularly the 5 and 7-day protocols, have allowed for tight synchronization of estrus, the implementation of TAI, and reduced animal handling when compared to the Ovsynch protocol (Geary and Whittier, 1998). The addition of CIDR inserts to synchronization protocols act to tighten estrous synchrony by preventing premature ovulation (Savio et al., 1993; Galvão et al., 2004; Stevenson et al., 2008; Bisinotto et al., 2010), and/or luteolysis of a CL between the initial GnRH and $PGF_{2\alpha}$ 5 to 7 days later (Vasconcelos et al., 1999; Xu and Burton, 2000; Lima et al., 2009).

Researchers have demonstrated that P/AI is improved or remains similar if the duration of CIDR treatment in a CIDR-Cosynch protocol is shortened from 7 to 5 days, and the interval from CIDR removal and $PGF_{2\alpha}$ to the second GnRH is extended to 72 hours (Bridges et al., 2008; Kasimanickam et al., 2009; Lopes et al., 2013; Santos et al., 2010; Whittier et al., 2010; Wilson et al., 2010; Ahmadzadeh et al., 2015). Specifically, by shortening CIDR treatment from 7 to 5 days the incidence of prolonged follicular dominance, which results in the development and ovulation of an aged and potentially inviable oocyte, is reduced (Bridges et al., 2008; Lopes et al., 2013; Santos et al., 2010). Ovulation of an aged oocyte reduces P/AI, and decreases embryo quality (Cerri et al., 2004; 2009b). For these reasons, reducing the duration of CIDR treatment from 7 to 5 days appears to improve reproductive performance. Nevertheless, there is a limitation when the CIDR treatment is reduced from 7 to 5 days.

It is well documented that in a 5-day protocol, two $PGF_{2\alpha}$ injections (on day 5), either 7 to 24 hours apart, are required to cause complete luteolysis in beef cows (Bridges et al., 2008; Kasimanickan et al., 2009; Whittier et al., 2010; Bridges et al., 2015), dairy cows (Archbald et al., 1993; Repasi et al., 2005; Santos et al., 2010; Ribeiro et al., 2012), beef heifers (Peterson et al., 2011), and dairy heifers (Lima et al., 2013). These researchers argued that in this protocol, one injection of $PGF_{2\alpha}$ on d 5 of the protocol is not sufficient to reliably induce luteal regression by the time of AI. In other words, the success of a 5-day CIDR-Cosynch protocol in term of pregnancy relies on the ability of exogenous $PGF_{2\alpha}$ to successfully regress a GnRH-induced, newly formed CL, and reduce P_4 to an optimal concentrations (P_4 < 0.5 ng/mL) by the time of AI (Santos et al., 2010; Wiltbank et al., 2014; Stevenson, 2016). If complete luteolysis does not occur by the time of AI, fertility and pregnancy outcomes may be compromised. Incomplete luteolysis may be due to the refractoriness of a newly developed CL (< 5 days) that is not able to respond to a single $PGF_{2\alpha}$ injection with complete luteolysis (Tsai and Wiltbank, 1998; Diaz et al., 2002) in a 5-day CIDR-Cosynch protocol, thus compromising pregnancy outcomes. Miyamoto et al. (2009) identified the discrepancies in cellular mechanisms within an early CL, which is unresponsive to $PGF_{2\alpha}$, and a mid-cycle CL, which is sensitive to $PGF_{2\alpha}$. It was determined that the CL of cattle are refractory to $PGF_{2\alpha}$ especially during the first 5 days of the estrous cycle (Tsai and Wiltbank, 1998; Miyamoto et al., 2009). As a result, it has been concluded that a single dose of $PGF_{2\alpha}$ may be not sufficient to induce complete luteolysis in an immature CL. Therefore, two $PGF_{2\alpha}$ injections (7 to 24 hours apart) are needed to reliably reduce P_4 concentrations and cause complete luteolysis before the time of AI (Bridges et al., 2008; Kasimanickam et al., 2009; Santos et al., 2010; Whittier et al., 2010; Peterson et al., 2011; Ribeiro et al., 2012).

One vs. Two Prostaglandin $F_{2\alpha}$ in a 5-day CIDR-Cosynch

If ovulation to the initial GnRH occurs, a new CL, which will be only 5 days old and unable to fully respond to one $PGF_{2\alpha}$ injection with complete luteolysis. This incomplete luteolysis results in a suboptimal P_4 concentration (at the time AI) and may contribute to lower fertility. Thus, it has been postulated that the apparent improved P/AI in a 5-day CIDR protocol is attributed to the effectiveness of a double $PGF_{2\alpha}$ injection scheme causing complete luteolysis and optimal P_4 concentrations at the time of AI.

Santos et al. (2010) argues that the second $PGF_{2\alpha}$ dose in the 5-day protocol may be particularly critical in cows that are presynchronized before enrolling in the timed AI protocol because of an increased incidence of ovulation to the first GnRH and occurrence of a newly formed CL. Interestingly, none of the above mentioned studies that were conducted in beef cattle (Bridges et al, 2008, Kasimanickam et al., 2009, Peterson et al., 2011) measured blood P_4 concentrations, however P/AI were greater in cows receiving two doses of $PGF_{2\alpha}$ in these studies In dairy cows, the use of a 5-day CIDR-Cosynch protocol produced optimal P_4 concentrations (defined by these researchers as $P_4 < 1$ ng/mL) at the time of AI, for cows that received two injections of $PGF_{2\alpha}$ 24 hours apart than those that received only one $PGF_{2\alpha}$ injection at CIDR removal (Santos et al., 2010). In contrast, Ribeiro et al. (2012) demonstrated that dairy cows (not presynchronized) subjected to a 5-day CIDR Cosynch protocol had lower P_4 concentrations at TAI when administered two injections of $PGF_{2\alpha}$ (24 hours apart), though P/AI was not different between cows that received one or two $PGF_{2\alpha}$ injections.

As for dairy heifers, the results have been more variable. For example, Lima et al. (2013) suggested that, in a 5-day CIDR-Cosynch protocol, two $PGF_{2\alpha}$ injections 24 hours apart optimizes luteolysis and P/AI. However, Rabaglino et al. (2010) showed no difference in P/AI for heifers using either one or two $PGF_{2\alpha}$ 12 hours apart in a 5-day CIDR-Cosynch. Nevertheless, the direct effects of one or two $PGF_{2\alpha}$ on P_4 profiles and luteolysis have not been examined in beef or dairy cows subjected to a 5-day CIDR-Cosynch estrous synchronization protocol.

RATIONALE

Synchronization protocols that result in greater synchronized ovulation and P/AI will ultimately facilitate the use of AI, and may increase its adoption among beef and dairy producers. The use of Cosynch protocols has allowed for synchronization of ovulation and TAI, as well as reduced number of handlings when compared to Ovsynch (Geary and Whittier, 1998). In addition, CIDR inserts between the initial GnRH and $PGF_{2\alpha}$ improve synchronization and P/AI by preventing premature estrus and ovulation before the $PGF_{2\alpha}$ injection (Vasconcelos et al., 1999; Xu and Burton, 2000; Larson et al., 2006). Nevertheless,

current literature alludes to the necessity of a second $PGF_{2\alpha}$ injection in a 5-day CIDR-Cosynch protocol (Archbald et al., 1993; Repasi et al., 2005; Bridges et al., 2008; Kasimanickan et al., 2009; Santos et al., 2010; Whittier et al., 2010; Ribeiro et al., 2012; Bridges et al., 2015). The investigation of the use of one or two doses of $PGF_{2\alpha}$ in the 5-day CIDR-Cosynch protocol was developed to gain further knowledge regarding luteolysis and P_4 concentration in beef and dairy cows; however, the potential benefits associated with P/AI should be weighed against the additional cattle handling and expenses associated with a second administration of $PGF_{2\alpha}$.

HYPOTHESIS

One Lutalyse HighCon (dinoprost tromethamine; 12.5 mg/mL) dose, a high concentration of conventional Lutalyse (dinoprost tromethamine; 5 mg/mL), is as effective as one or two conventional Lutalyse injections (12 hours apart) in reducing P_4 concentrations and causing complete luteolysis by the time of AI, in suckling beef cows and lactating dairy cows when subjected to a 5-day CIDR Cosynch.

OBJECTIVES

To examine the effects of one HighCon Lutalyse injection, one conventional Lutalyse, or two conventional Lutalyse injections (12 hours apart) after CIDR removal in a 5-day CIDR-Cosynch protocol in beef and lactating dairy cows on:

1.) Progesterone concentrations after CIDR removal and treatment administration until the time of AI.
2.) Complete luteolysis ($P_4 \leq 0.5$ ng/mL).

MATERIALS & METHODS

CHARACTERISTICS OF RESEARCH COWS

This study was conducted from 2016 to 2017 at the University of Idaho Dairy and Beef Research and Education centers located in Moscow, Idaho. All animal handling procedures and treatment protocols were approved prior to initiation of the experiment by the University of Idaho, Institutional Animal Care and Use Committee (Appendix 3).

In experiment 1, 87 lactating Holstein cows were initially enrolled into this study and synchronized using a 5-day CIDR-Cosynch protocol (Bridges et al., 2008). Twenty cows

were removed as they did not have a CL at and (or) elevated concentrations of P_4 on day 5 before treatment (7 cows), or they were acyclic and blood samples from day -7, 0, and 5 were all < 0.5 ng/mL (13 cows). On this dairy, cows are inseminated four times a year (3 months apart), and all cows were housed in free stall barns and milked twice daily. Cows had access to a total mixed ration for more than 20 hours per day and ad libitum access to water. The TMR was balanced that meet or exceeded the nutritional requirements for high producing dairy cows (NRC, 2001).

In experiment 2, 78 suckling Charolais beef cows were initially enrolled into the study and synchronized for the first insemination using a 5-day CIDR-Cosynch protocol. Twenty-four cows were removed as they did not have a CL and (or) elevated P_4 concentrations before treatment (3 cows), or they were acyclic and blood samples from day -7, 0, and 5 were all < 0.5 ng/mL (21 cows). Cows were housed on pasture and had access to supplemental hay, a mineral supplementation, and water.

EXPERIMENTAL PROCEDURES & TREATMENTS

Seven days before synchronization, blood samples were collected via puncture of the medial caudal vein or artery (Experiment 1) or jugular veinpuncture (Experiment 2) from all cows (Figure 1). On day 0 (initiation of synchronization), all cows received GnRH (100 μg, i.m.; Factrel; Fort Dodge Animal Health, Fort Dodge, IA), and a blood sample was obtained. Simultaneously, a CIDR (1.38 g P_4; Eazi-Breed CIDR, Zoetis, Florham Park, NJ) was inserted (Figure 1) and all cows were subjected to transrectal ultrasonography (Aloka SSD-500 V; Aloka, Tokyo, Japan), and categorized as having the presence or absence of a CL. Five days later, CIDR inserts were removed, and one hour after CIDR removal, blood samples were taken and ultrasonography was conducted to confirm the presence of luteal tissue. Cows were stratified on day 5 first by the presence or absence of a CL on day 0, then by parity and days postpartum. Following stratification cows were assigned randomly to one of three treatments: a) one $PGF_{2\alpha}$ (1PG; Exp. 1 n = 24; Exp. 2 n = 19; 25 mg i.m.; Lutalyse, Zoetis, Florham Park, NJ), b) two $PGF_{2\alpha}$ 12 hours apart (2PG; Exp. 1 n = 22; Exp. 2 n = 17; 2 × 25 mg i.m.) or c) one high concentration, long-lasting $PGF_{2\alpha}$ (HighCon; Exp. 1 n = 21; Exp. 2 n = 18; 25 mg i.m.; Lutalyse HighCon, Zoetis, Florham Park, NJ) (Figure 1). Following treatment, blood samples were collected every 12 hours from day 5 to 8 to measure P_4 concentrations (Figure 1). In both experiments, between day 5 and 8, cows were

monitored every 4 to 6 hours for estrous behavior via removal of tail paint. Regardless of estrus expression, all cows received a second GnRH (100 μg) and were inseminated by the same technician on day 8 (Figure 3.3). Additionally, on day 8, ovarian structures were examined by ultrasongraphy. Blood samples were taken 11 days later (day 19 of experiment) for P_4 concentrations, and pregnancy status was confirmed 32 days post-AI (day 40 of experiment) using pregnancy-specific protein B analysis (BioPRYN; BioTracking, Inc., Moscow, Idaho).

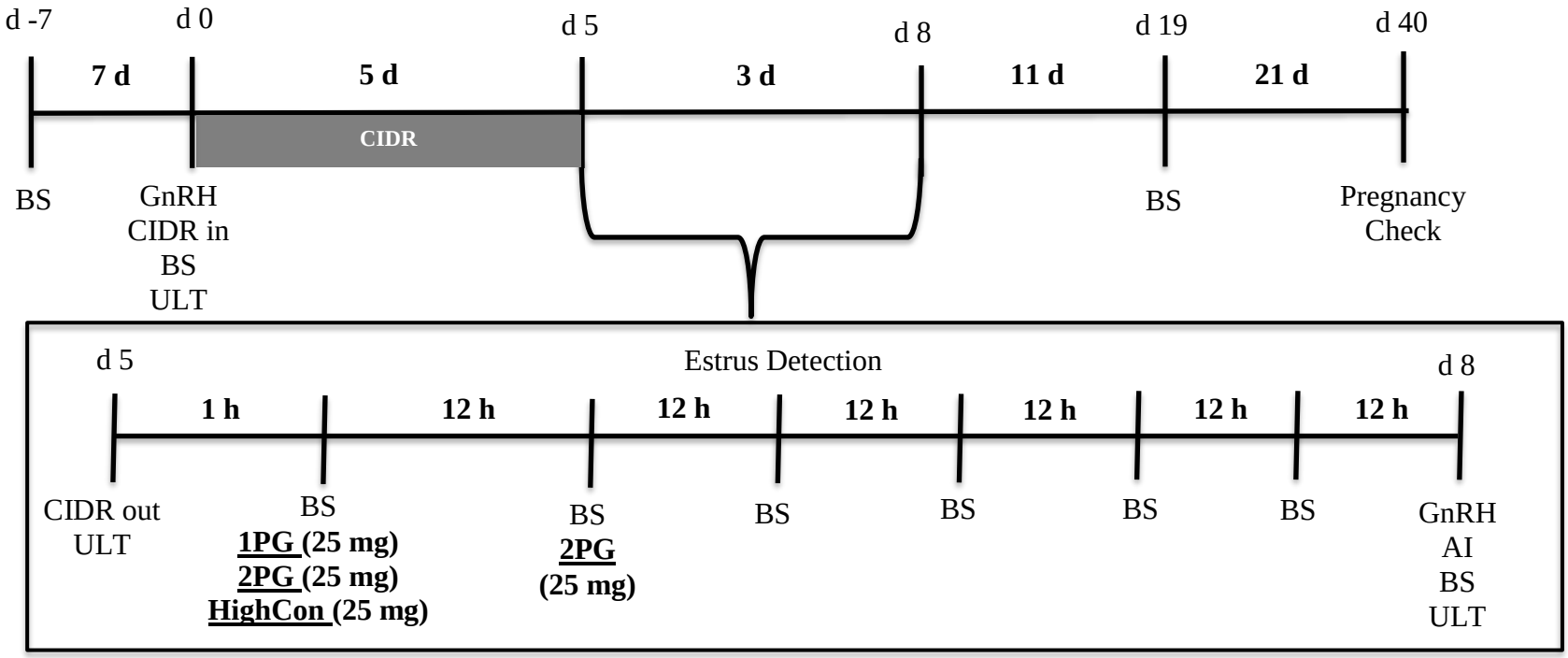

Figure 3.3 Schematic of the experimental design to examine the effect of one $PGF_{2\alpha}$ (1PG; 25 mg i.m), two $PGF_{2\alpha}$ (2PG; 50 mg i.m.), or one high concentration, long-lasting $PGF_{2\alpha}$ (HighCon; 25 mg i.m) on progesterone (P_4) concentrations in lactating dairy cows (Experiment 1) and suckling beef cows (Experiment 2) subjected to a 5-day controlled internal drug release (CIDR)-Cosynch protocol. On day 0, 5, and 8, ovarian structures were recorded for all cows via transrectal ultrasonography (ULT). On day 5, cows were stratified by presence or absence of a corpus luteum (CL) on day 0, then by parity and days postpartum. One hour after CIDR removal, treatments were administered, and coccygeal (Experiment 1) or jugular (Experiment 2) blood samples (BS) were taken every 12 hours until day 8, and additionally a BS was collected on day 19. All BS were analyzed for P_4 concentrations. A single BS was collected 32 days after AI for pregnancy specific protein B (PSPB) to determine pregnancy status.

Ultrasonography

On day 0, 5, and 8, transrectal ultrasonography (Aloka SSD-500 V; Aloka, Tokyo, Japan) was conducted to assess ovarian structures. Cows were stratified into treatments on day 5 based on the presence or absence of a CL on day 0, and cows with two CL on day 5 (existing CL and ovulated to initial GnRH) were evenly distributed between treatments.

Cows were categorized as developing a new CL, or possessing an old CL at the time of treatment on day 5. The presence of a dominant follicle (≥ 10 mm diameter) on day 0, and the presence of a CL on day 5, in the same location, identified cows that ovulated to the initial GnRH and formed a new CL. Cows presenting a CL on day 0 and day 5 in the same location were classified as possessing an old CL at the time of treatment. On day 8 remnants of luteal tissue were recorded. In addition, the presence of a dominant follicle (≥ 10 mm diameter), indicating a potential ovulatory follicle at the time of the second GnRH and AI, were recorded.

Blood Collection, Processing, & Progesterone Quantification

Blood samples were collected via puncture of the medial caudal vein or artery (Experiment 1), or jugular venipuncture (Experiment 2) using a 20 gauge or 18 gauge, 1 ½" single use blood collection needle. Blood samples were collected using a 10 mL evacuated tube (Covidien LLC, Mansfield, MA). All samples were placed on ice and stored at 4°C for 18 to 24 hours. Samples were then centrifuged for 20 minutes at 1,300 × *g* at 4°C. Serum was harvested and stored at -20°C until assayed for P_4 concentrations.

Progesterone concentrations were analyzed using a double antibody, radioimmunoassay (RIA; MP Biomedicals, Costa Mesa, CA) under equilibrium conditions. The standard curve ranged from 0.05 to 25 ng/mL. All samples and standards were run in duplicate, with an intra- and inter-assay coefficient of variation of 3.6% and 6.7%, respectively. Concentrations of PSPB were determined using BioPRYN, an enzyme-linked immunosorbent assay (ELISA; BioPRYN, BioTracking, Moscow, ID), at the BioTracking laboratory in Moscow, ID. Optical density (OD) concentrations were assessed and each sample was categorized as pregnant, high recheck, low recheck, or non-pregnant. Any high or low recheck OD values of PSPB were re-evaluated for verification of pregnancy approximately 7 days later.

STATISTICAL ANALYSIS

In Experiment 1, data for weekly milk production during the experiment, parity, days in milk (DIM) at the time of experiment, number of times bred (TBRD) prior to treatment, and body condition score (BCS) were analyzed using the analysis of variance procedure to test for treatment effects. Similarly, in Experiment 2, days postpartum, body weight (BW) at initiation of synchronization, and BCS were analyzed using the analysis of variance procedures. Analysis of variance was also used to compare mean P_4 concentrations on day 0, 5, and 8 between treatments.

The differences in proportion of cows between treatments with the presence of a CL on day 0, development of a new CL (ovulatory follicle ≥ 10mm on day 0 and presence of CL on day 5 in same location), remnants of a CL on day 8, and P_4 concentrations ≤ 0.5 ng/mL on day 7 (60 hours after CIDR removal) and day 8 (72 hours after CIDR removal) were analyzed using a generalized linear model, assuming a binomial distribution.

A generalized linear mixed model repeated measures analysis of variance was used to determine differences in P_4 concentrations across time, assuming a lognormal distribution (Stroup, 2014). The model included the fixed effects of treatment, time, and time by treatment interaction. Initial P_4 concentrations prior to treatments (day 5) were included as a covariate in the model, and cow within treatment was considered the random effect. The correlation for the repeated measures followed an ARMA (1,1) structure.

Non-orthogonal predetermined contrasts were made between groups for P_4 concentrations (60 and 72 hours after treatments) and ovarian structures on day 0 (presence or absence of CL), 5 (development of new CL), and 8 (remnants of a CL). Using Bonferroni t-test (Games, 1971), differences between 1PG vs. 2PG, 1PG vs. HighCon, and 2PG vs. HighCon were examined. All statistical computations were carried out using SAS (v. 9.4; SAS Institute Inc. 2015). Significance was declared at $P < 0.05$ and a tendency at $P \leq 0.1$.

RESULTS

EXPERIMENT 1 – LACTATING DAIRY COWS

Descriptive Data

Sixty-seven primiparous and multiparous lactating Holstein cows were used in this study. Cows were stratified on day 5 (Figure 3.1) based on the presence or absence of a CL on day 0 (initiation of synchronization and CIDR insertion), parity, days postpartum and then assigned randomly to one of three treatments 1PG (n = 24), 2PG (n = 22), or HighCon (n = 21). There were no differences in parity ($P = 0.28$) or TBRD ($P = 0.61$) between treatments. There were differences in daily milk production ($P < 0.01$), and BCS ($P < 0.01$), between treatments, although DIM tended ($P = 0.07$) to differ (Table 3.1).

Table 3.1 Mean ± SE for daily milk production (Milk), parity, days in milk (DIM), number of times bred before experiment, and body condition score (BCS) for 67 lactating Holstein dairy cows subjected to a 5-day CIDR-Cosynch protocol with differing doses of prostaglandin $F_{2\alpha}$ at CIDR removal.

Treatment [1]	Milk (kg) [2]	Parity	DIM (d)	TBRD [3]	BCS [4]
1PG (n = 24)	37.3 ± 0.6 [a]	2.0 ± 0.1	113 ± 7 [d]	0.46 ± 0.06	2.4 ± 0.04 [a]
2PG (n = 22)	34.6 ± 0.6 [b]	2.1 ± 0.1	137 ± 8 [e]	0.55 ± 0.06	2.2 ± 0.06 [b]
HighCon (n = 21)	36.1 ± 0.6 [a,b]	1.9 ± 0.1	131 ± 8 [d,e]	0.48 ± 0.07	2.6 ± 0.05 [c]

[1] Cows were assigned randomly to receive one $PGF_{2\alpha}$ injection (1PG), two $PGF_{2\alpha}$ injections given 12 hours apart (2PG) 12 hours apart, or one high-concentration $PGF_{2\alpha}$ (HighCon) injection upon CIDR removal.
[2] Average daily milk production during experiment.
[3] Number of inseminations prior to experiment.
[4] BCS on a scale of 1 to 5 in 0.25 increments (1 = emaciated; 5 = over conditioned).
[a,b,c] Differs between treatments within columns ($P < 0.05$).
[d,e] Tends to differ between treatments within columns ($P = 0.07$).

Ovarian Structures

The percentage of cows with an existing CL on day 0 of the experiment tended ($P = 0.09$) to differ between treatments (Table 3.2). More 2PG cows had a CL present on day 0 (initiation of synchronization) than HighCon, but did not differ from 1PG. In addition, the presence of a CL on day 0 did not differ between 1PG and HighCon treatments.

On day 5, all cows had a CL present on the ovary, and the proportion of cows that ovulated to the initial GnRH and developed a new CL did not differ between groups ($P =$

0.39). Overall, ovulation to the initial GnRH injection was 40% regardless of treatment. On day 8, there was a difference in proportion of cows with CL remnants ($P = 0.04$). More 1PG cows had CL remnants compared with 2PG, but HighCon did not differ from 1PG and 2PG treatments (Table 3.2). Based on ultrasonography, 21 cows (12 in 1PG, 3 in 2PG, and 6 in HighCon) had remnants of luteal tissue on day 8.

Progesterone Concentrations and Profiles

The P_4 data from 12 to 72 hours after treatment were skewed indicating a non-normal distribution with an unstable variance. Univariate analyses confirmed a large variation, and hence, P_4 data were assumed to follow a lognormal distribution. Concentrations on day 5 were used as a covariate in the model to account for concentrations before treatment administration. There was no significant difference ($P = 0.26$) in P_4 concentrations on day 5; however, day 5 P_4 concentrations were left in the model as a covariate to help explain some of the residual error.

Mean P_4 concentrations on day 0 (initiation of synchronization) did not differ between treatments ($P = 0.98$) and were 3.21 ± 0.78 for 1PG, 3.25 ± 0.82 for 2PG, and 3.40 ± 0.84 ng/mL for HighCon (Figure 3.4). Similarly, mean P_4 concentrations did not differ ($P = 0.26$) between treatments on day 5. Mean concentrations on day 5 were 4.16 ± 0.67, 3.07 ± 0.70, and 2.60 ± 0.72 ng/mL for 1PG, 2PG, and HighCon, respectively (Figure 3.4).

As expected, P_4 concentrations after treatments decreased over time ($P < 0.01$) in all treatments. There was an effect of treatment ($P < 0.01$) and treatment by time ($P = 0.05$) on P_4 concentrations from 12 to 72 hours after treatments (Figure 3.5). These results provide evidence that the decline in P_4 concentrations over time were not similar among treatments. From 24 to 72 h after initial treatment, P_4 concentrations were lower in 2PG and HighCon compared with 1PG; however, P_4 concentrations during this sampling period did not differ between 2PG and HighCon. The serum P_4 concentrations (ng/mL) from day 5 to 8 are shown in Figure 3.6.

Mean P_4 concentrations on day 8 (day of AI) differed between treatments ($P < 0.01$). The 1PG had the highest P_4 concentrations (1.02 ± 0.15 ng/mL), followed by HighCon (0.22 ± 0.16 ng/mL), and 2PG had the lowest P_4 concentrations at the time of AI (day 8; 0.05 ± 0.15 ng/mL) (Figure 3.4). On day 19, the average P_4 concentrations were 3.71, 9.53, and 6.62 ng/mL for 1PG, 2PG, and HighCon, respectively.

The differences in P_4 concentrations 60 and 72 hours (day 8) after treatments were further examined to determine the proportion of cows with $P_4 \leq 0.5$ ng/mL, indicating functional luteolysis, between treatments. There was a difference ($P < 0.01$) between treatments for the proportion of cows with $P_4 \leq 0.5$ ng/mL at both 60 and 72 hours after treatments (Table 3.2). At 60 and 72 hours, 54 and 50% of 1PG cows exhibited $P_4 < 0.5$ ng/mL. The HighCon treatment had a greater proportion of cows with $P_4 < 0.5$ ng/mL at 60 and 72 hours with 90 and 81%, respectively. The HighCon treatment did not differ from 2PG, where 100% of cows had $P_4 < 0.5$ ng/mL at 60 and 72 hours (Table 3.2).

Table 3.2 Proportions of cows with progesterone concentrations ≤ 0.5 ng/mL at the time of AI and corpus luteum (CL) presence in lactating Holstein dairy cows subjected to one conventional prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection (1PG), two conventional $PGF_{2\alpha}$ injections administered 12 hours apart, or one high concentration $PGF_{2\alpha}$ injection (HighCon) in a 5-day controlled-internal drug release (CIDR)-Cosynch protocol.

Variable	**Treatments**[1]			***P*-value**
	1PG	2PG	HighCon	
Progesterone (≤ 0.5 ng/mL)[2]				
60 h, % (no./no.)	54 (13/24) [a]	100 (22/22) [b]	90 (19/21) [b]	< 0.01
72 h, % (no./no.)	50 (12/24) [a]	100 (22/22) [b]	81 (17/21) [b]	< 0.01
Presence of CL[3]				
Day 0, % (no./no.)	71 (17/24) [x,y]	89 (17/19) [x]	60 (12/20) [z]	= 0.09
Day 5, % (no./no.)	35 (8/23)	33 (7/21)	53 (10/19)	= 0.39
Day 8, % (no./no.)	52 (12/23) [a]	17 (3/18) [b]	30 (6/20) [a,b]	= 0.04

[1] In a 5-day CIDR-Cosynch protocol, cows were assigned randomly to receive one $PGF_{2\alpha}$ injection(1PG), two $PGF_{2\alpha}$ injections given 12 hours apart (2PG), or one high-concentration $PGF_{2\alpha}$ (HighCon) injection upon CIDR removal.
[2] Progesterone concentrations ≤ 0.5 ng/mL 60 and 72 hours after CIDR removal and initial treatment.
[3] Transrectal ultrasonography. Day 0 = existing CL, Day 5 = development of a new CL, Day 8 = remnants of a CL.
[a,b] Proportions with different superscripts within rows differ ($P < 0.05$).
[x,y,z] Proportions with different superscript within rows tend to differ ($P \leq 0.1$).

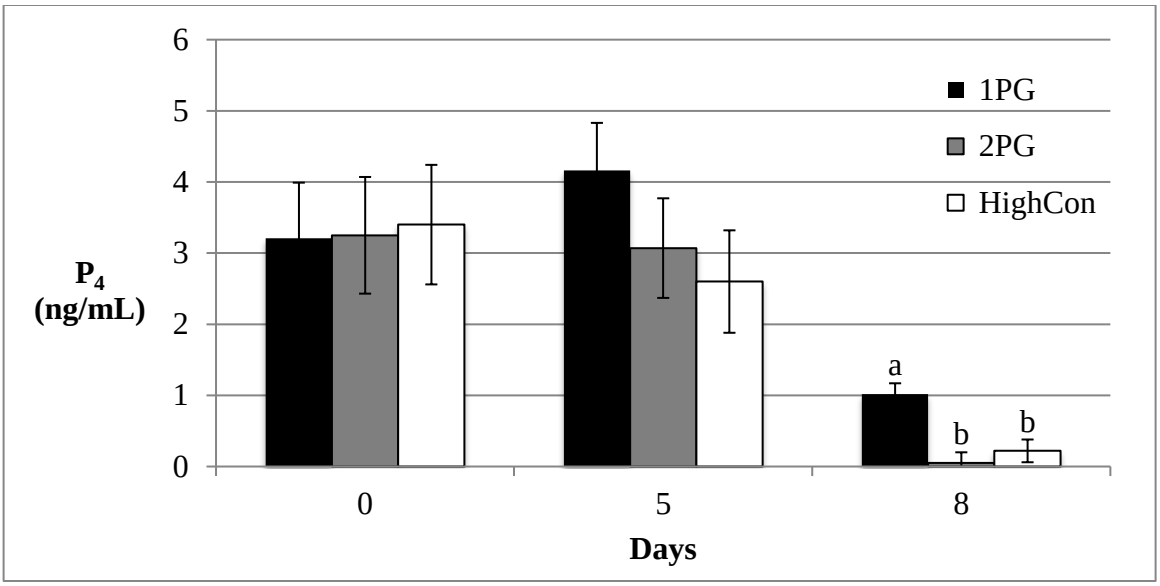

Figure 3.4 Mean progesterone concentrations (ng/mL) on day 0, 5, and 8 of the experimental protocol for lactating dairy cows subjected to a 5-day controlled internal drug release (CIDR)-Cosynch protocol. One hour after CIDR removal (day 5) cows were treated with either one prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 1PG; n = 24), two $PGF_{2\alpha}$ (2PG; n = 22) injections 12 hours apart, or one high concentration $PGF_{2\alpha}$ (HighCon; n = 21) injection.
[a,b] Bars with different letters within days differ $P \leq 0.05$.

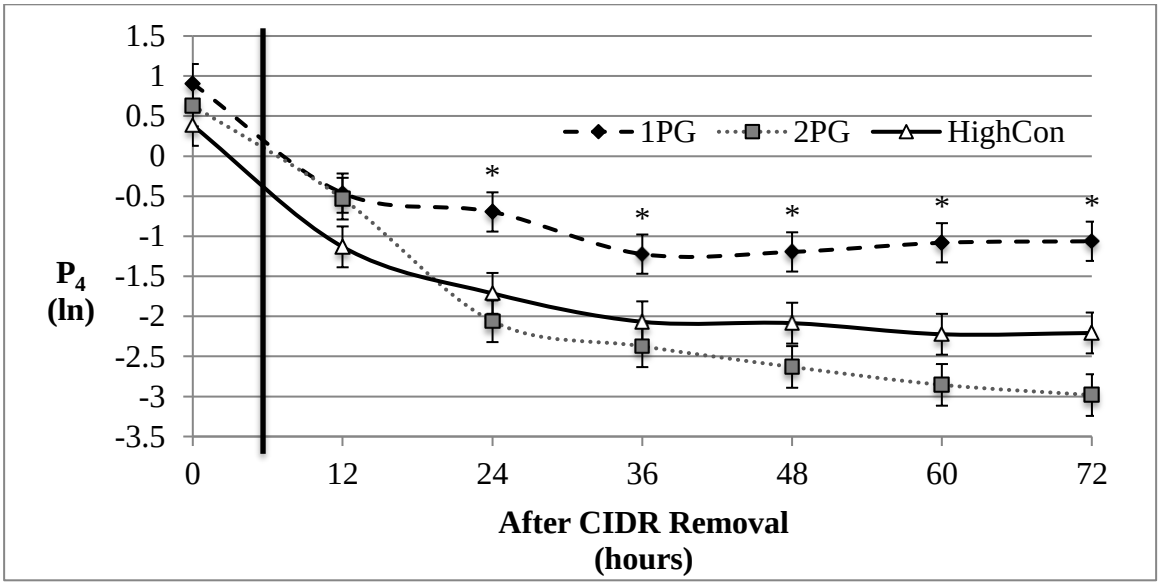

Figure 3.5 Mean blood progesterone (P_4) concentrations after lognormal data transformation between lactating dairy cows receiving one prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1PG; n = 24), two $PGF_{2\alpha}$ 12 hours apart (2PG; n = 22), or one high concentration $PGF_{2\alpha}$ (HighCon; n = 21) in a 5-day controlled internal drug release (CIDR)-Cosynch protocol. Time 0 is day 5, after CIDR removal before treatments were administered and used as a covariate. Blood samples were collected every 12 hours for three days after treatment administration.
* Means differ between 1PG and 2PG or HighCon treatments ($P < 0.05$).

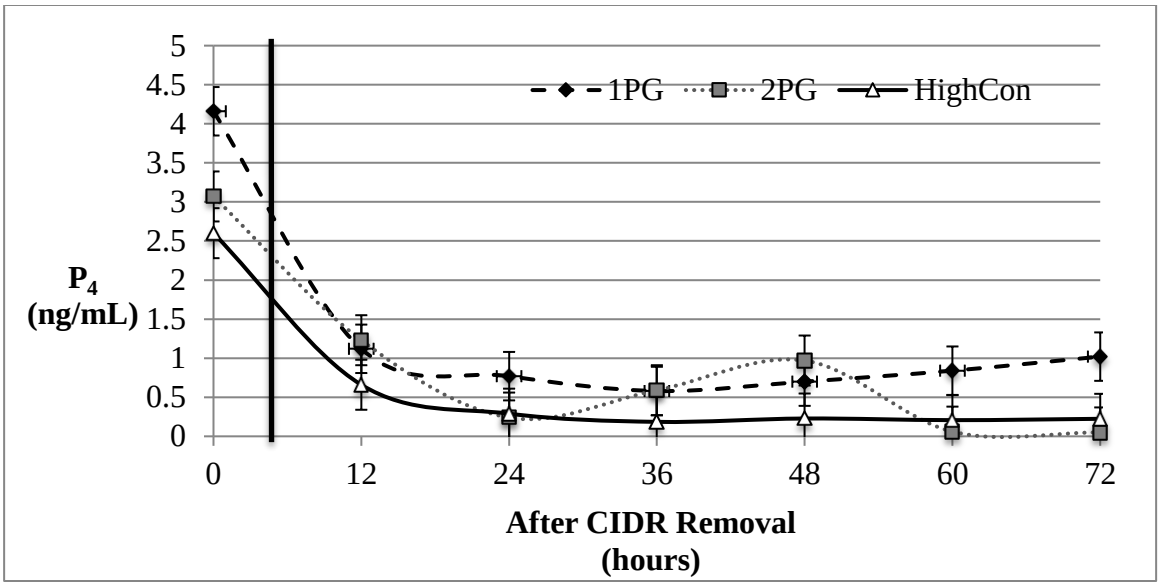

Figure 3.6 Serum progesterone (P_4) concentrations between lactating dairy cows receiving one prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1PG; n = 24), two $PGF_{2\alpha}$ 12 hours apart (2PG; n = 22), or one high concentration $PGF_{2\alpha}$ (HighCon; n = 21) in a 5-day controlled internal drug release (CIDR)-Cosynch protocol. Time 0 is day 5, after CIDR removal before treatments were administered and used as a covariate. Blood samples were collected every 12 hours for three days after treatment administration.

EXPERIMENT 2 – SUCKLING BEEF COWS

Descriptive Data

Fifty-four primiparous and multiparous suckling Charolais beef cows were used in this study and synchronized using a 5-day CIDR-Cosynch protocol. On day 5 and at the time of CIDR removal, cows were stratified by the presence or absence of a CL on day 0, parity, and days postpartum then assigned randomly to 1PG (n = 19), 2PG (administered 12 hours apart; n = 17), or HighCon (n = 18) treatments. There were no differences in BW ($P = 0.48$) between treatments; however, there were differences in average days postpartum ($P < 0.01$) and BCS ($P < 0.01$) between treatments (Table 3.3). The average days postpartum in the HighCon treatment was 6 days more than 1PG and 10 days more than the 2PG treatment (Table 3.3). Body condition score was calculated using the average of two evaluators and were between 6 and 7 (Table 3.3). Nevertheless, mean BCS were greater ($P < 0.05$) for 1PG when compared to 2PG and HighCon.

Table 3.3 Mean ± SE for days postpartum, body weight (BW), and body condition score (BCS) in Charolais suckling beef cows subjected to a 5-day CIDR-Cosynch protocol with differing doses of prostaglandin $F_{2\alpha}$ at CIDR removal for first AI.

Treatment [1]	Postpartum (d)	BW (kg)	BCS [2]
1PG	68 ± 1 [a]	688.78 ± 11.44	6.62 ± 0.11 [a]
2PG	64 ± 1 [a]	668.94 ± 12.06	6.00 ± 0.11 [b]
HighCon	74 ± 1 [b]	688.70 ± 14.77	6.21 ± 0.11 [b]

[1] Cows were assigned randomly to receive one $PGF_{2\alpha}$ injection (1PG), two $PGF_{2\alpha}$ injections given 12 hours apart (2PG), or one high-concentration $PGF_{2\alpha}$ (HighCon) injection upon CIDR removal.
[2] BCS on a scale of 1 to 9 in 0.5 increments (1 = emaciated; 9 = over conditioned).
[a,b] Means with different superscript within column differ ($P < 0.05$).

Ovarian Structures

The proportion of cows with an existing CL on day 0, ovulation to the initial GnRH (new CL on day 5), or remnants of a CL on day 8 are represented in Table 3.4. The proportion of cows with an existing CL on day 0 of the experiment did not differ ($P = 0.47$) between treatments (Table 3.4). All cows were cyclic and had a CL prior to administration of treatment on day 5. On day 5, the proportion of cows that ovulated to the initial GnRH did not differ ($P = 0.35$) between treatments. Overall, ovulation to the initial GnRH injection was 48% regardless of the treatment. On day 8, there was no difference ($P = 0.70$) in proportion of cows that had CL remnants based on ultrasonography and P_4 concentrations (Table 3.4). From 31 out of 54 cows, luteal tissue was detected on the ovaries of nine cows (4/11 in 1PG, 3/10 in 2PG, and 2/10 in HighCon) on day 8 (Table 3.4).

Progesterone Concentrations and Profiles

The P_4 data after treatments (12 to 72 hours) were skewed indicating a non-normal distribution with an unstable variance. Univariate analyses confirmed a large variation, and hence, P_4 data were assumed to follow a lognormal distribution. Concentrations on day 5 were used as a covariate in the model to account for concentrations before treatment administration. There was no significant difference ($P = 0.50$) on P_4 concentrations on day 5; however, day 5 P_4 concentrations were left in the model as a covariate to help explain some of the residual error.

Mean P_4 concentrations on day 0 (initiation of synchronization) did not differ between treatments ($P = 0.37$) and were 0.88 ± 0.38 for 1PG, 1.20 ± 0.40 for 2PG, and 1.64

± 0.39 ng/mL for HighCon (Figure 3.7). Similarly, mean P_4 concentrations did not differ ($P = 0.50$) between treatments on day 5. Mean concentrations on day 5 were 2.39 ± 0.43, 1.74 ± 0.45, and 1.77 ± 0.44 ng/mL for 1PG, 2PG, and HighCon, respectively (Figure 3.7).

As expected, P_4 concentrations decreased over time ($P < 0.01$) in all treatments. There were no effects of treatments on P_4 profiles over time ($P = 0.46$); however, there was an effect of treatment by time ($P = 0.02$) as P_4 profiles differ over time between treatments (Figure 3.8). Mean P_4 concentrations on day 8 (day of AI) did not differ ($P = 0.16$) between treatments (Figure 3.7). Progesterone concentrations at the time of AI were 0.50 ± 0.12 ng/mL for 1PG, 0.18 ± 0.12 ng/mL for 2PG, and 0.25 ± 0.11 ng/mL for HighCon treated cows (Figure 3.7). The average P_4 concentrations from day 5 to 8 are shown in Figure 3.9. On day 19, the average P_4 concentrations were 1.94, 3.20, and 2.29 ng/mL for 1PG, 2PG, and HighCon respectively.

The differences in P_4 concentrations 60 and 72 hours (day 8) after treatments were further examined to determine the proportion of cows with $P_4 \leq 0.5$ ng/mL, indicating functional luteolysis, between treatments (Table 3.4). Based on the results, there was a tendency ($P = 0.09$) for the proportion of cows with $P_4 \leq 0.5$ ng/mL at both 60 and 72 hours to differ between treatments (Table 3.4). At 60 hours, 1PG and HighCon did not differ ($P > 0.05$) in the proportion of cows $P_4 < 0.5$ ng/mL (Table 3.4). Additionally, at 60 hours, HighCon and 2PG did not differ ($P > 0.05$) in the proportion of cows with $P_4 \leq 0.5$ ng/mL (Table 3.4). At 72 hours, there was a tendency for more 2PG and HighCon cows to have $P_4 \leq 0.5$ ng/mL compared to cows in the 1PG treatment (Table 3.4).

Table 3.4 Proportions of cows with progesterone concentrations ≤ 0.5 ng/mL at the time of AI and corpus luteum (CL) presence in suckling Charolais beef cows subjected to one conventional prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection (1PG), two conventional $PGF_{2\alpha}$ injections administered 12 hours apart, or one high concentration $PGF_{2\alpha}$ injection (HighCon) in a 5-day controlled-internal drug release (CIDR)-Cosynch protocol for first insemination.

Variable	**Treatments**[1]			***P*-value**
	1PG	2PG	HighCon	
Progesterone (≤ 0.5 ng/mL)[2]				
60 h, % (no./no.)	68 (13/19) [x]	94 (16/17) [y]	89 (16/18) [x,y]	= 0.09
72 h, % (no./no.)	63 (12/19) [x]	88 (15/17) [y]	89 (16/18) [x,y]	= 0.09
Presence of CL[3]				
Day 0, % (no./no.)	63 (12/19)	50 (8/16)	70 (12/17)	= 0.47
Day 5, % (no./no.)	55 (6/11)	60 (6/10)	30 (3/10)	= 0.35
Day 8, % (no./no.)	36 (4/11)	30 (3/10)	20 (2/10)	= 0.70

[1] In a 5-day CIDR-Cosynch protocol, cows were assigned randomly to receive one $PGF_{2\alpha}$ injection (1PG), two $PGF_{2\alpha}$ injections given 12 hours apart (2PG), or one high-concentration $PGF_{2\alpha}$ (HighCon) injection upon CIDR removal.
[2] Progesterone concentrations ≤ 0.5 ng/mL 60 and 72 hours after CIDR removal and initial treatment.
[3] Transrectal ultrasonography. Day 0 = existing CL, Day 5 = development of a new CL, Day 8 = remnants of a CL.
[x,y] Proportions with different superscripts within rows tend to differ ($P < 0.1$).

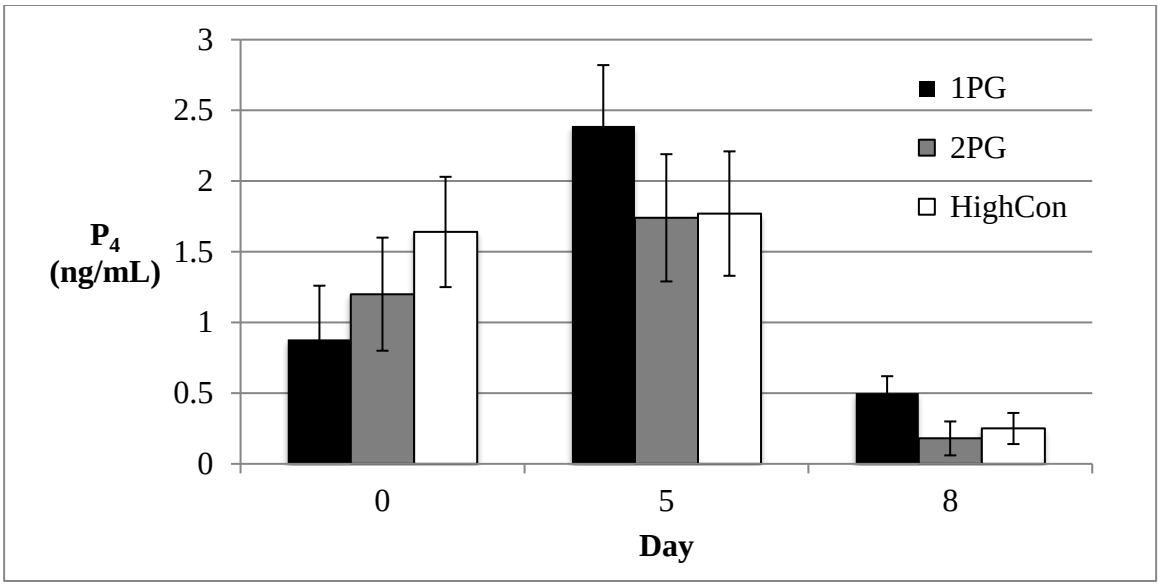

Figure 3.7 Mean progesterone (P_4) concentrations (ng/mL) on day 0, 5, and 8 of the experimental protocol for suckling beef cows subjected to a 5-day controlled internal drug release (CIDR)-Cosynch protocol. One hour after CIDR removal (day 5) cows were treated with either one prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 1PG; n = 19), two $PGF_{2\alpha}$ (2PG; n = 17) injections 12 hours apart, or one high concentration $PGF_{2\alpha}$ (HighCon; n = 18).

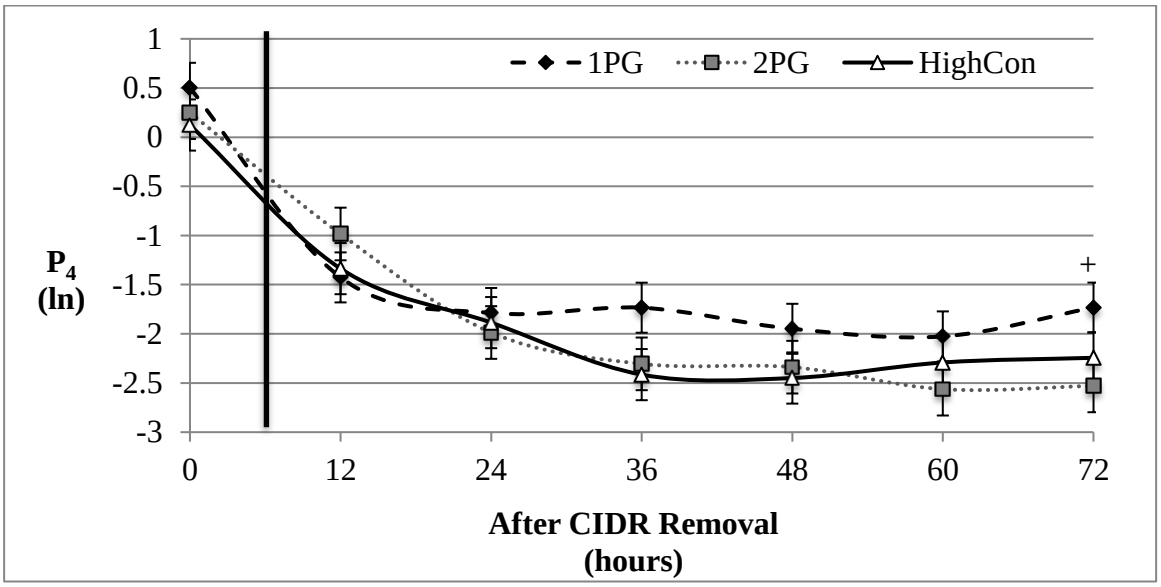

Figure 3.8 Mean blood progesterone (P_4) concentrations after lognormal data transformation in suckling beef cows receiving one prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1PG; n = 19), two $PGF_{2\alpha}$ 12 hours apart (2PG; n = 17), or one high concentration $PGF_{2\alpha}$ (HighCon; n = 18) in a 5-day controlled internal drug release (CIDR)-Cosynch protocol. Time 0 is considered to be after CIDR removal before treatments were administered. Blood samples were collected every 12 hours for three days after treatment administration.
+ Means tend to differ between 1PG and 2PG or HighCon ($P \leq 0.1$).

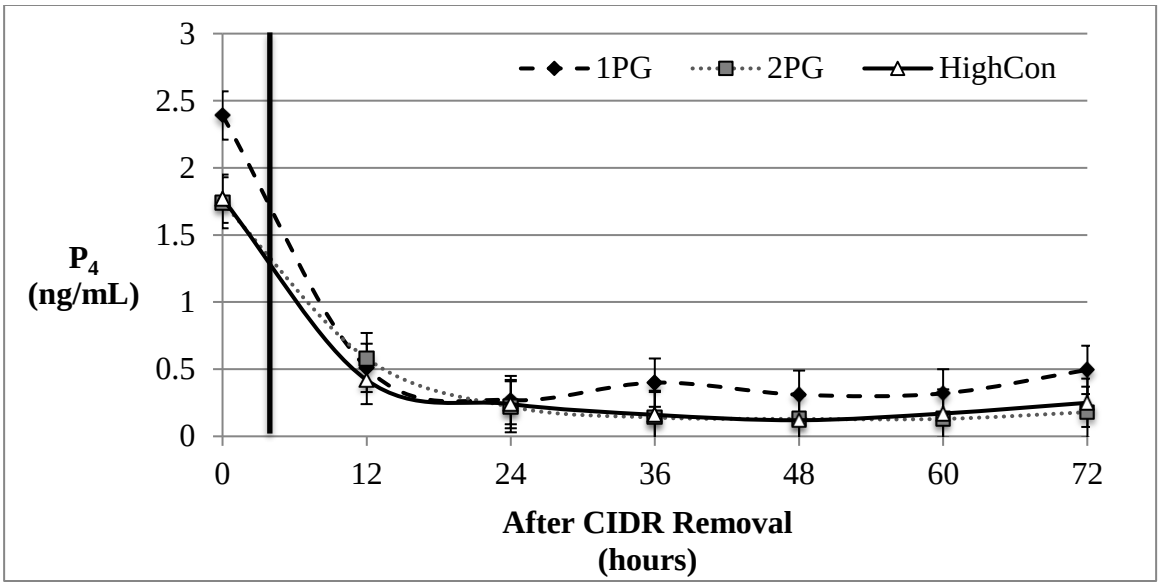

Figure 3.9 Serum progesterone (P_4) concentrations between suckling beef cows receiving one prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (1PG; n = 19), two $PGF_{2\alpha}$ 12 hours apart (2PG; n = 17), or one high concentration $PGF_{2\alpha}$ (HighCon; n = 18) in a 5-day controlled internal drug release (CIDR)-Cosynch protocol. Time 0 is day 5, after CIDR removal before treatments were administered and used as a covariate. Blood samples were collected every 12 hours for three days after treatment administration.

DISCUSSION

Synchronization protocols are useful reproductive tools to aid in synchronization of ovulation, facilitate the use of AI, and improve reproductive efficiency. Many estrous synchronization protocols exist, and the success of these protocols relies on the ability of exogenous hormones to mimic complex and sequential physiological events that occur in cows during the estrous cycle. In TAI protocols, several pharmaceuticals are used to control the timing of estrus or ovulation by altering the length of the estrous cycle and/or through manipulation of follicular growth. These protocols make the occurrence of ovulation more predictable and allow for appointment breeding without estrous detection.

There are three factors during a TAI estrous synchronization protocol that greatly influence fertility. The most important factor influencing fertility is ovulation to the final GnRH at the time of AI. The next important factor is that P_4 concentrations must be high (> 1 ng/mL) before $PGF_{2\alpha}$ is administered. The last important factor is the ability of $PGF_{2\alpha}$ to cause complete luteolysis within 48 hours after administration and optimize P_4

concentrations by the time of AI (Stevenson, 2016). The purpose of this study was to investigate the effects of one or two doses of conventional $PGF_{2\alpha}$ and one high-concentrated $PGF_{2\alpha}$ on P_4 profiles and luteolysis in lactating dairy cows and suckling beef cows subjected to a 5-day CIDR-Cosynch protocol.

Researchers have demonstrated that P/AI is improved or remains similar if the duration of CIDR treatment in a CIDR-Cosynch protocol is shortened from 7 to 5 days (interval between initial GnRH injection and CIDR insertion, to CIDR removal and $PGF_{2\alpha}$ injection), and the interval from CIDR removal and $PGF_{2\alpha}$ to the second GnRH is extended to 72 hours (Bridges et al., 2008; Kasimanickam et al., 2009; Lopes et al., 2013; Santos et al., 2010; Whittier et al., 2010; Wilson et al., 2010; Ahmadzadeh et al., 2015). However, by shortening the interval between initial GnRH and $PGF_{2\alpha}$ injections, the success of pregnancy relies on the ability of exogenous $PGF_{2\alpha}$ to successfully regress a GnRH-induced newly formed CL, and reduce P_4 concentrations to optimal concentrations ($P_4 < 0.5$ ng/mL) by the time of AI.

The inability of $PGF_{2\alpha}$ to successfully induce luteolysis and cause reduced P_4 to < 0.5 ng/mL by the time of AI has been termed incomplete luteolysis. One study defined incomplete luteolysis as a reduction in P_4 concentrations within 24 hours after $PGF_{2\alpha}$ and then no further decline in P_4 concentrations or even an increase in P_4 concentrations (Ferraz Junior et al., 2016). Others have defined incomplete luteolysis based on P_4 concentrations at the time of AI, with $P_4 > 0.5$ ng/mL (Brusveen et al., 2009; Carvalho et al., 2015). Incomplete luteolysis has been observed in 10 to 25% of cows that are subjected to an Ovsynch protocol, and is associated with reduced P/AI (Brusveen et al., 2009; Martins et al., 2011; Giordano et al., 2012; Carvalho et al., 2015; Wiltbank and Pursley, 2015). Interestingly, P_4 profiles and ovarian structures following $PGF_{2\alpha}$ injections in a 5-day CIDR-Cosynch protocol have not been studied well for lactating dairy cows or suckling beef cows. Therefore, the purpose of this study was to examine the necessity to of two $PGF_{2\alpha}$ injections in a 5-day CIDR-Cosynch protocol for lactating dairy cows (Experiment 1) and suckling beef cows (Experiment 2) by profiling P_4 concentrations from CIDR removal and $PGF_{2\alpha}$ to the time of AI and examining ovarian structures during the protocol.

OVARIAN DYNAMICS

In the curent study, 40% of lactating dairy cows and 48% of suckling beef cows ovulated to the initial GnRH injection. Within each experiment, the percentage of cows that ovulated to the initial GnRH did not differ between treatments. These results are consistent with other studies, which have indicated that approximately 50% of cows will ovulate to the initial GnRH when synchronization is initiated without presynchronization and during random stages of the estrous cycle (Vasconcelos et al., 1999; Stevenson, 2016).

LACTATING DAIRY COWS – PROGESTERONE PROFILES

It appears that incomplete luteolysis is the major contributing factor that influences fertility in dairy cows (Souza et al., 2007; Brusveen et al., 2009; Giordano et al., 2012; Carvalho et al., 2015). Most studies have examined P_4 concentrations at the initiation of an estrous synchronization protocol, at the time of $PGF_{2\alpha}$ administration, and at the time of AI (Brusveen et al., 2009; Santos et al., 2010; Stevenson, 2016; Colazo et al., 2017), however, the P_4 profile from $PGF_{2\alpha}$ injection until the time of AI has not been examined, and thus the effect of $PGF_{2\alpha}$ dose on rate of P_4 concentration decline has not been extensively investigated.

Understanding the P_4 profile following $PGF_{2\alpha}$ is important. At least in dairy cows, it has been shown that cows are more fertile if the rate of P_4 decline is more rapid after $PGF_{2\alpha}$ administration (Brusveen et al., 2009). Physiologically, this rapid reduction in P_4 concentrations would remove the negative feedback on the hypothalamus allowing secretion of GnRH and LH for final follicular development and subsequent ovulation (McCracken et al., 1999; Senger, 2012). To our knowledge there are only two studies in dairy cows that have examined effectiveness of one or two $PGF_{2\alpha}$ injections in a 5-day Cosynch protocol.

Santos et al. (2010) examined the effects of one or two $PGF_{2\alpha}$ injections 24 hours apart in a 5-day Cosynch protocol in lactating dairy cows. In this study, luteolysis was defined as $P_4 > 1$ ng/mL at $PGF_{2\alpha}$ injection (day 5) and $P_4 < 1$ ng/mL at time of AI (day 8). These authors concluded two $PGF_{2\alpha}$ injections are needed on day 5 and 6 of the protocol to successfully cause luteal regression, as the incidence of luteolysis was greater for two $PGF_{2\alpha}$ (95.7%) compared with one $PGF_{2\alpha}$ (59.1%) treatment. In addition, P_4 concentrations at the time of AI were lesser for two $PGF_{2\alpha}$ compared with one $PGF_{2\alpha}$ treatment (0.77 vs. 1.80 ng/mL). It should be noted that cows in the two $PGF_{2\alpha}$ injections group still had P_4

concentrations > 0.5 ng/mL at the time of AI, and may not have been at optimal P_4 concentrations to maximize pregnancy. Additionally, a CIDR was not included during the 5-day Cosynch protocol in the study by Santos et al. (2010), nor were P/AI between one and two $PGF_{2\alpha}$ injections.

Another study by Ribeiro et al. (2012), however, did examine the effects of one or two $PGF_{2\alpha}$ injections 24 hours apart on P/AI in a 5-day CIDR-Cosynch protocol in dairy cows. In this study, luteolysis was again defined as P_4 concentrations > 1 ng/mL at CIDR removal and < 1 ng/mL at time of AI. Although the incidence of luteolysis was numerically greater for two $PGF_{2\alpha}$ compared with one $PGF_{2\alpha}$ (95.7% vs. 82%), there were no differences in day 35 P/AI between one and two $PGF_{2\alpha}$ (30.2% vs. 34.3%) injections. It should be noted that these researchers did not compare the effects of one versus two $PGF_{2\alpha}$ specifically in the 5-day CIDR-Cosynch protocol. Rather, they reported the luteolytic effects of one versus two $PGF_{2\alpha}$ injections by combining the results of two different protocols. Therefore, it cannot be determined if there were any differences between one versus two $PGF_{2\alpha}$ in a 5-day CIDR-Cosynch protocol on P_4 concentrations, luteolysis, and P/AI.

In our study, one $PGF_{2\alpha}$ injection at the time of CIDR removal was not effective in causing complete luteolysis and reducing P_4 concentrations to ≤ 0.5 ng/mL by the time of AI. This finding is similar to other studies that have shown a reduced incidence, and increased variability in the proportion of cows that undergo luteolysis when a single dose of $PGF_{2\alpha}$ is given 5 days after the initial GnRH (Kasimanickan et al., 2009; Whittier et al., 2010; Ribeiro et al., 2012). Thus, it could be hypothesized that if ovulation to the initial GnRH (at CIDR insertion) occurs, the GnRH-induced CL will be immature and unable to respond to a single injection of $PGF_{2\alpha}$ with complete luteolysis in dairy cows.

It should be noted that in the previously mentioned studies in dairy cows (Santos et al., 2010; Ribiero et al., 2012) complete luteolysis was defined as cows having < 1 ng/mL P_4 concentration by the time of AI. If we redefined complete luteolysis from $P_4 \leq 0.5$ ng/mL to $P_4 < 1$ ng/mL at the time of AI, the proportion of cows with complete luteolysis would only increase by < 10% in one $PGF_{2\alpha}$ and still be less than two $PGF_{2\alpha}$ and HighCon (data not shown). This provides further evidence that in dairy cows one injection of $PGF_{2\alpha}$ may not be effective to cause lutelolysis and maximize P/AI when the 5-day CIDR-Cosynch protocol is used.

Administration of two $PGF_{2\alpha}$ injections 12 hours apart, or one HighCon $PGF_{2\alpha}$ injection, however, were effective in reducing P_4 concentrations to ≤ 0.5 ng/mL by the time of AI and causing complete luteolysis. Our findings are similar to Ribeiro et al. (2012) in that two injections of $PGF_{2\alpha}$ reduced P_4 concentration to 0.27 ng/mL and less than 0.5 ng/mL by the time of AI. Interestingly, in our study, mean serum P_4 concentrations for the HighCon treatment (0.22 ng/mL) were also similar to the P_4 concentrations observed for the cows that received two $PGF_{2\alpha}$ injections in the Ribeiro et al. (2012) study. The fact that the proportion of cows with complete luteolysis were not different between the 2PG and HighCon (100% vs. 81%) treatments lends further support to the hypothesis that HighCon is as effective as two $PGF_{2\alpha}$ in causing luteolysis in a 5-day CIDR-Cosynch protocol. In addition, if we were to redefine our definition of complete luteolysis from $P_4 \leq 0.5$ ng/mL to $P_4 < 1$ ng/mL at the time of AI, the proportion of cows with complete luteolysis would increase to 95% in HighCon (data not shown). Thus, for the first time we were able to demonstrate that one injection of HighCon (dinoprost tromethamine), a high concentration of Lutalyse (dinoprost tromethamine), is as effective as two $PGF_{2\alpha}$ (12 hours apart) injections in decreasing P_4 concentrations (causing complete luteolysis) by the time of AI in lactating dairy cows subjected to a 5-day CIDR-Cosynch.

Researchers have found that 58% of rounds from dairy carcasses had at least one injection-site lesion (DACQA). Approval for s.c. drug administration for HighCon allows producers and veterinarians to abide by beef quality assurance (BQA) and Dairy Animal Care and Quality Assurance (DACQA) standards, which aim to diminish injection site blemishes and muscle damage observed with i.m. drug administration. If one dose of HighCon is as effective as two doses of $PGF_{2\alpha}$ in causing complete luteolysis, there is an incentive for producers to use one dose of HighCon instead of two doses of Lutalyse. Therefore, producers may be able to administer only one $PGF_{2\alpha}$ injection at the time of CIDR removal and effectively cause complete luteolysis and maximize fertility in lactating dairy cows. Nevertheless, the association between incomplete and complete luteolysis and fertility in dairy cows warrants further investigation.

SUCKLING BEEF COWS – PROGESTERONE PROFILES

Currently, there is a limited amount of evidence about the effectiveness of one or two $PGF_{2\alpha}$ injections on P_4 concentrations for suckling beef cows subjected to a 5-day CIDR-

Cosynch protocol. It does appear that P_4 concentrations at the $PGF_{2\alpha}$ injection during a TAI estrous synchronization protocol are less important on maximizing fertility in beef cows when compared with dairy cows (Stevenson and Lamb, 2016). However, the ability of $PGF_{2\alpha}$ to cause complete luteolysis and reduce P_4 concentrations to less than 0.5 ng/mL by the time of AI does aid in maximizing the fertility of beef cows (Stevenson and Lamb, 2016). To our knowledge there are only two studies (Kasimanickam et al., 2009; Bridges et al., 2015) that have examined the effects of one or two $PGF_{2\alpha}$ injections in a 5-day CIDR-Cosynch protocol on P/AI in beef cows, and only one study examined P_4 concentrations at $PGF_{2\alpha}$ and AI to determine luteolysis (Bridges et al., 2015).

A study by Kasimanickam et al. (2009) found P/AI for TAI cows were greater for two $PGF_{2\alpha}$ injections (69%) given 7 hours apart compared with only one $PGF_{2\alpha}$ injection (52%) at CIDR removal in a 5-day CIDR-Cosynch protocol. Nevertheless, this study did not measure P_4 concentrations, and therefore the relationship between improved P/AI and complete luteolysis cannot be established. In a more recent study by Bridges et al. (2015), beef cows were treated with one $PGF_{2\alpha}$, a double dose (50 mg) of $PGF_{2\alpha}$, or two $PGF_{2\alpha}$ injections administered 8 hours apart in a 5-day CIDR-Cosynch protocol. In this study, luteolysis was defined as P_4 concentrations > 2 ng/mL at CIDR removal and < 1 ng/mL at the time of AI. These researchers found that luteolysis tended to differ between groups with two $PGF_{2\alpha}$ injections (97%) 8 hours apart having greater luteolysis when compared to a double dose (88%) or one $PGF_{2\alpha}$ (87%) injection at CIDR removal. Additionally, these researchers observed a difference in P/AI between treatments. The P/AI were greater for the two $PGF_{2\alpha}$ (55%) and double dose $PGF_{2\alpha}$ (51%) when compared with the one $PGF_{2\alpha}$ (48%) treatment. Although that study concluded that P/AI were superior in two $PGF_{2\alpha}$ injections given 8 hours apart or simultaneously, there was a farm effect. Only 6 out of 13 farms had greater P/AI for two $PGF_{2\alpha}$ injections given 8 hours apart compared with the one $PGF_{2\alpha}$ treatment. In addition, in 5 of the 13 farms, one $PGF_{2\alpha}$ had similar or even greater P/AI when compared with two $PGF_{2\alpha}$ injections. Therefore, it is not clear if two $PGF_{2\alpha}$ are needed in a 5-day CIDR-Cosynch protocol for beef cows, nor can it be determined that the improvement in P/AI is due to complete luteolysis.

Our study provides the first evidence on P_4 concentration profiles, and ovarian dynamics following one $PGF_{2\alpha}$, two $PGF_{2\alpha}$ 12 hours apart, or one HighCon in suckling beef

cows subjected to a 5-day CIDR-Cosynch protocol. These results provide evidence the administration one or two doses of conventional $PGF_{2\alpha}$, or one HighCon $PGF_{2\alpha}$ do not differ in serum P_4 concentrations by the time of AI. Although one $PGF_{2\alpha}$ does not appear to be as effective as two $PGF_{2\alpha}$ or one HighCon $PGF_{2\alpha}$ in rapidly reducing P_4 concentrations, the P_4 concentrations in all treatments were ≤ 0.5 ng/mL. Interestingly, one injection of HighCon is as effective as two $PGF_{2\alpha}$ in causing complete luteolysis and lowering P_4 concentrations rapidly. Thus, the apparent improved P/AI in cows that received two $PGF_{2\alpha}$ in some studies (Kasimanickam et al., 2009; Bridges et al., 2012) may not be due to reducing P_4 by the time of AI, but rather other underlying factors such as the rate of P_4 decline that influence P/AI outcomes.

Our findings are similar to those observed by Ahmadzadeh et al. (2011). In that study, the researchers investigated the effects of one $PGF_{2\alpha}$ (25 mg), 1.5 $PGF_{2\alpha}$ (37.5 mg) administered once, or two 0.5 $PGF_{2\alpha}$ (25 mg total) injections given 7 hours apart following CIDR removal in a 5-d CIDR-Cosynch protocol in beef cows (Ahmadzadeh et al., 2011). Progesterone concentrations were measured at CIDR insertion and at the time of AI. Ahmadzadeh et al. (2011) found no difference in P_4 concentrations regardless of treatment, and all treatments had < 0.5 ng/mL by the time of AI.

These experiments provide evidence on P_4 concentration profiles following one or two (12 hours apart) conventional $PGF_{2\alpha}$, or one high-concentrated, longer-lasting $PGF_{2\alpha}$ in lactating dairy and suckling beef cows subjected to a 5-day CIDR-Cosynch protocol. Furthermore, it appears that HighCon (dinoprost tromethamine), is as effective as one $PGF_{2\alpha}$, or two $PGF_{2\alpha}$ (12 hours apart) injections in beef cows and as effective as two $PGF_{2\alpha}$ injections in dairy cows in decreasing progesterone concentrations (causing complete luteolysis) by the time of artificial insemination when subjected to a 5-day CIDR-Cosynch.

The investigation of the use of one or more doses of $PGF_{2\alpha}$ in the 5-day CIDR-Cosynch protocol was developed to gain further insight concerning luteolysis and P_4 concentrations following $PGF_{2\alpha}$ treatment, and to weigh the potential influence on pregnancy against additional cattle handling and expenses associated with a second $PGF_{2\alpha}$. Considering P_4 concentrations and its relationship to fertility, the administration of one $PGF_{2\alpha}$ is more practical for synchronizing dairy and beef cows, as it reduces animal handling, labor, and drug costs. Further, the addition of a second $PGF_{2\alpha}$ treatment within the

5-day CIDR-Cosynch program increases labor associated with animal handlings, and may decrease protocol compliance, ultimately reducing the potential benefits of two $PGF_{2\alpha}$ injections in a 5-day CIDR-Cosynch protocol. Future research is needed to compare the effects of P_4 profiles on P/AI in order to further support the use of one high concentrated $PGF_{2\alpha}$ injection in a 5-day CIDR-Cosynch protocol for dairy and beef cows.

REFERENCES

Ahmadzadeh, A., D. Gunn, J. B. Hall, and B. Glaze. 2015. Evaluation of 5-day versus 7-day CIDR treatment on reproductive outcomes of beef heifers using a modified timed-AI protocol. J. Anim. Sci. 88(Suppl. 1):394.

Ahmadzadeh, A., K. Carnahan, T. Robison, and C. Autran. 2011. Effect of various doses of prostaglandin $F_{2\alpha}$ on estrous behavior and blood progesterone in beef cows. J. Anim. Sci. 89:E-Suppl 1, 253.

Archbald, L.F., C. Risco, P. Chavatte, S. Constant, T. Tran, E. Klapstein, and J. Elliot. 1993. Estrus and pregnancy rate of dairy cows given one or two doses of prostaglandin F2 alpha 8 or 24 hours apart. Theriogenology 40:873-884.

Bello, N. M., J. P. Steibel, and J. R. Pursley. 2006. Optimizing ovulation to first GnRH improved outcomes to each hormonal injection of Ovsynch in lactating dairy cows. J. Dairy Sci. 89:3413-3424.

Bilby, T. R., R. G. S. Bruno, K. J. Lager, R. C. Chebel, J. G. N. Moraes, P. M. Fricke, G. Lopes Jr., J. P. Giordano, J. E. P. Santos, F. S. Lima, J. S. Stevenson, and S. L. Pulley. 2013. Supplemental progesterone and timing of resynchronization on pregnancy outcomes in lactating dairy cows. J. Dairy Sci. 96:7032-7042.

Bisinotto, R. S., L. O. Castro, M. B. Pansani, C. D. Narciso, N. Martinez, L. D. P. Sinedino, T. L. C. Pinto, N. S. Van de Burgwal, H. M. Bosman, R. S. Surjus, W. W. Thatcher, and J. E. P. Santos. 2015a. Progesterone supplementation to lactating dairy cows without a corpus luteum at initiation of the Ovsynch protocol. J. Dairy Sci. 98:2515-2528.

Bisinotto, R. S., I. J. Lean, W. W. Thatcher, and J. E. P. Santos. 2015b. Meta-analysis of progesterone supplementation during timed artificial insemination programs in dairy cows. J. Dairy Sci. 98:2472-2487.

Bisinotto, R. S., E. S. Ribeiro, F. S. Lima, N. Martinez, L. F. Greco, L. F. S. P. Barbosa, P. P. Bueno, L. F. S. Scagion, W. W. Thatcher, and J. E. P. Santos. 2013. Targeted progesterone supplementation improves fertility in lactating dairy cows without a corpus luteum at the initiation of the timed artificial insemination protocol. J. Dairy Sci. 96(4):2214-2225.

Bisinotto, R. S., E. S. Ribeiro, L. T. Martins, R. S. Marsola, L. F. Greco, M. G. Favoreto, C. A. Risco, W. W. Thatcher, and J. E. P. Santos. 2010. Effect of interval between induction of ovulation and artificial insemination (AI) and supplemental progesterone for resynchronization on fertility of dairy cows subjected to a 5-d timed AI program. J. Dairy Sci. 93(12):5798-5808.

Bridges, G. A., L. A. Helser, D. E. Grum, M. L. Mussard, C. L. Gasser, and M. L. Day. 2008. Decreasing the interval between GnRH and $PGF_{2\alpha}$ from 7 to 5 days and

lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology 69:843-851.

Bridges, G. A., J. K. Ahola, C. Brauner, L. H. Cruppe, J. C. Currin, M. L. Day, P. J. Gunn, J. R. Jaeger, S. L. Lake, G. C. Lamb, and G. H. L. Marquezini. 2012. Determination of the appropriate delivery of prostaglandin F2α in the five-day CO-Synch+ controlled intravaginal drug release protocol in suckled beef cows. J. Anim. Sci. 90:4814-4822.

Brusveen, D. J. A., P. Cunha, C. D. Silva, P. M. Cunha, R. A. Sterry, E. P. Silva, J. N. Guenther, and M. C. Wiltbank. 2008. Altering the time of the second gonadotropin-releasing hormone injection and artificial insemination (AI) during Ovsynch affects pregnancies per AI in lactating dairy cows. J. Dairy Sci. 91:1044:1052.

Carvalho, P. D., M. J. Fuenzalida, A. Ricci, A. H. Souza, R. Barletta, M. C. Wiltbank, and P. M. Fricke. 2015a. Modifications of Ovsynch improve fertility during resynchronization: Evaluation of presynchronization with gonadotropin-releasing hormone 6 d before initiation of Ovsynch and addition of a second prostaglandin $F_{2\alpha}$ treatment. J. Dairy Sci. 98:8741-8752.

Carvalho, P. D., A. H. Souza, M. C. Wiltbank, and P. M. Fricke. 2015b. Progesterone concentration at each treatment during an Ovsynch protocol affects fertility to timed AI in lactating Holstein cows. J. Dairy Sci. 98(Suppl. 2):92. (Abstr.)

Cerri, R. L., H. M. Rutigliano, R. G. S. Bruno, and J. E. Santos. 2009a. Progesterone concentration, follicular development and induction of cyclicity in dairy cows receiving intravaginal progesterone inserts. Anim. Reprod. Sci. 110(1-2):56-70.

Cerri, R. L. A., H. M. Rutigliano, R. C. Chebel, and J. E. P. Santos. 2009b. Period of dominance of the ovulatory follicle influences embryo quality in lactating dairy cows. Soc. Reprod. Fertil. 137:813-823.

Cerri, R. L. A., J. E. P. Santos, S. O. Juchem, K. N. Galvão, and R. C. Chebel. 2004. Timed artificial insemination with estradiol cypionate or insemination at estrus in high-producing dairy cows. J. Dairy Sci. 87:3704-3715.

Chebel, R. C., M. J. Al-Hassan, P. M. Fricke, J. E. P. Santos, J. R. Lima, C. A. Martel, J. S. Stevenson, R. Garcia, and R. L. Ax. 2010. Supplementation of progesterone via controlled internal drug release inserts during ovulation synchronization protocols in lactating dairy cows. J. Dairy Sci. 93(3):922-931.

Chebel, R. C., J. E. P. Santos, R. L. Cerri, H. M. Rutigliano, and R. G. S. Bruno. 2006. Reproduction in dairy cows following progesterone insert presynchronization and resynchronization protocols. J. Dairy Sci. 89(11):4205-4219.

Chenault, J. R., J. F. Boucher, K. J. Dame, J. A. Meyer, and S. L. Wood-Follis. 2003. Intravaginal progesterone insert to synchronize return to estrus of previously inseminated dairy cows. J. Dairy Sci. 86:2039-2049.

Colazo, M. G., I. L. Helguera, A. Behrouzi, D. J. Ambrose, and R. J. Mapletoft. 2017. Relationship between circulating progesterone at timed-AI and fertility in dairy cows subjected to GnRH-based protocols. Theriogenology 94:15-20.

Colazo M. G., J. A. Small, D. R. Ward, N. E. Erickson, J. P. Kastelic, and R. J. Mapletoft. 2004. The effect of presynchronization on pregnancy rate to fixed-time AI in beef heifers subjected to a Cosynch protocol. Reprod. Fertil. Dev. 16:128.

de Vries, A., and C. A. Risco. 2005. Trends and seasonality of reproductive performance in Florida and Georgia dairy herds from 1976 to 2002. J. Dairy Sci. 88:3155-3165.

Dewey, S. T., L. G. Mendonça, G. Lopes Jr., F. A. Rivera, F. Guagnini, R. C. Chebel, and T. R. Bilby. 2010. Resynchronization strategies to improve fertility in lactating dairy cows utilizing a presynchronization injection of GnRH or supplemental progesterone: I. Pregnancy rates and ovarian responses. J. Dairy Sci. 93:4086–4095.

Diaz, F. J., L. E. Anderson, Y. L. Wu, A. Rabot, S. J. Tsai, and M. C. Wiltbank. 2002. Regulation of progesterone and prostaglandin F-2 alpha production in the CL. Mol. Cell. Endocrinol. 191:65–80.

El-Zarkouny, S. Z., J. A. Cartmill, B. A. Hensley, and J. S. Stevenson. 2004. Pregnancy in dairy cows after synchronized ovulation regimens with or without presynchronization and progesterone. J. Dairy Sci. 87:1024-1037.

Ferraz Junior, M. V. C., A. V. Pires, M. V. Biehl, M. H. Santos, D. M. Polizel, D. D. Nepomuceno, R. Sartori, J. B. B. Filho, J. R. S. Gonçalves, and M. L. Day. 2016. Luteolysis in Bos indicus cows on Days 5 and 7 of the estrous cycle with varying doses of $PGF_{2\alpha}$. Theriogenology 86:1268-1274.

Galvão, K. N., M. F. Sá Filho, and J. E. P. Santos. 2007. Reducing the interval from presynchronization to initiation of timed AI improves fertility in dairy cows. J. Dairy Sci. 90:4212-4218.

Galvão, K. N., J. E. Santos, S. O. Juchem, R. L. Cerri, A. C. Coscioni, and M. Villaseñor. 2004. Effect of addition of a progesterone intravaginal insert to a timed insemination protocol using estradiol cypionate on ovulation rate, pregnancy rate, and late embryonic loss in lactating dairy cows. J. Anim. Sci. 82:3508–3517.

Games, P.A. 1971. Multiple comparisons of means. American Educational Research Journal 8(3):531-565.

Geary, T. W., and J. C. Whittier. 1998. Effects of timed insemination following synchronization of ovulation using the Ovsynch or CO-synch protocol in beef cows. PAS. 14(4), 217-220.

Ginther, O. J., M. C. Wiltbank, P. M. Fricke, J. R. Gibbons, and K. Kot. 1996. Selection of the dominant follicle in cattle. Biol. Reprod. 55:1187-1194.

Giordano, J. O., M. C. Wiltbank, P. M. Fricke, S. Bas, R. Pawlisch, J. N. Guenther, and A. B. Nascimento. 2014. Effect of increasing GnRH and PGF2α dose during Double-Ovsynch on ovulatory response, luteal regression, and fertility of lactating dairy cows. Theriogenology 80:773–783.

Giordano, J. O., M. C. Wiltbank, J. N. Guenther, R. Pawlisch, S. Bas, A. P. Cunha, P. M. Fricke. 2012. Increased fertility in lactating dairy cows resynchronized with Double-Ovsynch compared with Ovsynch initiated 32 d after timed artificial insemination. J. Dairy Sci. 95:639–653.

Hermas, S. A., C. W. Young, and J. W. Rust. 1987. Effects of mild inbreeding on productive and reproductive performance of Guernsey cattle. J. Dairy Sci. 70:712-715.

Inskeep, E. K. 1973. Potential uses of prostaglandins in control of reproductive cycles of domestic animals 1. J. Anim. Sci. 36(6):1149-1157.

Inskeep, E. K. 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. J. Anim. Sci. 82 (E-Suppl):E24-39.

Johnson S. K., and R. Jones. 2004. Costs and comparisons of estrous synchronization systems. Proceed. Applied Reproductive Strategies in Beef Cattle, 103-115.

Kasimanickam, R., M. L. Day, J. S. Rudolph, J. B. Hall, and W. D. Whittier. 2009. Two doses of prostaglandin improve pregnancy rates to timed-AI in a 5-day progesterone-based synchronization protocol in beef cows. Theriogenology 71(5):762-767.

Lamb, G. C., C. R. Dahlen, J. E. Larson, G. Marquezini, and J. S. Stevenson. 2010. Control of the estrous cycle to improve fertility for fixed-time artificial insemination in beef cattle: A review 1. J. Anim. Sci. 88(13):181-192.

Lamb, G. C., J. S. Stevenson, D. J. Kesler, H. A. Garverick, D. R. Brown, and B. E. Salfen. 2001. Inclusion of an intravaginal progesterone insert plus GnRH and prostaglandin F2α for ovulation control in postpartum suckled beef cows. J. Anim. Sci. 79(9):2253-2259.

Lansford, A. C., T. L. Meyer, and R. N. Funston. 2018. Comparison of two alternative prostaglandin products in yearling beef heifers. Nebraska Beef Cattle Report 12-14.

Larson, J. A., G. C. Lamb, J. S. Stevenson, S. K. Johnson, M. L. Day, T. W. Geary, D. J. Kesler, J. M. DeJarnette, F. N. Schrick, A. DiCostanzo, and J. D. Arseneau. 2006. Synchronization of estrus in suckled beef cows for detected estrus and artificial insemination and artificial insemination using gonadotropin-releasing hormone, prostaglandin F, and progesterone. J. Anim. Sci. 84(2):332-342.

Lima, F.S., E.S. Ribeiro, R.S. Bisinotto, L.F. Greco, N. Martinez, M. Amstalden, W.W. Thatcher, and J.E.P. Santos. 2013. Hormonal manipulations in the 5-day timed artificial insemination protocol to optimize estrous cycle synchrony and fertility in

dairy heifers. J. Dairy Sci. 96:7054-7065.

Lima, J. R., F. A. Rivera, C. D. Narciso, R. Oliveira, R. C. Chebel, J. E. P. Santos. 2009. Effect of increasing amounts of supplemental progesterone on timed artificial insemination protocol on fertility of lactating dairy cows. J. Dairy Sci. 92:5436-5446.

Lopes, Jr. G., C. R. Johnson, L. G. D. Mendonça, P. B. Silvia, J. G. N. Moraes, A. Ahmadzadeh, J. C. Dalton, and R. C. Chebel. 2013. Evaluation of reproductive and economic outcomes of dairy heifers inseminated at induction of estrus or at fixed time after a 5-day or 7-day progesterone insert-based ovulation synchronization protocol. J. Dairy Sci. 96:1612-1622.

Lucy, M. C. 2001. Reproductive loss in high-producing dairy cattle: where will it end? J. Dairy Sci. 84:1277-1293.

Martins, J. P. N., R. K. Policelli, L. M. Neuder, W. Raphael, and J. R. Pursley. 2011. Effects of cloprostenol sodium at final prostaglandin $F_{2\alpha}$ of Ovsynch on complete luteolysis and pregnancy per artificial insemination in lactating dairy cows. J. Dairy Sci. 94:2815-2824.

McCracken, J. A., E. E. Custer, and J. C. Lamsa. 1999. Luteolysis: A neuroendocrine-mediated event. Physiol. Rev. 79(2):263-323.

Miyamoto, A., K. Shirasuna, and K. Sasahara. 2009. Local regulation of corpus luteum development and regression in the cow: impact of angiogenic and vasoactive factors. Domest. Anim. Endocrin. 37(3):159-169.

Nebel, R. L., and S. M. Jobst. 1998. Evaluation of systematic breeding programs for lactating dairy cows: a review. J. Dairy Sci. 81:1169-1174.

Norman, H. D., J. R. Wright, S. M. Hubbard, R. H. Miller, and J. L. Hutchison. 2009. Reproductive status of Holstein and Jersey cows in the United States. J. Dairy Sci. 92(7):3517-3528.

NRC. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Press, Washington DC; 2001.

Oosthuizen, N., L. B. Canal, P. L. P. Fontes, C. D. Sanford, D. DiLorenzo, C. R. Dahlen, and G. C. Lamb. 2017. Administration of subcutaneous high concentrate prostaglandin F2alpha in replacement beef heifers and the effects on estrus response and pregnancy rates. J. Dairy Sci. 95(Suppl. 4):228.

Peterson, C., A. Alkar, S. Smith, S. Kerr, J. B. Hall, D. Moore, and R. Kasimanickam. 2011. Effects of one versus two doses of prostaglandin F2alpha on AI pregnancy rates in a 5-day, progesterone-based, CO-Synch protocol in crossbred beef heifers. Theriogenology 75(8):1536-1542.

Pursley, J. R., M. C. Wiltbank, J. S. Stevenson, J. S. Ottobre, H. A. Garverick, and L. L. Anderson. 1997. Pregnancy rates per artificial insemination for cows and heifers inseminated at a synchronized ovulation or synchronized estrus1. J. Dairy Sci. 80(2):295-300.

Pursley, J. R., R. W. Silcox, and M. C. Wiltbank. 1998. Effect of time of artificial insemination on pregnancy rates, calving rates, pregnancy loss, and gender ratio after synchronization of ovulation in lactating dairy cows. J. Dairy Sci. 81:2139-2144.

Rabaglino, M. B., C. A. Risco, M. J. Thatcher, I. H. Kim, J. E. P. Santos, and W. W. Thatcher. 2010. Application of one injection of prostaglandin $F_{2\alpha}$ in the five-day Co-Synch + CIDR protocol for estrous synchronization and resynchronization of dairy heifers. J. Dairy Sci. 93:1050-1058.

Rathbone, M. J., C. R. Bunt, C. R. Ogle, S. Burggraaf, K. L. Macmillan, C. R. Burke, and K. L. Pickering. 2002. Reengineering of a commercially available bovine intravaginal insert (CIDR insert) containing progesterone. J. Controlled Release 85:105-115.

Repasi, A., J. F. Beckers, J. Sulon, A. Karen, J. Reiczigel, and O. Szenci. 2005. Effect of the type and number of prostaglandin treatments on corpus luteum, the largest follicle and progesterone concentrations in dairy cows. Reprod. Domest. Anim. 40:436-42.

Ribeiro, E. S., R. S. Bisinotto, M. G. Favoreto, L. T. Martins, R. L. A. Cerri, F. T. Silvestre, L. F. Greco, W. W. Thatcher, and J. E. P. Santos. (2012). Fertility in dairy cows following presynchronization and administering twice the luteolytic dose of prostaglandin F 2α as one or two injections in the 5-day timed artificial insemination protocol. Theriogenology 78(2):273-284.

SAS Institute. 2015. SAS 9.4, Version 2. SAS Institute Inc. Cary, NC.

Santos, J. E. P., C. D. Narciso, F. Rivera, W. W. Thatcher, and R. C. Chebel. 2010. Effect of reducing the period of follicle dominance in a timed artificial insemination protocol on reproduction of dairy cows. J. Dairy Sci. 93:2976-2988.

Savio, J. D., W. W. Thatcher, G. R. Morris, K. Entwistle, M. Drost, and M. R. Mattiacci. 1993. Effects of induction of low plasma progesterone concentrations with a progesterone-releasing intravaginal device on follicular turnover and fertility in cattle. J. Reprod. Fertil. 98:77-84.

Senger, P. L. (2012). Pathways to Pregnancy and Parturition. (3rd Ed.). Redmond, OR: Current Conceptions, Inc.

Silvia, W. J., G. S. Lewis, J. A. McCracken, W. W. Thatcher, and L. Jr. Wilson. 1991. Hormonal regulation of uterine secretion of prostaglandin F2α during luteolysis in ruminants. Biol. Reprod. 45(5):655-663.

Souza, A. H., A. Gümen, E. P. B. Silva, A. P. Cunha, J. N. Guenther, C. M. Peto, D. Z. Caraviello, and M. C. Wiltbank. 2007. Supplementation with estradiol-17β before

the last gonadotropin-releasing hormone injection of the Ovsynch protocol in lactating dairy cows. J. Dairy Sci. 90:4623-4634.

Stevenson, J. S. 2016. Physiological predictors of ovulation and pregnancy risk in a fixed-time artificial insemination program. J. Dairy Sci. 99:1007-10092.

Stevenson, J. S., and G. C. Lamb. 2016. Contrasting effects of progesterone on fertility of dairy and beef cows. J. Dairy Sci. 99:5951-5964.

Stevenson, J. S., G. C. Lamb, S. K. Johnson, M. A. Medina-Britos, D. M. Grieger, K. R. Harmoney, J. A. Cartmill, S. Z. El-Zarkouny, C. R. Dahlen, and T. J. Marple. 2003. Supplemental norgestomet, progesterone, or melengestrol acetate increases pregnancy rates in suckled beef cows after timed inseminations 1. J. Anim. Sci. 81(3):571-586.

Stevenson, J. S., M. O. Mee, and R. E. Stewart. 1989. Conception rates and calving intervals after prostaglandin F2α or prebreeding progesterone in dairy cows. J. Dairy Sci. 72(1):208-218.

Stevenson, J. S., D. E. Tenhouse, R. L. Krisher, G. C. Lamb, J. E. Larson, C. R. Dahlen, J. R. Pursley, N. M. Bello, P. M. Fricke, M. C. Wiltbank, D. J. Brusveen, M. Burkhart, R. S. Youngquist, and H. A. Garverick. 2008. Detection of anovulation by heatmount detectors and transrectal ultrasonography before treatment with progesterone in a timed insemination protocol. J. Dairy Sci. 91:2901–2915.

Taylor, C. and R. Rajamahendran. 1991. Follicular dynamics, corpus luteum growth and regression in lactating dairy cattle. Canadian J. Anim. Sci. 71(1):61-68.

Tsai, S. J., and M. C. Wiltbank. 1998. Prostaglandin F2alpha regulates distinct physiological changes in early and mid-cycle bovine corpora lutea. Biol. Reprod. 58:346-352.

Vasconcelos, J. L. M., R. W. Silcox, G. L. M. Rosa, J. R. Pursley, and M. C. Wiltbank. 1999. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology 52:1067-1078.

Washburn, S. P., W. J. Silvia, C. H. Brown, B. T. McDaniel, and A. J. McAllister. 2002. Trends in reproductive performance in Southeastern Holstein and Jersey DHI herds. J. Dairy Sci. 85:244–251.

Weems, C. W., Y. S. Weems, and R. D. Randel. 2006. Prostaglandins and reproduction in female farm animals. Vet. J. 171:206-228.

Whittier, W. D., R. K. Kasimanickam, J. F. Currin, H. H. Schramm, and M. Vlcek. 2010. Effect of timing of second prostaglandin F 2 alpha administration in a 5-day, progesterone-based CO-Synch protocol on AI pregnancy rates in beef cows. Theriogenology 74:1002-9.

Wilson, D. J., D. A. Mallory, D. C. Busch, N. R. Leitman, J. K. Haden, D. J. Schafer, M. R. Ellersieck, M. F. S. Smith, and D. J. Patterson. 2010. Comparison of short-term progestin-based protocols to synchronize estrus in postpartum beef cows. J. Anim. Sci. 88(6):2045-54.

Wiltbank, M.C., and J. R. Pursley. 2014. The cow as an induced ovulator: Timed AI after synchronization of ovulation. Theriogenology 81:170–185.

Wiltbank, M. C., A. H. Souza, P. D. Carvalho, A. P. Cunha, J. O. Giordano, P. M. Fricke, G. M. Baez, and M. G. Diskin. 2014. Physiological and practical effects of progesterone on reproduction in dairy cattle. Animal 8:70–81.

Xu, Z. Z, and L. J. Burton. 2000. Estrus synchronization of lactating dairy cows with GnRH, progesterone and prostaglandin $F_{2\alpha}$. J. Dairy Sci. 83:471-476.

Xu, Z. Z., L. J. Burton, and K. L. Macmillan. 1997. Treatment of post-partum anoestrous dairy cows with progesterone, oestradiol and equine chorionic gonadotrophin. New Zealand Vet. J. 45(5):205-207.

Zoetis Animal Health. 2018. Lutalyse (HighCon) for Cattle. Valley Vet Supply. Accessed May 5, 2018. https://www.valleyvet.com/ct_detail.html?pgguid=30428b54-40a4-43da-b17f-2fea1b9cee59.

APPENDIX 1

CHAPTER 1
Preparation and Preparation of Cell Culture Materials

Ham's F-12 Media

1. Place 900 mL of distilled distilled water in 1,000 mL beaker
2. Put on stir plate with stir stick on level 5
3. Pour in one package of powdered medium (10.6 g) from fridge
4. Fill beaker to 1,000 mL with distilled water
5. Add 1.176 g of sodium bicarbonate
6. Mix until dissolved but do not over stir
7. pH to 7.1
8. Filter sterilize into two 500 mL bottles under cell culture hood

Minimum Essential Medium (MEM)

1. Place 900 mL of distilled water in 1,000 mL beaker
2. Put on stir plate with stir stick on level 5
3. Pour in one package of powdered medium (9.53 g) from fridge
4. Fill beaker to 1,000 mL with distilled water
5. Add 2.2 g of sodium bicarbonate
6. Mix until dissolved but do not over stir
7. pH to 7.1
8. Filter sterilize into two 500 mL bottles under cell culture hood

Complete Media

1. 400 mL Ham's F-12
2. 400 mL MEM
3. 100 mL Equine Serum
4. 100 mL Fetal Bovine Serum
5. 746 µl Insulin
6. 500 µl D-valine
7. 10 mL antibiotic-antimycotic (ABAM)
    - pH to 7.3 and filter sterilize under hood.
    - Place in fridge.

Phosphate-buffered saline (PBSα)

1. Measure to one liter on stir plate with a stir bar.
2. Pour some distilled water out and mix in the following
    a. Sodium chloride 10 g
    b. Potassium chloride 0.224 g
    c. Sodium dibasic phosphate 1.42 g
    d. Potassium phosphate monobasic 0.272 g
3. Once mixed in, fill to one liter.
4. pH solution to 7.4 and autoclave for 45 minutes at 121°F.

10× Tris Buffered Saline (TBS)

1. For 500 mL add:
    a. Tris Base 15.15 g
    b. Sodium chloride 38 g
2. pH solution to 7.4.

1× Tris Buffered Saline (TBS)

1. 450 mL of distilled water
2. 50 mL 10× TBS

1× Tris Buffered Saline with Tween 20 (TBST)

1. 450 mL of distilled water
2. 50 mL 10× TBS
3. 500 µl Tween 20

Western Running Buffer

1. Make sure wearing gloves and mask.
2. For one liter of 10× Separating Buffer add 900 mL distilled water and:
    a. Tris base 30.2 g
    b. Glycine 144 g
    c. Sodium Dodecyl Sulfate (SDS) 10 g
        i. Make sure solution is mixed well and fill to one liter.
3. For one liter 1× Seperating Buffer:
    a. 100 mL 10× Running Buffer
    b. 900 mL distilled water

Western Transfer Buffer

1. For one liter, add the following into 700 mL distilled water:
    a. Tris base 3.03 g
    b. Glycine 14.4 g
        i. Make sure solution is mixed well and fill to 800 mL.
2. Add 200 mL methanol and store in 4°C.

Progesterone (P_4) Stock

1. Mother stock (P_4 10^{-3} M):
    a. 31.446 g of P_4
    b. 100 mL distilled water
    c. Filter sterilize under cell culture hood
2. Intermediate stock (P_4 10^{-5} M):
    a. 1 mL of P_4 10^{-3} M
    b. 99 mL complete media
3. Culture stock (P_4 10^{-7} M):
    a. 495 mL complete media
        i. pH to 7.1 as P_4 will increase culture media and want to be 7.3.
    b. 5 mL intermediate stock (P_4 10^{-5} M)

Urea

1. Make stock of 1000 m*M*. Molecular weight is 60.06 g/L (or *M*), therefore, 0.6006 g urea in 10 mL distilled water.
    a. Weight out urea, use gloves because it is an irritant.
    b. Add to small beaker and use pipette to add 10 mL distilled water.
    c. Place on stirring plate with stirring bar.
        i. Takes awhile to go into solution.
    d. Filter sterilize using a syringe into a 15 mL conicle tube and label.

Dimethadione (DMD)

1. Add 2.5 mL of autoclaved sterile water to a 15 mL conicle tube and mark line.
2. Add 0.645 g of DMD to conicle tube.
3. Add water back into 15 mL conicle tube to line.
4. Vortex tube until DMD is completely dissolved in the sterile water.
5. If it does not mix heat in the water bath for a few seconds and continue to vortex.

Measuring pH (every 6 hours)

1. Wipe pH meter down with 70% ethanol and place under hood.
    a. Calibrate and record slope.
    b. Dip probe in 15 mL conicle tube containing 5 mL PBSα.
    c. Wipe with large kim wipe.
    d. Dip probe in 15 mL conicle tube containing 5 mL of 70% ethanol.
    e. When ethanol is dry tilt plate away and place probe in plate, completely submerge.
    f. Record pH
    g. Dip probe in PBSα, wipe and then in 70% ethanol.
2. Record all plates without IFNτ and then all with IFNτ and go from low to high DMD concentrations (0, 10, 15, and 20 m*M*).
3. Repeat for all plates two times for each plate.

APPENDIX 2

CHAPTER 1
Preliminary Study

CELL VIABILITY

A preliminary experiment was performed to assess the viability and morphology of BEND cells in the presence of various urea concentrations and various pH levels of the media after incubation (0, 2, 4, 6, 8, 10, 12 hours) at 37°C and 5% CO_2. Cells were placed in media containing 0, 2.5, 5 or 10 m*M* of urea, which correspond to physiological BUN concentrations in cows, and fed low (~14% CP) to high (≥ 20% CP) dietary protein, respectively (Ferguson et al., 1988; Butler et al., 1996; Wright et al., 1998; Ocon and Hansen, 2003; Rhoads et al., 2006). Cells were examined (photomicroscopy) using an optical microscope every 2 hours from 0 to 12 hours of incubation with urea. Urea at all concentrations did not significantly change the pH of the media. To regulate extracellular pH, DMD was added to the culture medium. As indicated earlier, DMD is a non-metabolizable weak acid that lowers the pH of the culture medium and has been used to study the effects of pH on development embryos *in vitro* (Bavister et al., 1983; Carney and Bavister, 1987; Edwards et al., 1998; Ocon and Hansen, 2003). Cells were placed in media containing 0, 10, 15, 20, and 40 m*M* DMD, and media was tested for pH and cells were examined after 0, 2, 4, 6, 8, 10, and 12 hours of incubation.

To further assess the effect of urea and pH on BEND cell viability, CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was used. Viable cells were determined by quantification of ATP, which indicates the metabolic activity of cells. The amount of ATP was determined using a thermostable luciferase (UltraGlo™ Recombinant Luciferase). Cells were grown in opaque tissue culture-treated 96 well plates for 24 hours, and thereafter treated with culture media containing urea (0, 5, 10 m*M*) or DMD (0, 15, 20 m*M*, corresponding to media pH 7.3, 6.9, and 6.8). Cells were incubated for 24 or 48 hours. Subsequently, cells were processed by adding reagent (50 μL)/well and shaken for 2 minutes to induce cell lysis. After a 10-minute stabilization period, the plates were read in a Victor2 plate reader (PerkinElmer, MA).

Urea Results

Based on photomicroscopy, cell growth (based on rate of proliferation) and morphology (determined by visualization of cell structure and integrity) were not altered by any concentration of urea tested (0, 2.5, 5 or 10 m*M*). These urea concentrations did not alter the media pH. The results from the cell viability assay indicated that there was no detrimental effect of urea (P = 0.65) on cell viability [167, 140, 188, 206 (SE ± 29) mmol ATP for 0, 2.5, 5 or 10 m*M* urea, respectively].

pH Results

The pH values for media with 0, 10, 15, 20, and 40 m*M* of DMD were 7.3, 7.1, 6.9, 6.8, and 6.4, respectively. Photomicroscopy results indicated that 40 m*M* DMD was detrimental to cell viability as cell growth was inhibited and morphology was abnormal; thus that concentration was not used in cell viability assay. Based on the cell viability assay, cell growth and morphology were not altered by 0 to 20 m*M* DMD concentrations. As previously discussed a pH of 6.9 was achieved when a 19% crude protein diet was fed and BUN concentration was 8.9 m*M* (Elrod and Butler, 1993; Elrod et al., 1993; Dawuda et al., 2002). There was a time × concentration interaction effect ($P < 0.05$) on cell viability. At any concentration, DMD did not affect cell viability during 24 hours incubation [205, 157, 227 (S.E. ± 80) mmol ATP] for 0, 15, and 20 m*M* DMD, respectively. However, cell viability was negatively affected by all DMD concentrations at 48 hours [806, 218, 170 (SE ± 80) mmol ATP].

BEND CELL RESPONSE TO IFNτ

In an additional study, the ability of BEND cells to express Mx1and ISG-15 proteins in response to IFNτ was examined. Bovine endometrial cells were cultured in T_{75} flasks for 0, 8, 16, 24, 32, 40, and 48 hours with 10,000 IU of IFNτ. Cells were harvested by the addition of Protein Extraction Reagent (M-PER, Pierce, Rockford, IL), placed on a rocker plate for 15 minutes, collected and placed in -20ºC. Post-collection cell lysates were quantified using a BCA™ Protein Assay Kit (Pierce, IL), and all time points were subjected to a Western blot procedure to detect Mx1 or ISG-15 expression as described in the experimental procedures (see Chapter 1 materials and methods).

Protein Expression

Figure 1 illustrates the presence of Mx1 (70 KDa) in BEND cells every 8 hours for

48 hours after cells were challenged with 10,000 IU recombinant IFNτ. However, Mx1 expression did not appear to be strong at 8 hours post IFNτ challenge. The expression of Mx1 appeared to increase over time and was maximal at 24 and 32 hours. Moreover, we were able to demonstrate (as shown by others; Austin et al., 1996; Johnson et al., 1999; Vallet et al., 1991) that ISG-15 production was induced by IFNτ (10,000 IU) in BEND cells (Figure 2).

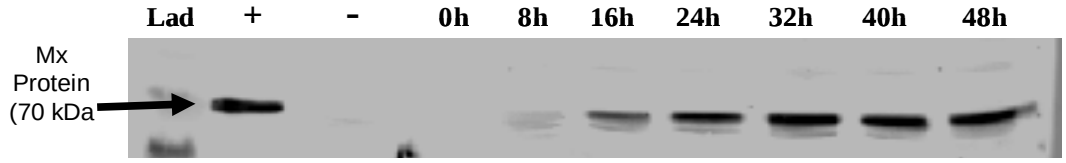

Figure 1 Effects of interferon-tau (IFNτ) (10,000 U/mL) on Mx1 protein expression in cultured bovine endometrial (BEND) cells during 48 hours

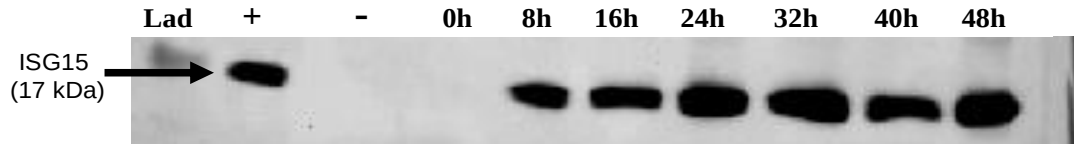

Figure 2 Effects of interferon-tau (IFNτ) (10,000 U/mL) on ISG-15 protein expression in cultured bovine endometrial (BEND) cells during 48 hours

APPENDIX 3

CHAPTER 2
Institutional Animal Care & Use Committee Approval

University of Idaho
Institutional Animal Care and Use Committee
Annual Protocol Review

Date: Wednesday, June 1, 2016
To: Amin Ahmadzadeh
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2015-28
Effect of aspirin on conception and embryonic loss in lactating dairy cows

Original Approval:	5/21/2015	**Annual Expiration:**	5/21/2016	**3 Year Expiration:**	5/21/2018

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. This brief renewal application will provide the basis for an annual review for projects that have not changed or may have only minor modifications from year to year. A new protocol must be submit ted for new projects, major changes in existing protocols, and for all protocols every three years.

1) Please indicate the present status of your project by checking one of the statements below:

____ This project is no longer active, please withdraw.

_XX_ This project is pending/active and there have been no changes in procedures with respect to animal use or personnel.

____ This project is active and there have been changes in the personnel or experimental procedures. (Describe any such changes below.)

2) Please provide a description of any changes in procedures with respect to animal care and use since your protocol was originally approved. Attach additional pages if necessary.

N/A

3) List all changes in personnel involved in your project:

Add	Delete	Name	Department	Email	Training & Experience
N/A					

Signature: _____ *Amin Ahmadzadeh*_____ Date: __6/1/2016___

Send this completed form to iacuc@uidaho.edu or IACUC, Office of Research Assurances, University of Idaho, Moscow, Idaho 83844-3010. **You must immed iately cease all live animal activities described under this protocol on or before the renewal date of this protocol, 5/21/2016, unless it is reviewed and approved by the Institutional Animal Care and Use Committee prior to this date.** If you have any questions, please contact the Office of Research Assurances at (208) 885-6162.

APPENDIX 4

CHAPTER 3
Institutional Animal Care & Use Committee Approval

Ahmadzadeh, Amin (amin@uidaho.edu)

From: Institutional Animal Care and Use Committee (iacuc@uidaho.edu)
Sent: Wednesday, April 27, 2016 8:35 AM
To: Ahmadzadeh, Amin (amin@uidaho.edu)
Subject: IACUC Protocol 2016-17 Approved

University of Idaho
Institutional Animal Care and Use Committee

Date: Wednesday, April 27, 2016
To: Amin Ahmadzadeh
From: University of Idaho
Institutional Animal Care and Use Committee
Re: Protocol 2016-17
The use of two injections of prostaglandin F2α in a 5-d CIDR timed-AI breeding protocols

Your animal care and use protocol for the project shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Wednesday, April 27, 2016.

This protocol was originally submitted for review on: Monday, January 1, 1900
The original approval date for this protocol is: Wednesday, April 27, 2016
This approval will remain in effect until: Thursday, April 27, 2017
The protocol may be continued by annual updates until: Saturday, April 27, 2019

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Barrie Robison, IACUC Chair