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AN EVALUATION OF BOVINE REPRODUCTIVE FUNCTION FROM THE MICRORNA EXPRESSION IN THE FEMALE TO PERFORMANCE TESTING IN THE MALE

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Animal and Veterinary Sciences

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by
Erin Marie Amiss
December 2022

Accepted by:
Nathan M. Long, Committee Chair
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Charles F. Rosenkrans Jr.

ABSTRACT

Reproductive function is crucial to the success of a beef or dairy herd. Both the female and male play significant roles in the outcome of bovine reproduction. The bull contributes to reproductive success in many ways, such as genetics. The initial role that the bull has in reproductive success is fertilization, whether natural or by artificial insemination. One method of assessing bull performance are central bull test stations, which are traditionally administered by a university, extension center, or are privately owned. Central bull tests assess the performance of bulls from various management systems, breeds, and ages. Some measurements taken during a central bull test are body weight, and a breeding soundness exam (BSE) is usually administered at some point during the test. Bull BSEs are the golden standard for predicting breeding potential in bulls by assessing structural soundness, semen motility, semen morphology, and SC. These factors are significant to ensuring that the bull has the ability to breed.

Many events can occur making reproduction unsuccessful such as, failure of bull to breed, anestrous, fertilization failure, and pregnancy loss. Pregnancy loss varies between beef and dairy cattle with lactating dairy and beef cattle having a pregnancy loss rate of 50-60% and 10.8-48% respectively (Stevenson et al., 2003; J. E.P. Santos et al., 2004; Wiltbank et al., 2016; S T Reese et al., 2020). Detecting pregnancy loss early in gestation is crucial to the reproductive success of cattle. Standard methods of bovine pregnancy diagnosis include rectal palpation, transrectal ultrasonography, enzyme linked immunoabsorbant assays for pregnancy association glycoproteins. Other methods being examined for bovine pregnancy diagnosis include interferon-tau (INF-T), maternal

progesterone concentrations, and microRNA (miRNA) expression. Profiled miRNAs have been associated with pregnancy and pregnancy loss, specifically, uterine miRNAs are implicated with embryo adhesion and attachment. MicroRNAs (miRNA) are a class of small, non-coding, endogenous, and evolutionarily conserved RNAs that range in size from 19 to 24 nucleotides in length (Lagos-Quintana et al., 2003; Bhaskaran and Mohan, 2014). MiRNAs have been used as biomarkers for various health issues and physiological states in both humans and animals (Kroh et al., 2007).

The objective of this study is to evaluate aspects of bovine reproduction function pertaining to the male using performance data and microRNA expression in females at d 30 of gestation.

DEDICATION

This thesis is dedicated to my family, who have supported my passions and aided in my achieving my goals. I would not be the person I am today without the opportunities they have given me. To my grandfather, David Coleman, who started my love for cattle and agriculture, and gave me opportunities to grow in the world of animal science. To my grandmother, who has supported me throughout my life continuously. To my mother, Dawn Amiss, who showed me how to be a strong woman and strive to be the best version of myself. To my father, Phillip Amiss, for also encouraging me to never give up. To my significant other, Hunter Womack, you have been with me nearly every day since I started this journey, and you always supported me and encouraged me, and I could not have completed this without you. To my siblings, Elly and Grant Amiss, for being the best friends I could have asked for. Finally, to my canine companion, Hallie, for always being there for me.

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TABLE OF CONTENTS

Page

Table of Contents (Continued)

LIST OF TABLES

CHAPTER 1

REVIEW OF LITERATURE

BOVINE PREGNANCY LOSS

Introduction

Reproductive performance in cattle is significant to the success of a producer's business. One of the detriments to reproductive performance in cattle is pregnancy loss. Producing a live and healthy calf is very important both monetarily and for genetic improvement to both beef producers and dairy producers. Although fertilization rates may be as high as 95% in beef cattle,here and throughout; however, pregnancy rates may only be 68% following one insemination (Perry et al., 2005; S. T. Reese et al., 2020). High fertilization rates and lower pregnancy rates show that one of the most detrimental aspects of reproductive success in cattle is pregnancy loss. Pregnancy loss varies between beef and dairy cattle with lactating dairy and beef cattle having a pregnancy loss rate of 50-60% and 10.8-48% respectively (Stevenson et al., 2003; J. E.P. Santos et al., 2004; Wiltbank et al., 2016; S T Reese et al., 2020).

Timing of pregnancy loss

Pregnancy loss can be seen in the early embryonic period, which occurs from day 0 to approximately day 30, late embryonic period, day 30 to between days 60, and fetal period, which is from the end of the late embryonic period to parturition (Reese et al., 2020). Most pregnancy loss occurs in the first month of gestation (Reese et al., 2020) or the early embryonic period, with nearly half of all pregnancy loss occurring in the first two weeks following insemination, as confirmed by return to estrus or pregnancy

diagnosis (Dunne et al., 2000; Diskin et al., 2016; Wiltbank et al., 2016). Other studies report that nearly half of pregnancy loss occurs between day 27 and 42, as confirmed by pregnancy diagnosis (Diskin and Morris, 2008; Reese et al., 2020).

Reese et al. (2020) reported pregnancy losses in beef cattle of 28.4% by day 7, 3.9% between days 7 and 16, and 15.6% between days 16-32. From day 28 and on, pregnancy loss rates vary, with Reese et al. (2020) reporting a 5.8% loss and Silke et al., (2002) reporting a 7.2% loss for dairy cows and 6.1% loss for dairy heifers between days 28 and 84. Another study compiled data of lactating dairy cows from 46 studies and reported that 20% of embryonic loss occurs from day 8-28, and another 5-20% loss occurs from day 28-60, leaving a 2% loss for the remainder of the first trimester (Wiltbank et al., 2016). Pregnancy losses in the fetal period are usually less than 10% of total pregnancy losses for both beef and dairy cattle (J. E.P. Santos et al., 2004).

Cost of pregnancy loss

The cost of bovine pregnancy loss varies depending on stage of gestation, animal quality, and management. De Vries (2006) reported that the average cost of one pregnancy in Holstein cows was $555. The costs of losing a pregnancy ranged from $0 to $373, where the $0 cost is when a cow was culled (de Vries, 2006). The cost of pregnancy loss after one month of gestation was greater than the value of a new pregnancy. Stevenson (2001) reported that at a 40% conception rate the value of an additional pregnancy is $253, whereas at a 50% conception rate it is $140 in high producing dairy cows. Using a Markov chain algorithm, Cabrera (2012) reported that the cost of pregnancy loss ranged from $128 to $897, and the cost of a new pregnancy ranged

from $128 to $232. Studies estimate that pregnancy failure may cost the beef industry $2.8 billion, with the added lost revenue of a live calf and breeding expenses this number could be closer to $21 billion (Mercadante et al., 2020). Pregnancy loss can cost both beef and dairy producers and have detrimental effects on revenue.

Pregnancy loss caused by diseases

Often, we see pregnancy loss associated with infectious, non-infectious disease, and toxins. Diseases such as trichomoniasis, leptospirosis, Bovine Viral Diarrhea (BVD), bovine herpesvirus (Infectious Bovine Rhinotracheitis or IBR), vibriosis, brucellosis, ureaplasma, and mycoplasma, are some of the most common causes of pregnancy loss in cattle (Corbeil et al., 2006; BonDurant, 2007; Rani et al., 2018).

Tritrichomonas foetus or trichomoniasis is a venereally transmitted protozoan pathogen found on the epithelium penis and prepuce of bulls (BonDurant, 2005; BonDurant, 2007). The embryo usually survives past the maternal recognition of pregnancy, but pregnancy loss will most often occur after 42 days of gestation but not later than 4 months (BonDurant, 2005). Breeding with artificial insemination instead of natural service is a way that producers can decrease their chances of trichomoniasis infection (BonDurant, 2005). There is not a treatment for trichomoniasis, however vaccination for trichomoniasis has been found to be efficient in females, but the efficacy is unknown for bulls (BonDurant, 2005; BonDurant, 2007). In bred dairy heifers aged 18 months, it was found that 18% of heifers vaccinated for trichomoniasis were infected and 27% remained pregnant until approximately 100 d post-breeding by an infected bull (Kvasnicka et al., 1989). In the same study, 42% of heifers not vaccinated for

trichomoniasis were infected, and none of these heifers were pregnant at 100 d post-breeding to an infected bull (Kvasnicka et al., 1989).

Leptospira hardjo and *Leptospira pomana* are two of the most common serovars seen in the zoonotic disease, Leptospirosis (Barr and Anderson, 1993). Leptospirosis, a spirochetes, shed through the urine of infected cattle and can often cause persistent infections (Barr and Anderson, 1993; Bondurant, 2007). *Leptospira hardjo* infection has a herd fetal loss rate of 10% and can lead to pregnancy losses starting at four months of gestation (Barr and Anderson, 1993), whereas *Leptospira pomana* infections have a fetal loss rates of around 50% and usually cause pregnancy losses in the last trimester (Barr and Anderson, 1993; BonDurant, 2007). A maternal infection usually won't result in pregnancy loss until several weeks or months later (Barr and Anderson, 1993). Leptospirosis can be treated with antibiotics and prevention is available through vaccinations (Barr and Anderson, 1993).

Bovine Viral Diarrhea Virus (BVDV) is a common virus seen in cattle herds. Acute infection is usually caused by the non-cytopathic type, however both cytopathic and non-cytopathic types, can lead to pregnancy loss. (Barr and Anderson, 1993; BonDurant, 2007). Pregnancy loss is usually seen in the first trimester, between days 0 and 125, but can occur at any point in gestation (Barr and Anderson, 1993; BonDurant, 2007). If the maternal non-cytopathic infection occurs between days 18-125, the conceptus can survive but will be viremic and be persistently infected (PI) with BVDV throughout their life (Barr and Anderson, 1993; Grooms, 2004; BonDurant, 2007). Prior to placentation, the zona pellucida has been shown to possibly protect the embryo from

the effects of BVDV infection, however, following placentation, transplacental infections are not uncommon (Grooms, 2004). Between days 42 and 125 of gestation pregnancies may withstand infection but congenital abnormalities with the nervous system can occur (Grooms, 2004). Following day 125 of gestation the fetus's immune system is usually functional and able to withstand infection, although pregnancy loss can still occur (Grooms, 2004). Controlling BVDV composes of eradication of contact with PI animals and vaccination (Barr and Anderson, 1993). Modified-live and killed vaccines are available on the market for both cytopathic and non-cytopathic BVDV (BonDurant, 2007).

Infectious bovine rhinotracheitis (IBR) or bovine herpesvirus type I is a virus transmitted by respiratory, ocular, and reproductive secretions of both beef and dairy cattle (Kahrs, 1981). In some beef and dairy herds the prevalence of IBR can be between 70-85% (Nettleton and Russell, 2017). The time from exposure to pregnancy loss varies, but can be between 15 days and several months (Nettleton and Russell, 2017). Pregnancy loss can occur at any point in gestation but most often occurs at five months of gestation or later (BonDurant, 2007; Nettleton and Russell, 2017). The most efficient way of avoiding IBR linked pregnancy loss is vaccination (Barr and Anderson, 1993).

Camplyobacter fetus, also known as vibriosis, is a gram-negative, S-shaped, bacteria that infects the epithelium of the female reproductive tract and the male's penis and prepuce (Grooms, 2004; BonDurant, 2005). *Campylobacter fetus* is composed of two subspecies, *C. fetus fetus* and *C. fetus venerealis* (Barr and Anderson, 1993). *C. fetus venerealis* is a venereally transmitted infection whereas *C. fetus fetus* is not, but both

subspecies can cause pregnancy loss (Barr and Anderson, 1993; BonDurant, 2005). Pregnancy loss can occur at any point in gestation but most observed after day 42 of gestation (BonDurant, 2005). Females infected with either subspecies of *C. fetus* often experience cervical, endometrial, inflammation as well as inflammation of the vaginal, and oviductal mucosae (BonDurant, 2005). *C. fetus* can also lead to pyometria following pregnancy loss (BonDurant, 2005). Antibiotic treatment is effective in treating *C. fetus* in both males and females (Barr and Anderson, 1993; BonDurant, 2005). Artificial insemination, vaccination (Barr and Anderson, 1993; BonDurant, 2005), and culling are methods of controlling and preventing *C. fetus* (BonDurant, 2005).

The zoonotic infection *Brucella abortus* or commonly called Brucellosis, is caused by a Gram-negative coccobacilli bacterium that can also induce embryonic and fetal death (Barr and Anderson, 1993; Neta et al., 2010). Brucellosis infections have been reduced in the United States but still occur (Barr and Anderson, 1993). Pregnancy loss usually occurs after the fifth month of gestation (Barr and Anderson, 1993). Infection of *B. abortus* is caused by the ingestion of infected tissues (Barr and Anderson, 1993; Anderson, 2007). Upon infection, large numbers of *B. abortus* organisms reside in the fetus as well as placenta (Anderson, 2007). Pregnancy loss can often occur due to destruction of the placenta and trophoblast cells (Anderson, 2007). Vaccination for brucellosis offers some protection and the proper disposal of infected tissues is important for prevention (Barr and Anderson, 1993).

Ureaplasma diversum are free-living, cell wall deficient microorganisms in the mollicutes class and can cause bovine pregnancy loss, although it is not common (Miller

et al., 1994). Ureaplasma diversum typically affects the reproductive tracts of both males and females (Miller et al., 1994) and upper respiratory tracts (Padua and Hansen, 2010). Infection with Ureaplasma diversum can damage cells and lead to destruction of the placenta (Miller et al., 1994).

In the same family of bacterium, mycoplasma, can be another cause of pregnancy loss (Miller et al., 1994). The most common mycoplasma found to cause infection in cattle is *Aspergillus fumigatus,* which has been found to be the cause of 64% of mycotic infections (Knudtson and Kirkbride, 1992). Other common mycoplasmas that can lead to pregnancy loss are *A. temeus* and *A. flavus* (Knudtson and Kirkbride, 1992). Myotic infection occurs more often in the winter months in areas where feed is stored (Anderson, 2007). Pregnancy loss typically occurs in the third trimester with mycotic infection and could be due to how mycoplasmas affect the placenta (Anderson, 2007). The best method of controlling mycoplasma is to store feed properly (Barr and Anderson, 1993).

Bluetongue is a non-contagious orbivirus (Osburn, 1994) that utilizes the *Culicoides* genus as a vector (Maclachlan et al., 2009; Kruse et al., 2017) and has an affinity to the platelets and erythrocytes of animals (Maclachlan et al., 2009). Bluetongue virus has also been found to possibly be shed through semen of viremic bulls due to erythrocyte contamination (Osburn, 1994). While there are 24 serotypes of bluetongue virus (Osburn, 1994), serotype 11 is responsible for 51% of cases in the United States (Osburn et al., 1982), however serotype 8 is the most common cause of pregnancy loss (Maclachlan et al., 2009). Early embryonic deaths can occur (Osburn, 1994), however fetal deaths are more common due to transplacental infection (Osburn et al., 1982). If

fetal death does not occur, fetuses can have neurological issues or be persistently infected where they will remain viremic throughout their life (Osburn et al., 1982). Bluetongue virus is controlled by vaccination and proper management (Rojas et al., 2021).

Chlamydophila abortus is a zoonotic, intracellular, gram-negative bacteria (Longbottom and Coulter, 2003) that can cause pregnancy loss and placentitis mostly during the sixth to eighth month of gestation, especially with first calf heifers (Borel et al., 2006). Chlamydiosis is most often transmitted through inhalation or consumption of feces (Borel et al., 2006; Kemmerling et al., 2009) but can also be venereally transmitted (Kauffold et al., 2007). There is no vaccine for chlamydiosis—the main method of reducing infection is through management of facility and animal cleanliness (Kemmerling et al., 2009).

Epizootic Bovine Abortion, also known as Foothill abortion, is caused by the deltaproteobacterium, *Pajaroellobacter abortivirus*, (King et al., 2005) and is spread through the argasid tick (*Ornithodorus coraceus*)(Anderson, 2007). Epizootic Bovine Abortion infections are seen in the foothill rangelands of California, Nevada, and Oregon (Anderson, 2007). Exposure to Epizootic Bovine Abortion during the first trimester can lead to late term pregnancy loss (Anderson, 2007). Some calves are born live but have gross lesions, inflammatory lesions, lymphoid organs of abnormal size, and elevated immunoglobin levels (Anderson, 2007).

Neospora caninum is a protozoan parasite (Dubey and Lindsay, 1996) that is spread through contamination of feed and water sources through its hosts which are dogs, sheep, goats, horses, and deer (Dubey and Lindsay, 1996; Anderson et al., 2000).

Neosporosis rarely causes pregnancy loss before 90 days of gestation (F. López-Gatius et al., 2004) as most loss occurs in the fourth to sixth months of gestation (Anderson et al., 2000). Infected animals can spread neosporosis transplacentally, causing tissue damage throughout the body (Anderson et al., 2000). Calves born with neosporosis infection can be chronically infected (F. López-Gatius et al., 2004). Vaccination and management strategies are the only ways of reducing the risk of neosporosis infection (Anderson et al., 2000).

Salmonella dublin is the pathogenic serotype that causes salmonellosis (Holschbach and Peek, 2018). Salmonellosis can cause pregnancy loss mostly during the third trimester of gestation by luteolysis from a release of prostaglandin due to inflammation or from high fevers during infection (Holschbach and Peek, 2018). Salmonella is transmitted through contaminated feces (Holschbach and Peek, 2018). Salmonellosis can be treated with fluid therapy, antibiotics, and antinflammatories, and can be prevented by decreasing contamination (Holschbach and Peek, 2018).

Listeriosis is caused by the gram positive, rod-shaped bacterium, *Listeria monocytogenes* (Low and Donachie, 1997). *Listeria monocytogenes* can be spread through ingestion of contaminated feed, feces, and other biological material (Low and Donachie, 1997; Nightingale et al., 2005). Listeriosis can cause transplacental infection during the last trimester of gestation (Daniel Givens and Marley, 2008). The only method of reducing listeriosis contamination is management practices that ensure the cleanliness of facilities and feed (Nightingale et al., 2005).

Other causes of pregnancy loss

Aside from diseases, many genetic, environmental, developmental, or physiological issues can arise during gestation leading to the loss of a pregnancy. Most pregnancy loss occurs in the first month of gestation. This first month includes the 10-50% of potential pregnancies that are lost in the first week due to fertilization failures and issues related to cell division (Wiltbank et al., 2016; Reese et al., 2020). Once initial cell cleavage is complete, the embryo will continue to divide and differentiate. During this stage of the 2-8 cell embryo, maternal mRNAs and proteins must be degraded to allow for the activation of the embryonic genome (Tadros and Lipshitz, 2009; Reese et al., 2020). During the first week of gestation, maternal nutrition is very important to embryo survival and development. Previous studies have shown that nutrient restricted cows and heifers yield lower quality embryos by day 7 post-artificial insemination (Bridges et al., 2012; Kruse et al., 2017). Maternal nutrition continues to be important for the development and survival of the conceptus throughout gestation.

Some possible mechanisms for nutritional deficiencies leading to pregnancy loss in the first month could be changes in the circulating progesterone concentration, IGF1 concentration, IGF binding protein concentration, or histotroph secretion (Bridges et al., 2012). However, excessive energy consumption can also cause pregnancy loss and be detrimental to embryo development possibly due to elevations in circulating insulin or increased luteinizing hormone (Wiltbank et al., 2014).

Maternal-zygotic transition is initiated first by maternal signals and then from zygotic transcription, where proteins and microRNAs enhance the degradation of maternal mRNA and proteins (Tadros and Lipshitz, 2009). If the zygote's genome is not activated, development beyond the 16-cell stage in cattle does not occur (Reese et al., 2020).

Persistent ovarian follicles can affect early embryo survival. Ahmed et al. (1995) found that a persistent follicle is associated with altering the ability of the embryo to reach the 16-cell stage because of impairment of embryo maturation and changes in the oviduct consistent with higher intrafollicular estradiol concentrations (E2). Cows with a persistent follicle are reported to have a lower pregnancy rate than those without (Monteiro et al., 2015).

The environment can affect pregnancy loss. Heat stress is an environmental factor that can lead to early pregnancy loss. Heat stress can reduce the number of four to eight cell embryos and the number of two-cell embryos reaching blastocyst stage (Lannett and Hansen, 1997). Some mechanisms for how heat stress can cause pregnancy loss is altered follicular growth, increase in the number of persistent dominant follicles, lowers circulating estradiol, and decreased progesterone concentrations (Ahmad et al., 1995; de Rensis & Scaramuzzi, 2003; Wolfenson et al., 2000). Some studies disagree with these mechanisms of pregnancy loss in heat stressed animals and found that circulating progesterone concentrations were not altered (Wise et al., 1988) or were increased (Roman-Ponce et al., 1981). Heat stress also could decrease blood flow to reproductive organs as seen in rabbits (Lublin and Wolfer-Son, 1996).

Following the 16-cell stage, cell division continues, the conceptus becomes a morula and is then differentiated into a blastocyst (Wiltbank et al., 2016). The blastocyst is composed of an inner cell mass, blastocoele cavity, and trophectoderm (Wiltbank et al., 2016). The blastocyst must undergo cell division and differentiation of the totipotent cells of the embryo (Wiltbank et al., 2016). Excess of proteins, carbohydrates, energy, and amino acids can also lead to pregnancy loss in the blastocyst stage (Wiltbank et al., 2014).

Following this stage, initial embryo elongation occurs between days 7-14 of gestation (Bardot and Hadjantonakis, 2020). The process of elongation is the change from the spherical conceptus shape to a tubular conceptus shape (Hue et al., 2012). Elongation is driven by uterine secretions, gene networks, and pathways (Hue et al., 2012). Any alteration of embryo elongation can lead to loss of the embryo in the second week of gestation including heat stress. Ryan et al. (1993) found that the embryo mortality rate increased between days 6-14 post-insemination during heat stress. Another common issue that can lead to pregnancy loss is negative energy balance (NEB), which is often seen in high-producing dairy cattle (Nebel and McGilliard, 1993). Post-partum NEB can lead to a reduced immune response to various uterine infections (LeBlanc, 2008). Negative energy balance can cause the uterine environment to be insufficient to meet the conceptuses needs, which can lead to pregnancy loss (LeBlanc, 2008). After initial embryo elongation, proper maternal communication and recognition with the embryo must occur.

Around days 14-17 luteolysis will start to occur as it normally does in the estrous cycle of a cow or heifer (Thatcher et al., 1995). The conceptus must be recognized by the

maternal body to maintain progesterone secretion, the pregnancy hormone. Maternal recognition of pregnancy occurs when the elongated conceptus produces chemical signals to prevent luteolysis. Estradiol-17β and prostaglandin E2 and E1, are produced by the conceptus at days 13-19, and have been found to be very important in the maintenance of pregnancy by suppressing luteolysis (Reynolds et al., 1983; Ford, 1985; Weems et al., 2011). Another proteins reported to be involved in maternal recognition of pregnancy is, interferon-tau (IFN-tau) (Bazer et al., 1969) . This protein, IFN-tau, is an antiluteolytic protein that suppresses secretion of prostaglandin-F2 alpha (PGF2a) and allows retention of the CL thus sustaining progesterone production (Thatcher et al., 1995; Spencer and Bazer, 2002). Maternal recognition must occur following elongation of the blastocyst or the pregnancy will be lost.

During maternal recognition of pregnancy, the major histocompatibility complex (MHC) class I genes are silenced to prevent immune rejection of the embryo (Choi et al., 2003). These molecules help the body recognize self and non-self (Choi et al., 2003). The MHC class I molecules present foreign antigens and peptides to cytotoxic T cells and T lymphocytes, allowing the body to recognize and kill them (Choi et al., 2003). The maternal immune system must be apt in protection of both itself and the toleration of the conceptus, which is regulated by interferons and regulatory T cells present in the uterus. (Wan et al., 1987; la Rocca et al., 2014). A lower number of T cells can lead to pregnancy loss (la Rocca et al., 2014). Trophoblast cells also assist in protecting the conceptus from the maternal immune response by serving as a barrier to the uterine epithelium (Choi et al., 2003). Steroid hormone induced proteins called uterine serpins

have also been found to play an important role in regulation of the maternal immune system (Padua and Hansen, 2010). An abnormal immune response in any of these processes can lead to loss of a pregnancy (Hansen, 1997; la Rocca et al., 2014).

Around day 20 the attachment of the uterus to the placental membranes begins to develop, and by day 21 to 30 the attachment is complete (King et al., 1981). This attachment leads to the shift in embryonic nutrition from the histotroph or choriovitelline nutrition to the chorioallantoic or placental nutrition (Wiltbank et al., 2016). Inadequate attachment of the allantoic tissue to maternal tissue and challenges with the nutritional shift can lead to pregnancy loss (Wiltbank et al., 2016). Placentation abnormalities are most often seen in in-vitro produced embryos (IVP) and somatic cell nuclear transfer (SCNT) embryos (Hill et al., 2000a). During this time, uterine blood flow increases to support the rapidly developing fetus (Ford et al., 1979). Inadequate blood flow can lead to pregnancy loss as the conceptus will not be able to develop properly (Kurjak et al., 1991).

Some pregnancy losses are a result of body condition score (BCS). For both beef and dairy animals, a low BCS at the time of insemination can yield a 10% less pregnancy rate in the first 30 days of gestation than those animals in good body condition (López-Gatius et al., 2003). Body condition score has been reported to affect pregnancy loss later in gestation too, as dairy cows may experience decreased pregnancy loss with a greater BCS (Mellado et al., 2019). Synchronization protocol can also affect pregnancy losses in the first 30 days of gestation. Cattle bred using a timed artificial insemination protocol

were found to have more pregnancy losses than those bred using estrus detection (Nebel and Jobst, 1998).

Twin bovine pregnancies not only can lead to dystocia and increased calf mortality, but they can also increase the risk of pregnancy loss, most often in the first trimester (López-Gatius et al., 2017). Twin pregnancies increase the risk of pregnancy loss by 3-7 times during the first 90 days of gestation, with many twin pregnancies being lost before day 60 post breeding, following initial pregnancy diagnosis at around days 28-30 (López-Gatius et al., 2017). López-Gatius et al. (2002) found that between transrectal ultrasonography at days 28-44 post breeding and 90-96 days post breeding, 8.2% of single pregnancies were lost, whereas 21.4% of twin pregnancies were lost. Unilateral twin pregnancies only develop a single corpus luteum, these pregnancies are 5-9 times more at risk than bilateral twin pregnancies for loss (López-Gatius et al., 2017). Spontaneous embryo reduction occurs when one conceptus of the twin pregnancy is lost (Lopez-Gatius et al., 2009). One study found that 62% of cows which undergo spontaneous embryo reduction will lose the total pregnancy following initial diagnosis of pregnancy at day 28-34 gestation (Lopez-Gatius et al., 2009).

Some studies have found that lactating dairy cows with clinical mastitis or high somatic cell counts had an increased risk of pregnancy loss in the first trimester of gestation (Risco et al., 1999; Pinedo et al., 2009). The mechanisms behind this pregnancy loss is thought to be due to mastitis causing inflammation or secretion of PGF2a, which could result in decreased luteal function (J E P Santos et al., 2004). However, a study conducted by Dahl et al. (2017) reviewing the results of eight different publications found

that the majority of studies were unable to establish a timely relationship between mastitis infection and pregnancy loss.

Genetics can play a part in pregnancy loss before and after day 30. Most genetic causes of pregnancy loss are due to homozygous recessive lethal genotypes (Bamber et al., 2009). Genetic causes of pregnancy loss can also be caused by chromosomal defects, individual genes, and gene interactions (VanRaden and Miller, 2006).

Signal transducer and activator (STAT) proteins are a family of proteins that are important to development of the conceptus as they are involved in gene transcription during the cellular encounter or cytokines and growth factors (Khatib et al., 2008). The STAT5A protein is involved in many signaling pathways associated with pregnancy maintenance and conceptus survival such as the IFN-T signaling pathway and choriosomatomammotropin pathway (Khatib et al., 2008). Pregnancy loss due to STAT5A gene mutations occurs earlier in gestation, however, the mechanisms for embryonic loss are unknown (Khatib et al., 2008).

Mutations in haplotypes can alter gene function for various cellular processes related to embryo development which can cause pregnancy loss (Mcclure et al., 2014). Additive gene variance in pregnancy loss in the fetal stage can be explained by seven genomic regions located on BTA2, BTA10, BTA14, BTA16, BTA21, BTA24, and BTA29 (Sigdel et al., 2021). The genes located on these regions play various roles in feto-placental growth, immune modulation, calcium signaling, vascularization, and organogenesis (Sigdel et al., 2021).

One homozygous recessive defect that is found in dairy cattle is the deficiency of uridine monophosphate synthase (DUMPS), which can cause embryonic mortality (Shanks and Robinson, 1989). Uridine-5'-monophosphate synthase is an enzyme that catalyzes the conversion of orotic acid to the precursor of all pyrimidine nucleotides, known as UMP (Shanks and Robinson, 1989). This condition causes a decreased amount of maternal pyrimidines going to the embryo, which can lead to pregnancy loss usually between days 40-50 post-insemination (Shanks and Robinson, 1989). Testing sires for DUMPS has significantly reduced the prevalence of heterozygous sires of homozygous recessive embryos (VanRaden and Miller, 2006).

Another recessive defect that can cause pregnancy loss is complex vertebral (CVM) malformation in Holstein cattle (Agerholm et al., 2001). Inbreeding can cause CVM to occur but CVM is generally caused by a mutated SLC35A3 gene (Thomsen et al., 2006). Developmental issues that arise with CVM in the conceptus are organ and skeletal malformations, vertebral lesions, symmetric arthrogryposis, and reduced birth weight (Agerholm et al., 2001). Pregnancy loss can also be caused by CVM, and it happens usually in the fetal period of gestation (Agerholm et al., 2001).

Pregnancy detection

Reproductive success is reliant on accurate pregnancy diagosises. Many methods of detecting pregnancy in cattle have been developed. Methods are continuing to be improved by providing easier, more accurate, and earlier pregnancy diagnosis.Common methods of bovine pregnancy diagnosis include rectal palpation, transrectal ultrasonography, and ELISA for pregnancy association glycoproteins.

Rectal palpation is the oldest method of diagnosing pregnancy in cattle as it does not require extensive equipment. Palpation via the rectum have been documented since the 1800s (Momont, 1990). Pregnancy can be diagnosed by the palpation of a trained technician with a change in uterine size or location, the presence of fluid in the uterine lumen, middle uterine artery hypertrophy, and/or the presence of a corpus luteum on the ovary (Momont, 1990). There are certain health conditions that can also produce these signs such as infections including pyometra (Momont, 1990).

Diagnosing pregnancy with rectal palpation can be done 30-35 days of gestation in cows and occasionally a few days earlier in heifers (White et al., 1989; Momont, 1990). Pregnancy diagnosis earlier in gestation, around 30-35 days, is usually done by palpation of fluid in amniotic vesicle (White et al., 1989). Zaied et al. (1979) reported an accurate pregnancy diagnosis of 69% and an accurate non-pregnancy diagnosis of 87% when performing rectal palpation at days 21 to 22 of gestation. Once in the fetal stage of gestation, pregnancy diagnosis is often done by confirming the presence of fluid or the fetus (White et al., 1989). It has commonly been documented that rectal palpation can lead to loss of the embryo or fetus (Paisley et al., 1978; Franco et al., 1987). Studies have shown that when performing rectal palpation, the possibility of damage to structures involved with conceptus survival such as the amniotic vesicle, placentomes, and the conceptus itself, especially when diagnosing pregnancy between days 30-42 (Paisley et al., 1978; Franco et al., 1987). These losses are often seen with pregnancies that were initially abnormal (Paisley et al., 1978). Some research has shown that rectal palpation has no effect on pregnancy loss in cows and heifers (Romano et al., 2011). Other

concerns with rectal palpation is the variation in technician accuracy and risk of diseases that are spread through feces (Momont, 1990).

Transrectal ultrasonography waa considered gold standard for diagnosing pregnancy, fetal sexing, monitoring the estrous cycle, and assessing health of reproductive organs, conceptus, and related structures (Fricke, 2002). Transrectal ultrasonography generally uses a B-mode linear or convex transducer that emits and receives high frequency ultrasound waves (Fricke, 2002). The range of frequency used for transrectal ultrasonography is 5-7.5 MHz, where 5 MHz produces an image with a greater depth of tissue but less detail, and a 7.5 MHz produces an image with lesser depth of tissue and more detail (Fricke, 2002). Pregnancy is usually diagnosed in cattle using transrectal ultrasonography by detecting the conceptus heartbeat or presence of allantoic fluid in the uterus using a linear transducer ultrasound (Pieterse et al., 1990; Beal et al., 1992; Fricke, 2002). Transrectal ultrasonography can detect an embryonic heartbeat at 20 days post artificial insemination but cannot be used past 90 days due to the size of the fetus (Beal et al., 1992; Fricke, 2002). Pregnancy diagnosis with transrectal ultasonography using the presence of an embryonic vesicle, corpus luteum, and the appearance of the uterus can be done around d 12, however another study found that pregnancy diagnosis before day 18 only had an accuracy of 50% (Pierson and Ginther, 1984; Curran et al., 1986; Kastelic et al., 1989).

Conceptus heartbeat can be detected at day 22, with one study reporting an accurate positive predictive value of 96.6% and 97.9% for days 26-27 and 29-30 post-insemination consecutively using a 7.5 MHz linear transducer (Szenci et al., 1998). After

days 33 post-insemination the positive predictive value is 100% (Szenci et al., 1998). The same study reported that conceptus heartrate will produce a 67.5%, 83,1%, 92.5%, 97.1%, 95.3%, 98.3%, and 100% negative predictive value for days 26-27, 29-30, 33-34, 37-38, 39-40, 44-45, and 53-58 post-insemination consecutively using a 7.5 MHz linear transducer (Szenci et al., 1998).

When using allantoic fluid presence as a means of diagnosing pregnancy, the positive predictive value in one study was 92.9%, 95.0%, 98.3%, and 98.2% from days 26-27, 29-30, 33-34, 37-38, and 100% following day 39 using a 7.5 MHz linear transducer (Szenci et al., 1998). They also reported a negative predictive value of 86.4%, 92.3%, and 97.3% for days 26-27, 29-30, 33-34, following day 34 the negative predictive value was 100% using a 7.5 MHz linear transducer (Szenci et al., 1998). Romano et al. (2006) reported a 100% negative pregnancy predictive value after day 29 post artificial insemination and 88% at day 24 post artificial insemination using a 5 MHz linear transducer to examine the presence of fluid in the uterine lumen and a corpus luteum on the ipsilateral ovary or the allantochorion and embryo. Another study reported that the specificity at day 30 of gestation of transrectal ultrasonography for pregnancy diagnosis, which is the number of animals diagnosed correctly as not pregnant divided by the combined number of animals that were correctly diagnosed as not pregnant and the number of pregnant animals diagnoses as not pregnant, was 92.3% using a 6.5 MHz linear transducer (Abdullah et al., 2014). The same study also reported results on sensitivity at day 30 of gestation, which is the number of animals correctly diagnosed as pregnant divided by the combined number of animals correctly diagnosed and pregnant

and the number of animals that were not pregnant but diagnosed as pregnant, which was 97.05% using a 6.5 MHz linear transducer (Abdullah et al., 2014). However, Romano et al. (2006) reported a 100% sensitivity at day 29 post artificial insemination of cows and at day 26 post artificial insemination for heifers when diagnosing pregnancy by presence of fluid in the uterine lumen and a corpus luteum on the ipsilateral ovary or the allantochorion and embryo using a 5 MHz linear transducer. Accuracies for pregnancy diagnosis using transrectal ultrasonography ranged from 93.9% to 97.8% on day 27 post artificial insemination as reported by Silva et al. (2007). When using the presence of embryonic heartbeat to diagnose pregnancy, fewer false positive diagnoses and more false negative diagnoses can be seen (Szenci et al., 1998). Pregnancy diagnosis using transrectal ultrasonography is most accurate following day 26 when the allantoic fluid and conceptus are present (Pieterse et al., 1990). Although transrectal ultrasonography is a commonly used method of pregnancy detection, one of the main detriments to using this method is cost of training, and equipment (Fricke, 2002).

The use of Doppler ultrasonography is used to diagnose pregnancy in cattle as well. The Doppler ultrasound is a B-mode pulse-waved color flow ultrasound tool that converts soundwaves into images of blood flow to organs and tissues in the body (Matsui and Miyamoto, 2009; Utt et al., 2009). The blood flow to the corpus luteum increases in early pregnancy, and decreases in non-pregnant cows as they come into heat around days 19-21 after ovulation (Lüttgenau and Bollwein, 2014). One study found that luteal blood flow was significantly higher in pregnant cattle than non-pregnant cattle at day 15 post artificial insemination (Lüttgenau and Bollwein, 2014). Utt et al. (2009) found that when

using corpus luteum blood flow to diagnose pregnancy, the correct pregnancy diagnosis percentage ranged from 60.9% to 91.3% at day 17 post estrus, 69.6% to 95.7% at day 19 post estrus, and 78.3% to 91.3% at day 21 post estrus. However, the percentage of correct non-pregnant diagnoses was lower at all dates, causing the overall correct diagnosis percentage to range from, 54% to 64% at day 17, 64% to 72% at day 19, and 62% to 74% at day 21 when using corpus luteum blood flow (Utt et al., 2009)

There are several biochemical methods of pregnancy diagnosis in cattle using blood or milk samples (Reese et al., 2016; Reese et al., 2018). Pregnancy specific proteins, also known as pregnancy associated glycoproteins (PAGs), are one of the more common methods of biochemical pregnancy diagnosis in cattle (Reese et al., 2018). Pregnancy associated glycoproteins include 20 individual proteins and over two dozen genes, and they belong to the aspartic proteinase family, however they are not active as proteinases due to their amino acid sequence (Xie et al., 1991; Reese et al., 2016; Northrop et al., 2019). The binucleate cells of the trophectoderm secrete PAGs into maternal circulation shortly after placentation of the bovine embryo (Breukelman et al., 2012; Reese et al., 2016). The exact function of PAGs is not entirely known (Green et al., 2005). Pregnancy specific protein B was first detected at the University of Idaho by (Sasser et al.). Since then, much research and development has been done on the protein, such as the use of radioimmunoassay and an ELISA to diagnose pregnancy. Pregnancy specific protein B (PSPb) was first identified in cattle in the 1980s, and used in a radioimmunoassay (RIA) for pregnancy diagnosis (Butler et al., 1982; Zoli et al., 1992). Since the initial development of RIA, enzyme-linked immunoabsorbant assays (ELISAs) have been

developed for PAG detection and pregnancy diagnosis starting at day 28 of gestation in Bos taurus and Bos indicus bovine species (Green et al., 2005; Reese et al., 2018). Concentrations of PAG increase around day 24 of gestation in pregnant animals and will peak at week five of gestation and the again at parturition, however sire effect may play a role in this (Kiracofe et al., 1993; Pohler et al., 2016). Several studies have tested PAG concentrations at various times during gestation to determine the optimal time for pregnancy diagnosis. Reese et al. (2018) found that only 51% of animals were correctly diagnosed as pregnant at day 17 post embryo transfer when comparing serum PAG concentrations using ELISA to the same cattle at day 24.

Diagnosing pregnancy at day 28 post artificial insemination, using ELISA to quantify plasma PAG concentrations, has yielded a sensitivity of 95.3% and a specificity of 88.3% (Thompson et al., 2010). Concentrations of PAG increase as gestation progresses, and by day 30 post artificial insemination PAG concentrations have a 100% sensitivity and 90.6% specificity using ELISA (Thompson et al., 2010). The same study also found that 90.6% of cows were correctly diagnosed as non-pregnant at both day 32 using transrectal ultrasonography and at day 30 using plasma PAG concentration (Thompson et al., 2010). Overall accuracy of diagnosing PAG concentrations using ELISA was reported to be between 93.7% and 96.2% on day 27 to day 28 post artificial insemination (Silva et al., 2007; Karen et al., 2015). Ricci et al. (2015) reported a 92% accuracy when using plasma PAG concentrations to detect pregnancy at day 32 post artificial insemination.

Determining PAG concentrations using ELISA is a commercial method of diagnosing pregnancy, PAG concentrations can also be determined using radioimmunoassay procedures (Butler et al., 1982). Serum PAG concentrations were estimated using the radioimmunoassay protocol as described by Perenyi et al. (2002), and were found to be 97% accurate at diagnosing pregnancy at day 28 post artificial insemination (Karen et al., 2015). Another study found the total accuracy of using radioimmunoassay protocols to diagnose pregnancy using PAG concentrations to be 94.65% (Zoli et al., 1992). The previous studies have used Bos Taurus cattle, diagnosis of pregnancy using PAG concentrations has also been found to be 96% accurate in *Bos Indicus* cattle (Pohler et al., 2016). Concentrations of PAGs have been found to differ amongst parity, with primiparous cows having a higher concentration (Pohler et al., 2016).

BioPRYN (BioTracking LLC. Moscow, ID USA) is an ELISA that can be used to diagnose pregnancy using PSPb concentrations. The sensitivity and specificity of using an ELISA test in dairy cows has been reported as 93.9% and 95.5% respectively at day 28 in plasma samples (Romano and Larson, 2010). The positive predictive values, negative predictive values, and accuracy for pregnancy detection using PSPb at day 28 were both 94.7%, based on transrectal ultrasonography pregnancy diagnosis, and increased as days post artificial insemination went on (Romano and Larson, 2010). One study found that the differences in using PAG versus PSPb concentrations included earlier increased concentration of PSPb in pregnant animals at day 22 post artificial insemination, whereas PAG concentrations increased by day 25 post artificial

insemination but were not significantly different to non-pregnant animals until day 27 (Giordano et al., 2012).

Milk samples can also be used to detect PAG concentrations in ELISA tests (LeBlanc, 2013; Ricci et al., 2015). Positive predictive value for one studied was reported as 99.8% in cows ranging from 60 to 230 days post artificial insemination (LeBlanc, 2013). The negative predictive value reported from the same study was 80.8% with cows ranging in 61 to 341 days post artificial insemination (LeBlanc, 2013). Ricci et al. (2015) found that the accuracy of using milk samples to diagnose pregnancy using a PAG ELISA was 89% at day 32 post artificial insemination. These lower accuracies are probably due to milk have a much lower PAG concentration than plasma (Ricci et al., 2015).

Other members of the PAG family, such as PSP-60, which is a form of PAG-1, had a 90% positive accuracy at day 28 with plasma samples in heifers using a radioimmunoassay procedure with pregnancy confirmed by rectal palpation at day 89 (Mialon et al., 1994). However, the accuracy of diagnosing cows as pregnant in the same study as only 74% at day 28 (Mialon et al., 1994). These positive accuracies in cows and heifers increased as gestation went on until it reached 100% and 99% in heifers and cows respectively, at the time of rectal palpation around day 90 (Mialon et al., 1994). Negative pregnancy diagnosis was greater than 90% accurate for both heifers and cows using PSP-60 concentrations starting at day 28 until rectal palpation (Mialon et al., 1994).

Pregnancy diagnosis using PAG can be utilized using commercial ELISA tests (Reese et al., 2016). The commercial PA ELISA tests available are BioPRYN

(BioTracking LLC. Moscow, ID USA), IDEXX (IDEXX Laboratories Inc. Westbrook, ME USA), and DG29 (Genex Cooperative Inc. Shawano, WI USA) (Reese et al., 2016). These tests are reported to have a 98% to 99% true positivity rate with a 1% to 5% false positivity rate when used at the recommended sample time (Reese et al., 2016). Low concentration of PAG in pregnant cattle can give a false negative result using commercial PAG tests, this can lead to the cow being culled or rebred, which then leads to loss of the embryo (Romano and Larson, 2010). False positives can also be seen with commercial PAG tests when females have a retained high concentration of PAG after undergoing pregnancy loss (Romano and Larson, 2010). Studies have reported that PAG concentration can remain high in cows for up to 90 days post-partum, which can limit the use of commercial PAG tests for pregnancy detection depending on the day post-partum a cow was bred (Kiracofe et al., 1993). Using PAG concentrations has proven to be an accurate and cost-effective method of diagnosing pregnancy in cattle with some limitations.

Progesterone hormone concentrations in the milk and blood of cows and heifers can be used to detect pregnancy at the early stages of gestation. Progesterone, which is produced by the corpus luteum, maintains pregnancy, and increases significantly in maternal plasma and milk around day 21 of gestation (Gillis et al., 2002; Breukelman et al., 2012). Pregnancy detection using progesterone concentrations was first utilized in the 1970s, with radioimmunoassay (Pennington et al., 1976). By the mid-1980s, ELISAs were developed to detect progesterone concentrations (Claycomb et al., 1998). Other studies have used biosensors such as BIACORE (Biacore Ab, Uppsala, Sweden) to detect

to measure bovine progesterone in milk (Gillis et al., 2002). The accuracy of progesterone concentration for detecting pregnancy vary, with correct negative diagnoses being more accurate than correct positive diagnoses (Zaied et al., 1979; Nebel, 1988).

Initial studies using progesterone RIA to diagnose pregnancy showed an accuracy of 77.6% and an accuracy to diagnose non-pregnancy of 98% in milk samples collected 21 to 22 days post-breeding (Pennington et al., 1976). Zaied et al. (1979) found an overall accuracy for detecting pregnancy and non-pregnancy to be 89% on days 21 and 22, but a 98% accuracy when including day 28 diagnoses. Another initial study found an accuracy to diagnose pregnancy of 85%, when using plasma samples at 19 days post artificial insemination. Nebel (1988) found an accuracy to correctly diagnose pregnancy using progesterone ELISA of 60% to 96% in milk samples collected days 20 to 24 post artificial insemination. The same study also found that detection of progesterone yielded a correct negative pregnancy diagnosis accuracy of 81% to 100% using an ELISA for milk samples collected on days 20 to 25 post-breeding (Nebel, 1988). A low progesterone concentration in plasma and milk samples has been shown to accurately diagnose non-pregnant animals, however, due to the increase in progesterone during the luteal phase of the natural estrous cycle the accuracy of correctly diagnosing pregnancy is lower, and therefore can not accurately and efficiently be used(Zaied et al., 1979; Nebel, 1988; Balhara et al., 2013).

Detecting and combatting pregnancy loss

Detecting and mitigating pregnancy loss in cattle early in gestation is fundamental in facilitating reproductive success. Many of the tools used to detect pregnancies are also

used to detect pregnancy loss, such as transrectal ultrasonography, and maternal concentration of pregnancy associated glycoproteins and progesterone (Starbuck et al., 2004; Moore et al., 2005; Gábor et al., 2016; Northrop et al., 2019; Szenci, 2021). The accuracy and efficiency of the current tools to detect pregnancy loss continues to improve as more methods are being studied and developed.

Transrectal ultrasonography can be used to detect pregnancy loss by either a B-mode linear or convex transducer or a Doppler ultrasound ((Fricke, 2002). Using a 6-8 MHz linear array transducer, Gábor et al., (2016) confirmed pregnancy at days 29-42 with the presence of an embryo, embryonic heartbeat, and corpus luteum, the presence of an embryo with the absence of embryonic heartbeat and decreased chorioallantoic fluid suggested that pregnancy loss was occurring. These ultrasound diagnoses were conducted 29-42 days following artificial insemination were confirmed using rectal palpation 60-70 days following artificial insemination, and those diagnosed with pregnancy loss initially using transrectal ultrasonography were confirmed 2 weeks later (Gábor et al., 2016). The pregnancy loss detected by ultrasound was 7.1% overall, and 72.9% of this pregnancy loss were from pregnancies that possessed abnormalities at the initial diagnosis and confirmed 2 weeks later (Gábor et al., 2016). Reduced crown rump length has been found to be associated with pregnancy loss in cattle as well (Hill et al., 2000b; Kelley et al., 2017). Corpus luteum blood flow has also been studied to assess pregnancy status using a color-flow Doppler ultrasound (Utt et al., 2009; Herzog et al., 2010; Kelley et al., 2017). Blood flow to the corpus luteum has been shown to be a predictor of progesterone concentration, which could make luteal blood flow a useful tool to assess corpus luteum

function, progesterone concentration, and possibly pregnancy loss (Utt et al., 2009; Herzog et al., 2010; Kelley et al., 2017). However, researchers have yet to develop an accurate method of using luteal blood flow and Doppler ultrasonography to detect or predict pregnancy loss (Utt et al., 2009; Kelley et al., 2017).

Pregnancy associated glycoprotein (PAG) concentrations are a commonly used method of diagnosing pregnancy in cattle and have also been studied in detecting pregnancy loss in the embryonic and early fetal period (Hill et al., 2000b; F López-Gatius et al., 2004; Gábor et al., 2007; Pohler et al., 2016). Following pregnancy loss, concentrations of PAGs have decreased and cows with lower PAG levels in early pregnancy have a higher chance of experiencing pregnancy loss (Northrop et al., 2019) . One study found that cows with a PAG concentration of less than 1.8ng/mL on day 31 of gestation had a 95% chance of losing their pregnancy by day 60 of gestation (Pohler et al., 2016). Gábor et al., (2016) found that cows with a PSPb concentration between 0.5-1.1ng/mL were at risk for pregnancy loss at 29-35 days post artificial insemination. Another study found that decreased PSPb concentrations on days 23 to 28 post artificial insemination were related to pregnancy loss between days 28 to 35 post artificial insemination, but they were not related to pregnancy losses following day 35 (Martins et al., 2018). Hill et al. (2000) found that the PSPb concentrations for cows who experienced pregnancy loss before day 90 were not significantly different than pregnant cows between days 30 and 50 of gestation. However, it has also been reported that high levels of PAGs during the early fetal period are also associated with pregnancy loss, which could be associated with various factors including interrupted feto-maternal

communication (López-Gatius et al., 2007). There has been many studies determining the accuracy and timing that PAGs can be used to detect or predict pregnancy loss. Gábor et al. (2016) found an 87.2% positive prediction rate for pregnancy loss using an ELISA for PSPb at days 29-34 after artificial insemination when the pregnancy diagnosis threshold was set at 0.65ng/mL, however when setting the threshold to 4ng/mL, the positive predictive value of pregnancy loss decreased to 18.4%. They found that the pregnancy loss detected by a PSPb range between 0.5-1.1ng/mL was 64.9%, which was similar to the transrectal ultrasonography pregnancy loss rate, 72.9%, by using the presence of abnormalities in transrectal ultrasonography (Gábor et al., 2016). Studies have clearly shown the relationship between PSPb and PAG levels and pregnancy loss and how concentrations of these proteins can be used to possibly detect and predict early pregnancy loss.

Progesterone hormone concentrations are very important in the maintenance of pregnancy and have been shown to be a tool in diagnosing pregnancy. Progesterone concentrations have also been studied for the use of detecting and predicting pregnancy loss. Kelley et al. (2017) found that cows who experienced pregnancy loss had decreased plasma progesterone concentrations at day 34. Cows at d 20 of gestation with a serum progesterone concentration higher than 5.99 ng/mL were nearly 5 times more likely to maintain pregnancy to day 27 over cows with a progesterone concentration less than 3.52 ng/mL (Moore et al., 2005). Another study found that late embryonic loss, between days 30 and 36 and confirmed at day 60 after artificial insemination using rectal palpation, is predictable using low serum progesterone concentrations, the total prediction rate of

embryonic loss across low, medium, and high progesterone levels was 57.7% (Gábor et al., 2007). However, the effectiveness of predicting pregnancy loss was much higher at 84.6% to 95.1% with lower progesterone concentrations, which are less than 2ng/mL for a pregnancy loss diagnosis (Gábor et al., 2007). Starbuck et al. (2004) also found that progesterone concentrations can be used to predict pregnancy loss, as cows with lower progesterone concentrations at week 5 of gestation were more likely to experience pregnancy loss, especially before week 7. Only 50% of pregnancies were not lost when serum progesterone concentrations where less than 2.8 ng/mL (Starbuck et al., 2004). Another study, however, found that pregnancy loss during the late embryonic and early fetal periods was not associated with serum progesterone concentrations (Pohler et al., 2016).

Detecting a lost pregnancy early in gestation is very significant in mitigating the damage from pregnancy loss. Besides pregnancy loss detection, other tools have been studied to decrease the possibility of pregnancy loss. Such tools include aspiration of subordinate follicles, supplementing progesterone, and proper herd management (Rajamahendran and Sianangama, 1992; F López-Gatius et al., 2004; Diskin et al., 2016; Monteiro et al., 2021).

The potential negative outcomes of twin pregnancies are something beef and dairy producers should be aware of, as cows with twin pregnancies are more likely to experience pregnancy loss. Some methods of preventing pregnancy loss involve preventing twin pregnancies. Follicular aspiration, puncture, and drainage is one method that has been researched to prevent twin pregnancies (López-Gatius and Hunter, 2018).

Follicular puncture and drainage involves aspirating and draining all subordinate follicles, which has been defined as follicles less than 10mm in some studies, but essentially is all follicles that are not dominant (López-Gatius and Hunter, 2018; 2019). Cows who had follicular puncture and drainage performed had an incidence of twin pregnancies of 0%, as compared to 50% in those who had not had follicular drainage (López-Gatius and Hunter, 2018). This study did not find a change in pregnancy rate, although decreasing the chance of twins can minimize the occurrence of pregnancy loss

Another more common method of mitigating the risk of pregnancy loss is supplementing progesterone. Cows fitted with a progesterone-releasing intravaginal device (PRID) for 28 days following pregnancy diagnosis had a pregnancy loss of 5.3% on day 90, whereas cows not receiving a PRID had a pregnancy loss of 12% (F López-Gatius et al., 2004). More recently, the use of an accessory corpus luteum has been researched as a method of preventing pregnancy loss.

In cows receiving embryos, the administration of GnRH at day 5 of the estrous cycle was shown to induce the formation of an accessory corpus luteum, thus increasing circulating progesterone concentrations (García-Guerra et al., 2020; Monteiro et al., 2021). The treatment of GnRH was found to reduce the incidence of pregnancy loss in from day 33 to 60 in cows receiving embryos at the expanded blastocyst stage, or stage 7 (García-Guerra et al., 2020). Another study found that the induction of an accessory corpus luteum using GnRH at day 5 reduced pregnancy loss from days 26 to 61, where cows induced with an accessory corpus luteum had a pregnancy loss of 6.6%, whereas cows that were not induced with accessory corpus luteum had a pregnancy loss of 13.7%

(Monteiro et al., 2021). However, the same study also found that induction of an accessory corpus luteum was only greatly reduced with an ipsilateral accessory corpus luteum, whereas a contralateral accessory corpus luteum had a much greater pregnancy loss rate and was like that of the not induced group between days 26 to 33 (Monteiro et al., 2021).

Another method of inducing an accessory corpus luteum uses the administration of human chorionic gonadotropin (hCG) hormone (Hazano et al., 2021). The administration of hCG occurs in the luteal phase of the estrous cycle and has been found to increase progesterone concentrations by inducing an accessory corpus luteum, and has been studied as a tool to reduce pregnancy loss since the 1990s (Hazano et al., 2021).

MICRORNAS

Background of microRNAs

MicroRNAs (miRNA) are a class of small, non-coding, endogenous, and evolutionarily conserved RNAs that range in size from 19 to 24 nucleotides in length (Lagos-Quintana et al., 2003; Bhaskaran and Mohan, 2014). MicroRNAs can be encoded in multiple locations in the genomes of plants, invertebrate, and vertebrate animals. (Lagos-Quintana et al., 2003; Almeida et al., 2011; Bhaskaran and Mohan, 2014). Biosynthesis of microRNAs begins in the nucleus, where miRNA genes are transcribed by RNA polymerase II into primary microRNAs (Bhaskaran and Mohan, 2014). Primary microRNAs are much longer than microRNAs, as they are usually over 100 nucleotides long, can be coding or non-coding, and contain a cap structure at the 5’ end as well has have a poly-adenylated tail at the 3’ end, which aids in stability (Lagos-Quintana et al.,

2003; Almeida et al., 2011; Bhaskaran and Mohan, 2014). A microprocessor complex, then processes the primary miRNA into a shorter, stem-loop sequence known as precursor miRNA, which is 60 to 120 nucleotides in length (Almeida et al., 2011; Bhaskaran and Mohan, 2014). Drosha is a ribonuclease III protein that forms the microprocessor complex with another protein complex known as DGCR8/Pasha (Kim et al., 2003; Kim, 2004; Kim, 2005). Following transcription of pre-miRNA, it is exported to the cytoplasm from the nucleus by an Exportin-5-dependant mechanism, where it is then cleaved by Dicer-1, an RNase III enzyme, along with proteins TRBP/PACT (Kim et al., 2003; Kim, 2004; Almeida et al., 2011). The result of this cleavage is a short double-stranded miRNA, which is then unwound by a helicase (Almeida et al., 2011). One strand of this miRNA, along with Argonaute (AGO) protein 2 that is within the RNA-induced silencing complex (RISC) will act as guide for the RISC, where it will be guided to complementary sites of the target messenger RNA (mRNA), targeting the untranslated region, and repression of translation or transcript destabilization will occur (Siomi and Siomi, 2010; Almeida et al., 2011).

Function of microRNAs

MicroRNAs mainly function as post-transcriptional regulators of gene expression, most often by repression, but they can also mediate activation (Bhaskaran and Mohan, 2014). Post-transcriptional regulation is facilitated by the binding of complementary sites in mRNA, where the miRNA will alter translation (Almeida et al., 2011; Bhaskaran and Mohan, 2014). Studies have also found that miRNAs can also alter expression levels by triggering mRNA destabilization and degradation (Almeida et al., 2011). Due to miRNAs

role in the synthesis of protein, they are fundamental to many biological processes and pathways within plant and animal species (Hannon et al., 2003; Bhaskaran and Mohan, 2014). A single miRNA can regulation multiple genes, and a single gene can be regulated by multiple miRNAs, which allows the expression of genes in these processes to fluctuate (Bhaskaran and Mohan, 2014). In animals, miRNAs play an important role in many metabolic and cellular processes, some of which include, pathogenesis, organogenesis, carcinogenesis, cell differentiation, cell proliferation, and apoptosis (Hwang and Mendell, 2006; Bhaskaran and Mohan, 2014).

Discovery of microRNAs

MicroRNAs were first discovered in 1993 by Lee and colleagues. MicroRNAs were first found in a nematode species, *C. elegans* (Lee et al., 1993). They found two small, 20 and 61 nucleotide long *lin*-4 gene transcripts that contained sequences complementary to a repeated sequence in the 3' untranslated region of mRNA of the LIN-14 protein (Lee et al., 1993). They concluded from these results that translation of LIN-14 must be by an antisense RNA-RNA interaction by *lin-4* (Lee et al., 1993). Following this initial discovery, Reinhart et al. (2000) discovered the second miRNA, *let*-7, in *C. elegans*. Following these initial identifications of these small RNAs, research into what we now know as miRNAs become very prevalent, to where we are today with thousands of miRNAs identified in numerous species (Bhaskaran and Mohan, 2014).

Initial utilization of microRNAs

Cancer research and treatment were some of the first biomedical usages of miRNAs in mammalian species. MicroRNAs were first found in blast crisis chronic

myelogenous leukemia, where loss of miR-328 expression was found in this cancer (Calin et al., 2002; Eiring et al., 2010). Following that, microRNAs were first used as a biomarker in lung cancer, with a reduced expression of *let*-7 in lung cancer (Takamizawa et al., 2004). Takamizawa et al., (2004) found that overexpression of *let*-7 in lung adenocarcinoma cell line inhibited the in-vitro lung cancer cell growth, which were very promising results for the future of cancer detection and treatment. MicroRNAs are present in most all tissues and cells as well as in extracellular forms most often carried by extracellular vesicles (EV) in fluids such as plasma, saliva, urine, amniotic fluid, colostrum, breast milk, bronchial lavage, cerebrospinal fluid, peritoneal fluid, pleural fluid, and seminal fluid (Chen et al., 2008; Weber et al., 2010). Following the initial identification of altered expression of miRNAs in cancer, research in that area has expanded drastically. Therapies including anti-miRNA oligonucleotides, microRNA sponges, miRNA masking, small molecule inhibitor, and restoring the function of some suppressor miRNAs, are some examples of cancer treatments (Ebert et al., 2007; Li et al., 2009; Eiring et al., 2010). Several microRNAs have also been identified as biomarkers for the risk, diagnosis, or prognosis, of heart disease as well as other diseases (HWANG and MENDELL, 2006; Cortez et al., 2011).

The public repository and online resource for microRNA sequences, annotation, nomenclature, and target prediction information is called miRBase, and was set up in 2002 (Griffiths-Jones, 2004). This online miRNA registry provides up-to-date information on miRNAs for 271 species in the 22.1 version and is commonly used in gene and target prediction for miRNA research (Griffiths-Jones, 2004). The miRNA

registry includes 1025 mature miRNA and 1064 pre-cursor miRNA (Griffith-Jones, 2004). MicroRNA nomenclature involves a three or four-letter species prefix along with a numeric suffix, (eg, has-miR-21) (Griffiths-Jones et al., 2008). Furthermore, mature miRNA sequences can be expressed in multiple hairpin precursor loci, where they will have yet another numeric suffix, and related hairpin loci expressing related mature miRNA sequences have lettered suffixes (Griffiths-Jones et al., 2008).

In target prediction of miRNAs, the model TargetScan, uses thermodynamics-based modeling of RNA:RNA duplex interactions with comparative sequence analysis to predict miRNA targets (Bartel, 2009; Agarwal et al., 2015). TargetScan predicts these targeted sites within mRNAs by finding the Watson-Crick complementarity to bases 2 to 8 of the miRNA from the 5' end, this segment is known as the "miRNA seed" (Bartel, 2009; Agarwal et al., 2015). The use of target prediction of miRNAs has extensively enhanced the usage and research of miRNAs as biomarkers.

Current and future utilization of microRNAs in reproduction

In humans, miRNAs have been found to be involved in various reproductive system functions such as oocyte maturation, folliculogenesis, steroid synthesis, and pregnancy (Mouillet et al., 2010; Li et al., 2013; Miura et al., 2015). MicroRNAs specific to the ovary and placenta have been detected in serum and have been used to diagnose ovarian cancer, diagnose preeclampsia, evaluate embryonic development, determine ovarian endocrine function, evaluate the efficacy of reproduction specific drugs, determine pregnancy status, as well as many other key reproductive system functions in both the male and female (Li et al., 2009; Li et al., 2013; Miura et al., 2015).

The utilization of microRNAs as biomarkers for reproductive function and status has also been extensive in food animal species, such as in beef and dairy cattle. Studies have shown the utilization of miRNAs to monitor cow cyclicity, oocyte and embryo quality, and pregnancy status (Miles et al., 2012; Abd El Naby et al., 2013; Sontakke et al., 2014; Perkel et al., 2015; Maalouf et al., 2016; McCarty et al., 2019; Jiang et al., 2022). Ioannidis and Donadeu, (2016a) first characterized the change in expression of plasma miRNAs during the bovine estrous cycle, where specific miRNAs such as let-7f, miR-125b, miR-99a-5p, and miR-145 increased during the time of estrus. They also identified changes in the levels of plasma miRNAs during early pregnancy in cows, where during days 16 to 25 of pregnancy miR-26a was increased in plasma (Ioannidis and Donadeu, 2016b).

MicroRNAs are reported to be expressed in oocytes, cumulus oocyte complexes, and granulosa cells, and differentially expressed at different stages of oogenesis, suggesting that miRNAs play an important role in oocyte development and maturation (Miles et al., 2012; Abd El Naby et al., 2013; Sontakke et al., 2014; Maalouf et al., 2016). One study found that the expression of certain miRNAs decreased in abundance with time of maturation (Abd El Naby et al., 2013). The same study also found that the expression of these same miRNAs in preimplantation stage embryos were highly abundant earlier in development and that these miRNA abundances decreased after the 8-cell stage to the blastocyst stage (Abd El Naby et al., 2013). Kropp et al., (2014)found that the expression of miR-25, miR-302c, miR-19a2, and miR-181a was higher in degenerate embryos than blastocysts when looking at in-vitro culture media. The

differences in expression between stages of oocyte maturation and blastocyst quality provide the strong potential of using miRNAs as biomarkers for assisted reproductive technologies.

Ovarian follicles have also been reported to express miRNAs at different levels of folliculogenesis (Sontakke et al., 2014). These miRNAs present in ovarian follicles are found to target signaling pathways involved in follicular cell proliferation, steroidogenesis, prevention of premature luteinization, and oocyte maturation (Sontakke et al., 2014). The miRNAs in ovarian follicles are involved in the regulation of many reproductive processes and could give insight on ovarian function.

As certain miRNAs are expressed at different levels in the mammalian reproductive system and throughout various biological states such as estrus and pregnancy, much recent research in the food animal species world involves pregnancy. MicroRNAs have been found to be differentially abundant in various stages of bovine gestation, both in the embryonic and fetal period. Various studies have found that circulating miRNA expression and abundance is significantly different in pregnant, non-pregnant, and non-pregnant due to embryonic mortality in serum samples, specifically within the first month of gestation (Pohler et al., 2017; Gebremedhn et al., 2018b; DeCarlo et al., 2020). Recent studies have also utilized the use of on-site detection of miRNAs in serum extracellular vesicles (EVs) using a biosensor that detects miR-16b, as it has been found to be related to embryonic mortality (Jiang et al., 2022). This onsite diagnosis for the detection of pregnancy loss related miRNAs yielded a 90% sensitivity and 88.89% specificity (Jiang et al., 2022). The increase in research in these relatively

newly discovered miRNAs opens the door for many possibilities to improve animal reproductive efficiency.

Conclusion

In conclusion, reproductive efficiency has continuously been a highly researched topic in animal science. Improving the pregnancy rate of cattle will significantly increase the success of a herd. With pregnancy loss being one of the most contributing factors to reproductive failure, it is important to take preventative measures and that it is detected early. MicroRNAs may be a solution to predicting and detecting pregnancy loss, as they are key indicators of physiological states in mammalian species.

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CHAPTER II

Effects of breed and age on beef bull performance in central bull tests and breeding soundness examinations across four years in South Carolina

ABSTRACT: The objective of this study was to explore how breed and age affect bull body weights and breeding soundness examination outcomes during a forage based bull test. This study included 221 bulls managed at the Edisto Bull Test from 2016 to 2019. The breed groups used in the study were British (BRT) and Continental (CONT). After a 14 d adjustment period that followed consignment to the test, the bulls were weighed and placed on pastures for the length of the test (168 d). From the end of the test until the sale of the bulls on d 332, bulls were grazed on a different forages. Bull body weight (BW) was measured every 28 d of the test and bull scrotal circumference (SC) was measured every 56 d until the end of the test and then again prior to sale. A breeding soundness exam (BSE) was performed on junior bulls averaging 404.9 ± 20.3 d in age at d 112 and a BSE of senior bulls averaging 414.6 ± 18.3 d in age at d 56 of the test. A final BSE was performed a month before the sale date. Each breed group's BW, age, SC, and first and second BSE results per year were analyzed using the mixed procedure of SAS (SAS Inst. Inc., Cary, NC), with a significance of $P < 0.05$. Bull BW differed for all four years at every 28 d measurement, with bulls in 2018 consistently having increased BW. Bull SC differed across years for d 56, 112, and 332, but did not differ with breed. Head abnormalities and total sperm was increased for bulls in 2019, and total sperm only was increased for bulls in 2016. Bulls in the CONT group had increased sperm concentration at the first BSE, but not second. Passing of BSE differed for bulls in 2016 and 2019 at the first BSE. Concentration was increased for bulls in 2016 and 2019 at the second BSE, but not the first BSE. In summary, bull performance at our central bull tests did not differ amongst breed but did differ each year. The results of first and second BSE

varied with both year and breed, with most factors contributing to BSE results being SC, total sperm, concentration, and head abnormalities. Central bull tests are an advantageous way to analyze bull performance in terms of BW and BSE, and are crucial to analyze bull fertility; more data from these two common assessments will aid in the production of beef bulls.

Keywords: beef, bull, fertility, performance,

INTRODUCTION

One of the most common historical ways that producers assess the performance of their breeding bulls was by a central bull test station. Most bull test programs were administered by a university, extension center, or are privately owned facilities. The lengths of these bull tests range from 56-168 d. These programs assess the performance of the bulls from different backgrounds and management systems and can also compare different breeds and ages. Body weights (BW) were taken frequently to feed accordingly and to assess average daily gain (ADG). The data collected at a central bull test are used to predict the performance of a bull and a breeding soundness examination (BSE) was often administered during the test following the guidelines established by the Society for Theriogenology (Kennedy et al., 2002; Koziol 1986-). The BSE is performed by a veterinarian or trained professional and is a method of predicting breeding potential in bulls by examining overall structural soundness, semen motility, semen morphology, and scrotal circumference (Kennedy et al., 2002; Spitzer and Hopkins, 1997). Semen morphology represents defects in the sperm and motility represents the movement capabilities of the sperm. Scrotal circumference (SC) can be an indicator of the puberty of a young bull (Lunstra et al., 1978). Bulls will be graded as either satisfactory, meaning that have met the minimum requirements of passing a BSE, unsatisfactory, meaning that they fail due to issues that may affect fertility, or deferred, meaning that they cannot be classified as satisfactory at this time, but a follow up BSE should be completed. It has been indicated that approximately 65-85% of beef bulls will be classified as satisfactory breeders, and that this number is affected by breed, age, management, environment,

forage, and genetics (Chenoweth and McPherson, 2016). Most bulls are classified as unsatisfactory breeders due to sperm motility or morphology (Kennedy et al., 2002). The objective of this study is to explore how breed and age affect bull body weights and BSE outcomes. Exploring how breed and age affects a bull's performance, such as SC, semen characteristics, and BW gain, in a central bull test station can allow producers and scientists to improve the implementation of these examinations.

MATERIALS AND METHODS

Animals and Experimental Design

All animal procedures were approved by Clemson University Animal Care and Use Committee (AUP #2015-060, AUP #2019-005).

All bulls were managed per the rules and regulations developed by the Edisto Bull Test Committee, following the guidelines of the Beef Improvement Federation (http://guidelines.beefimprovement.org/index.php/About_BIF) for forage bull tests.

This study was conducted with records from 221 bulls consigned to the Edisto Forage Bull Test in Blackville, SC for the years 2016, 2017, 2018 and 2019. The breed groups of bulls used in the data were British (BRT), which consisted of Angus, Polled Hereford, and Horned Hereford, and continental (CONT), consisting of Gelbvieh, Limousin, and Charolais, Simangus and Simmental. The breed breakdown per year of bulls is reported in Table 1. The bulls were consigned to the test in December of the previous year in which initial body weight (BW) was collected and the bulls were given a two-week adjustment period. Bulls were on test for 168 d and then sold at d 332. The bulls were managed at the following stocking densities, 1.45, 1.66, 1.50, and 1.47 head/acre for the years 2016, 2017, 2018, and 2019 respectively, on wheat, oat, and

ryegrass pastures until d 168 and then grazed pearl millet and Tifton 85 until sale on d 332. All bulls were also fed a concentrate ration (Average from previous four years: 97.5% DM; 15.35% CP; 73.95% TDN; 21.48% Crude Fiber) at 1% body weight from d 168 to sale and ration adjustments were made every 28 d. Bull scrotal circumferences (SC) were collected at d 0, d 56, d 112, d 168, and d 332. Bulls in the junior group (JR) had an average age of 404.9 ± 20.3 days when their first BSE was conducted at d 112 of the testing period. Bulls in the senior group (SR) had an average age of 414.6 ± 18.3 d when their first BSE was conducted at d 56 of the testing period. All bulls had a second BSE approximately 30-45 d before their sale date, d 332 of the testing period, in mid-late September.

Statistical Analysis

Data is presented as least square means ± SEM. Bull body weights and age amongst breeds per year were analyzed using the mixed procedure of SAS (SAS Inst. Inc., Cary, NC). The model statement included breed and year and their interaction. Differences are considered significant at $P \leq 0.05$, and a tendency indicated at $P \leq 0.10$.

RESULTS

Bull Body Weights

Bull BW throughout the testing period across all four years are reported in Table 2. Bull BW throughout 28-day intervals differed amongst all years, except for initial BW where a tendency ($P = 0.057$) was observed as bulls in 2018 followed by bulls in 2017 had increased BW compared to those in 2016 and 2019 ($P = 0.057$). Bull BW at d 0 was decreased for 2016 and 2017 ($P = 0.005$), compared to bulls in 2018 and 2019. Bull BW

at d 28 was increased (P = 0.0053) for years 2017 and 2018 compared to bulls in 2016 and 2019. Bull BW at D56 was increased for bulls in 2018, and decreased for bulls in 2019, compared to bulls in 2016 and 2017 (P < 0.0001). At d 84 and d 112, bull BW differed amongst all years, where in 2018 the bulls had increased BW followed by bulls in 2017, 2016, and 2019 (P < 0.001). Bull BW at d 140 were increased for bulls in 2018 followed by bulls in 2017 compared to bulls in years 2016 and 2019 (P < 0.0001). Bull BW at d 168 differed amongst all years where bulls in 2018 had increased BW followed by bulls in 2017, 2019, and 2016 (P = 0.0002). At d 332, BW for bulls in 2018 and 2019 was increased and bull BW in 2016 and 2017 was decreased (P = 0.015). Bull BW did not differ significantly amongst BRT and CONT groups (Table 3).

Bull Ages

Bull ages across years are reported in Table 4. Bull age did not differ significantly between years for d 0, 56, and 112, but was increased at d 332 for bulls in 2017 and decreased for bulls in 2016, compared to bull ages in 2018 and 2019 (P = 0.003). Ages of bulls did not differ significantly between BRT and CONT breed groups and are reported in Table 5. An interaction was observed between breed and year for both BRT and CONT at d 0, 56, 112, and 332.

Bull Scrotal Circumference

Bull SC across all four years is reported in Table 6. Bull SC at d 0 did not differ across years. At d 56, bull SC was decreased for bulls in 2018 and 2019 compared to bulls in 2016 and 2017 (P = 0.002). Bull SC at d 112 was increased for bulls in 2017 and 2018 compared to bulls in 2016 and 2019 (P = 0.001). Bull SC at d 168 did not differ

across years. At d 332, SC was increased for bulls in 2019 compared to bulls in 2016, 2017, and 2018 (P = 0.003). Bull SC across breed groups is reported in Table 7 and did not differ significantly between BRT and CONT.

Bull Breeding Soundness Examinations

At the first bull BSE, data across all four years is reported in Table 8. Bull age (P = 0.460), sperm concentration (P = 0.840), percent motile sperm (P = 0.266), percent normal sperm (P = 0.118), number of droplets (P = 0.395), number of tails abnormalities (P = 0.269), and number midpiece abnormalities (P = 0.137) did not differ at the first breeding soundness exam across years. Bulls in 2019 had an increased number of head abnormalities compared to bulls in 2016, 2017, and 2018 (P = 0.018). Bulls in 2019 and 2016 had an increased number of total sperm compared to bulls in 2017 and 2018, of which did not differ (P = 0.003). The first BSE data across breeds is reported in Table 9. Bull age (P = 0.460), motile sperm percentage, pass/fail, normal sperm percentage, number of droplets, number of tail abnormalities, number of midpiece abnormalities, number of head abnormalities, and total number of sperm did not differ (P > 0.05) For the first BSE, CONT bulls had a higher sperm concentration (P = 0.005).

At the second BSE, data across all four years are reported in Table 10. Bull age (P = 0.182), percent motile sperm (P = 0.315), percent motile sperm (P = 0.397), percent normal sperm (P = 0.654), number of droplets (P = 0.206), number of tail abnormalities (P = 0.287) did not differ between years. Sperm concentration was increased for bulls in 2016 compared to bulls in 2017, 2018, and 2019 (P = <0.0001). Pass/fail was increased for bulls in years 2016 and 2017, followed by bulls in 2018 and 2019 (P = 0.018). The

number of midpiece abnormalities was increased for bulls in 2016, followed by bulls in 2019, and the bulls in 2017 and 2018 did not differ (P = 0.0002). The number of head abnormalities was increased for bulls in 2019, whereas bulls in 2017 and 2016 did not differ, and bulls in 2018 and 2016 did not differ (P = 0.020). The second BSE data across BRT and CONT bulls throughout all four years did not differ significantly for bull age (P = 0.930), sperm concentration (P = 0.342), percent motile sperm (P = 0.455), pass/fail (P = 0.471), percent normal sperm (P = 0.636), number of droplets (P = 0.710), number of midpiece abnormalities (P = 0.831), and number of head abnormalities (P = 0.981) and is reported in Table 11. The number of tail abnormalities was increased in BRT bulls (P = 0.010). An interaction was observed for CONT bulls in 2017 and BRT bulls in 2019 for the number of droplets at the second BSE (P = 0.013).

DISCUSSION

The bull breeds used in this study are very typical of what is used across the U.S. beef herd. The bulls performed similar for BW for both BRT and CONT groups, however previous studies have indicated differences where Polled Hereford bulls, a BRT breed, were the heaviest at the end of the testing period, and others have reported increased BW for Continental breeds such as Simmental (Cain and Wilson, 1983). At d 0, bulls in 2018 and 2019 had increased BW compared to other years, however bulls in 2018 consistently had increased BW from d 0 to d 332, which suggests that these bulls performed better in terms of BW gain. Bull BW in 2017 were decreased compared to bulls in 2018 at all d and were increased compared to bulls in 2016 and 2019 at d 28, 84, 112, 140, and 168, but were similar to bulls in 2016 for d 56 and 332. This suggests that the bulls in 2017

had consistently increased BW compared to bulls in 2016 and 2019 for most of the testing period. The bulls in 2019 had decreased BW at d 28, 56, 140, and 168, but then had increased BW at d 332, suggesting that these bulls did not perform as well for BW during the testing period. The bulls in 2016 started with a d 0 BW decreased to that of bulls in other years but were increased to bulls in 2019 at d 84 and 112, and similar to bulls in 2017 at d 56 and 332, suggesting that that the bulls in 2016 had increased BW gain than bulls in 2019, but overall had decreased BW from the start of the test. Differences in bull BW across years could be due to a variety of factors, such as environment, and genetics, however age did not differ at the start of the test for bulls in all four years.

The ages of bulls at d 332 was increased for bulls in 2017 and decreased for bulls in 2016, but not different across all years for d 0, 56, and 112 (Table 4). An interaction ($P < 0.10$) between breed and year for age at d 56, 112, and 332 was observed.

Bull SC did not differ across breed groups in this study, contradicting previous studies in which Continental breed bulls had increased SC compared to British breed bulls, specifically Simmental bulls (Gipson et al., 1985; Coulter et al., 1987; Bruner et al., 1995; Kennedy et al., 2002; Menon et al., 2011). Bull SC did differ across years for d 56, 112, and 332, which may be attributed to BW as previous studies have reported that weight, particularly final weight, has a great effect on SC (Bruner et al., 1995). In the current study, BW and SC were increased for bulls in 2018 for d 56 and 112, and for 2019 at d 332. However, BW was decreased for bulls in 2016 and 2017, but the bulls in

these years had increased SC, which contradicts the previous data mentioned ((Bruner et al., 1995).

At the first BSE, CONT bulls had increased sperm concentration, but there were no differences in sperm motility or morphology between the breeds. Bulls in the 2019 test period had an increased number of head abnormalities for the first BSE, as well as total sperm, which could account for increased number of abnormalities. Some head abnormalities that could have been found include detached heads, size defects, shape defects, and acrosomal defects (Menon et al., 2011). At the second BSE, the bulls in 2016 had increased sperm concentration, but did not differ for other factors. These bulls had varying numbers of head, midpiece, and tail abnormalities, with BRT bulls having increased tail abnormalities across all years. The results from the second BSE, most importantly the pass/fail rates, show that the bulls with morphology and motility issues in the first BSE eventually grew out of these defects.

In conclusion, central bull tests have been prominent for the historical and current collection of bull performance data and the conduction of bull BSE is crucial to the production of beef cattle. The performance of bulls in central test periods vary with breed, age, starting weight, nutrition, and location (Cain and Wilson, 1983). Some of the same factors that affect the performance of bulls in central test stations can also influence BSE results such as breed, age, weight, sperm concentration, motility, morphology, and SC (Cain and Wilson, 1983; Gipson et al., 1985; Bourdon and Brinks, 1986; Coulter et al., 1987; Bruner et al., 1995; Kennedy et al., 2002; Menon et al., 2011). The current study suggests that both BRT and CONT breeds perform similarly on forage based

central bull tests, and the variation in performance may be due to other factors that differ between years. The BSE results in our study suggest that both BRT and CONT bulls also perform similarly for SC, sperm motility, and morphology, but CONT bulls may have higher sperm concentration. Central bull tests are an opportunity for more research in bull performance. To the Edisto Research and Education Center Staff, thank you for the support on this project.

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Tables and Figures

Table 1.1 Numbers of British (BRT) and Continental (CONT) bulls per year for 2016, 2017, 2018, 2019.

	Breed		Total bulls per year
Year	BRT	CONT	-
2016	47	12	59
2017	38	13	51
2018	35	18	53
2019	35	22	57

Table 1.2 Body weights of both breeds of bulls in each year throughout 28-day intervals.

	Year				*P*-Value
	2016	2017	2018	2019	Year
n	59	51	53	57	-
Initial BW[1], kg	750.57 ± 19.66[c]	793.66 ± 20.26[b]	817.50 ± 17.11[a]	770.08 ± 16.53[c]	0.057
D0 BW[2], kg	714.13 ± 17.59[c]	735.61 ± 19.28[b]	784.03 ± 16.28[a]	784.97 ± 15.28[a]	0.005
D28 BW, kg	790.90 ± 18.13[c]	819.58 ± 18.59[b]	867.65 ± 16.78[a]	795.41 ± 15.74[c]	0.0053
D56 BW, kg	849.35 ± 18.09[b]	859.01 ± 18.55[b]	919.03 ± 16.75[a]	805.37 ± 15.71[c]	<.0001
D84 BW, kg	920.89 ± 18.69[c]	1001.79 ± 19.75[b]	1050.34 ± 17.30[a]	875.52 ± 16.23[d]	<.0001
D112 BW, kg	1019.28 ± 19.42[c]	1075.04 ± 20.52[b]	1137.76 ± 17.97[a]	978.66 ± 16.86[d]	<.0001
D140 BW, kg	1118.34 ± 19.58[c]	1179.77 ± 21.46[b]	1236.30 ± 18.13[a]	1107.69 ± 17.00[c]	<.0001
D168 BW, kg	1162.91 ± 19.18[d]	1212.74 ± 21.03[b]	1273.52 ± 17.76[a]	1186.62 ± 16.66[c]	0.0002
D332 BW, kg	1325.67 ± 19.39[b]	1306.83 ± 21.16[b]	1384.29 ± 17.94[a]	1369.69 ± 16.99[a]	0.015

Data presented LSM ± SEM

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

[1]Initial body weight (BW) is the two-week adjustment period before the start of the test (D0)

[2]D0 body weight (BW) is the start of the 168 day test.

Table 1.3 Body weights of British (BRT) and Continental (CONT) bulls throughout 28-day intervals for all four years

	Breed		*P*-Value
	BRT	CONT	Breed
n	155	65	-
Initial BW[1], kg	783.05 ± 9.87	782.85 ± 15.60	0.991
D0 BW[2], kg	753.78 ± 9.12	755.59 ± 14.56	0.916
D28 BW, kg	820.62 ± 9.36	816.16 ± 14.60	0.797
D56 BW, kg	857.54 ± 9.34	858.84 ± 14.57	0.940
D84 BW, kg	958.25 ± 9.65	966.02 ± 15.24	0.667
D112 BW, kg	1050.45 ± 10.023	1054.92 ± 15.84	0.812
D140 BW, kg	1153.41 ± 10.15	1167.64 ± 16.20	0.457
D168 BW, kg	1199.93 ± 9.94	1217.97 ± 15.87	0.337
D332 BW, kg	1359.52 ± 10.39	1333.72 ± 15.83	0.175

Data presented LSM ± SEM

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

[1]Initial body weight (BW) is the two-week adjustment period before the start of the test (D0)

[2]D0 body weight (BW) is the start of the 168 day test.

Table 1.4 Ages of all bulls in each year at day 0, 56, 112, 332

	Year				*P*-Value
	2016	2017	2018	2019	Year
n	59	51	53	57	-
D0 age[1], d	486.49 ± 5.92	502.09 ± 6.07	501.18 ± 5.48	492.48 ± 5.14	0.181
D56 age, d	377.49 ± 5.92	389.63 ± 6.07	385.18 ± 5.48	374.48 ± 5.14	0.211
D112 age, d	433.49 ± 5.92	445.63 ± 6.07	445.18 ± 5.48	430.48 ± 5.14	0.115
D332 age, d	590.49 ± 5.95[c]	614.13 ± 6.28[a]	606.18 ± 5.50[b]	601.48 ± 5.15[b]	0.049

Data presented LSM ± SEM

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

[1]D0 age is the start of the 168 day test.

*An interaction was observed

Table 1.5 Ages of British (BRT) and Continental (CONT) bulls across all four years at day 0, 56, 112, and 332

	Breed		*P*-Value
	BRT	CONT	Breed
n	155	65	-
D0 age[1], d	499.48 ± 3.06	491.64 ± 4.77	0.168
D56 age, d	385.73 ± 3.06	377.66 ± 4.78	0.156
D112 age, d	442.73 ± 3.06	434.66 ± 4.77	0.156
D332 age, d	606.81 ± 3.07	599.44 ± 4.84	0.194

Data presented LSM ± SEM

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

[1]D0 age is the start of the 168 day test.

*An interaction was observed

Table 1.6 Scrotal circumferences of all bulls in each year at day 0, 56, 112, 168, and 332.

	Year				*P*-Value
	2016	2017	2018	2019	Year
n	59	51	53	57	-
D0 SC[1], cm	29.61 ± 0.57	30.31 ± 0.52	29.65 ± 0.47	29.17 ± 0.46	0.432
D56 SC, cm	32.59 ± 0.52[a]	32.47 ± 0.51[a]	31.33 ± 0.45[b]	30.28 ± 0.45[c]	0.002
D112 SC, cm	34.09 ± 0.46[c]	35.06 ± 0.46[b]	36.02 ± 0.39[a]	33.83 ± 0.39[c]	0.001
D168 SC, cm	35.99 ± 0.53	35.69 ± 0.52	36.47 ± 0.43	35.47 ± 0.43	0.388
D332 SC, cm	43.67 ± 12.75[b]	38.97 ± 13.33[b]	36.32 ± 10.67[b]	86.42± 10.39[a]	0.003

Data presented LSM ± SEM

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

[1]D0 scrotal circumference (SC) is the start of the 168 day test.

Table 1.7 Scrotal circumferences of British (BRT) and Continental (CONT) bulls at day 0, 56, 112, 168, and 332 throughout all four years.

	Breed		*P*-Value
	BRT	CONT	Breed
n	155	65	-
D0 SC[1], cm	30.01 ± 0.28	29.36 ± 0.42	0.197
D56 SC, cm	31.63 ± 0.26	31.70 ± 0.40	0.889
D112 SC, cm	34.66 ± 0.23	34.85 ± 0.36	0.653
D168 SC, cm	35.83 ± 0.26	35.98 ± 0.40	0.744
D332 SC, cm	47.41 ± 6.55	55.28 ± 9.88	0.508

Data presented LSM ± SEM

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

[1]D0 scrotal circumference (SC) is the start of the 168 day test.

Table 1.8 First bull breeding soundness exam of all bulls across all years conducted at d 56 for Senior bulls and d 112 for Junior bulls.

	Year				*P*-Value
	2016	2017	2018	2019	Year
n	59	51	53	57	-
Age, d	406.91 ± 8.67	414.15 ± 8.53	407.85 ± 7.53	395.96 ± 7.90	0.460
Concentration, m/mL	307.80 ± 54.25	278.67 ± 52.16	342.67 ± 47.15	313.66 ± 48.99	0.840
Motile sperm, %	71.92 ± 5.30	69.70 ± 4.70	60.38 ± 4.32	70.39 ± 4.76	0.266
Pass/Fail	8.72 ± 3.01[b]	0.81 ± 2.94[c]	2.24 ± 2.78[c]	12.39 ± 3.55[a]	0.035
Normal sperm, %	64.19 ± 4.30	74.14 ± 4.20	75.24 ± 4.00	64.99 ± 4.15	0.118
Droplets, n	8.42 ± 7.62	7.39 ± 7.44	2.91 ± 7.09	19.98 ± 7.35	0.395
Tails abnormalities, n	6.42 ± 1.19	9.38 ± 1.17	8.65 ± 1.11	9.19 ± 1.16	0.269
Midpiece abnormalities, n	1.63 ± 0.51	0.47 ± 0.50	0.62 ± 0.48	1.80 ± 0.50	0.137
Head abnormalities, n	14.53 ± 7.79[b]	7.93 ± 7.60[b]	12.98 ± 7.25[b]	38.86 ± 7.42[a]	0.018
Total, n	199.53 ± 25.06[a]	99.31 ± 24.46[b]	107.32 ± 23.15[b]	191.03 ± 23.89[a]	0.003

Data presented LSM ± SEM

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

Table 1.9 First bull breeding soundness exam British (BRT) and Continental (CONT) bulls across all four years conducted at d 56 for Senior bulls and d 112 for Junior bulls.

	Breed		*P*-Value
	BRT	CONT	Breed
n	155	65	-
Age, d	409.9 ± 4.4	402.5 ± 6.9	0.370
Concentration, m/mL	238.3 ± 28.3[b]	383.2 ± 42.1[a]	0.005
Motile sperm, %	64.4 ± 2.6	71.8 ± 4.0	0.128
Pass/Fail	6.7 ± 1.7	5.3 ± 2.6	0.650
Normal sperm, %	69.1 ± 2.3	70.2 ± 3.5	0.791
Droplets, n	12.0 ± 4.1	7.4 ± 6.1	0.530
Tails, n	9.0 ± 0.6	7.8 ± 1.0	0.336
Midpiece, n	1.1 ± 0.3	1.2 ± 0.4	0.861
Head, n	15.0 ± 4.2	22.2 ± 6.2	0.339
Total, n	147.0 ± 13.4	151.6 ± 20.1	0.851

Data presented LSM ± SEM

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

Table 1.10 Second bull breeding soundness exam of bulls across all years conducted before sale date.

	Year				*P*-Value
	2016	2017	2018	2019	Year
n	59	51	53	57	-
Age, d	575.85 ± 16.50	613.07 ± 15.33	618.44 ± 13.53	617.49 ± 14.78	0.182
Concentration, m/mL	978.14 ± 86.72[a]	519.49 ± 83.60[b]	462.07 ± 72.20[b]	527.21 ± 74.15[b]	<0.0001
Motile sperm, %	75.95 ± 3.92	81.60 ± 4.32	73.17 ± 3.55	72.95 ± 3.42	0.397
Pass/Fail	0.953 ± 0.06[a]	0.964 ± 0.07[a]	0.849 ± 0.05[b]	0.741 ± 0.05[c]	0.018
Normal sperm, %	84.31 ± 2.47	83.92 ± 2.73	84.63 ± 2.25	81.06 ± 2.17	0.654
Droplets, n	2.62 ± 0.726	4.11 ± 0.80	2.41 ± 0.66	3.93 ± 0.64	0.206
Tails, n	5.78 ± 1.22	5.04 ± 1.35	6.19 ± 1.11	8.09 ± 1.07	0.287
Midpiece, n	1.06 ± 0.18[a]	0.198 ± 0.196[c]	0.019 ± 0.161[c]	0.454 ± 0.155[b]	0.0002
Head, n	6.23 ± 1.18[bc]	6.76 ± 1.31[b]	5.28 ± 1.08[c]	9.77 ± 1.04[a]	0.020

Data presented LSM ± SEM

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

*An interaction was observed

Table 1.11. Second bull breeding soundness exam British (BRT) and Continental (CONT) bulls across all four years conducted before sale date.

	Breed		*P*-Value
	BRT	CONT	Breed
n	155	65	-
Age, d	605.6 ± 8.2	606.9 ± 12.7	0.930
Concentration, m/mL	583.92 ± 42.91	659.53 ± 66.81	0.342
Motile sperm, %	77.25 ± 2.11	74.58 ± 3.19	0.455
Pass/Fail	0.856 ± 0.03	0.898 ± 0.05	0.471
Normal sperm, %	82.90 ± 1.33	84.05 ± 2.02	0.636
Droplets, n	3.14 ± 0.39	3.40 ± 0.59	0.710
Tails, n	7.83 ± 0.65[a]	4.72 ± 1.00[b]	0.010
Midpiece, n	0.452 ± 0.09	0.415 ± 0.144	0.831
Head, n	7.0 ± 0.635	7.02 ± 0.97	0.981

Data presented LSM ± SEM

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

*An interaction was observed

CHAPTER III
The microRNA expression of ipsilateral pregnant or non-pregnant bovine uterus during early pregnancy in beef cows

ABSTRACT:

MicroRNAs (miRNAs) have been used as biomarkers for various health issues and physiological state; and could be utilized as a tool to detect pregnancy loss. The objective of this study was to determine the variation in miRNA expression between pregnant (P) and non-pregnant (NP) ipsilateral uteri of cows at day 30 post-insemination. Uterine samples were collected from pregnant (n = 4) and non-pregnant (n = 4) cows, and snap frozen in liquid N2. Isolation of uterine RNA was performed using the mirVANA kit and RNA quantification and quality assessed using a Nanodrop 1000 Spectrometer (Thermoscientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) respectively. Eight libraries were generated (4 biological replicates per group), subjected to RNAseq procedures, and bioinformatic analysis was conducted by LC Sciences (Houston, TX, USA). Raw sequence data were analyzed using the ACGT101-miR program (LC Sciences, Houston, TX) removing extraneous sequence information. The remaining sequences ranging in size for 18 to 26 nucleotides were mapped to known bovine miRNA sequences (miRbase 22.1) and the bovine genome. Significant and differentially abundant miRNAs were analyzed using normalized deep sequencing counts and were subjected to ANOVA. Fifty-five miRNA was differentially expression ($P < 0.05$), of which 35 were upregulated in open samples and 20 were upregulated in pregnant samples. Target prediction was performed using TargetScan for differentially abundant miRNA. Functional enrichment analysis of the miRNA target genes was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID 2021). Gene targets were analyzed using the Kyoto

Encyclopedia of Genes and Genomes (KEGG) database. The top 5 KEGG pathways across all significant and differentially abundant miRNAs were pathways in cancer, PI3K-Akt signaling pathway, MAPK signaling pathway, human papillomavirus infection, and metabolic pathways. These results show that there is variation in the expression of miRNAs between pregnant and open cows at day 30 post-insemination.

Key Words: beef cow, pregnancy, microRNA, KEGG, uterus

INTRODUCTION

Reproductive performance in cattle is significant in the economic success of a producer's business. One of the detriments to reproductive performance in cattle is pregnancy loss. Producing a live and healthy calf is very important both monetarily as well as from a genetic improvement standpoint to both beef producers and dairy producers. Fertilization rates can be as high as 95% in beef cattle, however pregnancy rates can be significantly lower at around 68% following one insemination (Perry et al., 2005; S. T. Reese et al., 2020). High fertilization rates and lower pregnancy rates show that one of the most detrimental aspects of reproductive success in cattle is pregnancy loss. Pregnancy loss varies between beef and dairy cattle with lactating dairy and beef cattle having a pregnancy loss rate of 50-60% and 10.8-48% respectively (Stevenson et al., 2003; J. E.P. Santos et al., 2004; Wiltbank et al., 2016; S T Reese et al., 2020). Pregnancy loss can be detected early in gestation using many methods such as rectal palpation, transrectal ultrasonography, pregnancy association glycoproteins, and microRNA expression. MicroRNAs (miRNA) are a class of small, non-coding, endogenous, and evolutionarily conserved RNAs that range in size from 19 to 24 nucleotides in length (Lagos-Quintana et al., 2003; Bhaskaran and Mohan, 2014). MiRNAs have been used as biomarkers for various health issues and physiological states in both humans and animals (Kroh et al., 2007). MicroRNAs have been reported to differ between various physiological states in mammals, such as pregnancy (Reese et al., 2016). MicroRNAs associated with pregnancy and reproductive status are in the maternal plasma and endometrium of humans, the endometrium of swine, the embryo culture

media of bovine and human preimplantation embryos, the bovine granulosa and cumulus cells, and in the luminal fluid of extracellular vesicles in the ovine uterus (X). Extracellular miRNAs, such as those in plasma and other fluids, can be carried through extracellular vesicles such as exosomes or proteins like Argonaute (AGO) (X,X). The objective of this study was to determine the variation in miRNA abundance between pregnant and non-pregnant uteri of cows at day (d) 30 post-insemination, in which the hypothesis is that differences in miRNA expression could be used as a tool to diagnose bovine pregnancy loss.

MATERIALS AND METHODS

Animals and Tissue Collection

All animal procedures were approved by Clemson University Animal Care and Use Committee (AUP #2018-048).

This study was conducted with 23 beef cows in 2018 and 2019. Following estrus detection, all cows were artificially inseminated (d 0) using cooled extended semen from a single Simmental Bull. All animals were harvested on d 30 following insemination where samples of ipsilateral uterine horn tissue were obtained from pregnant (n = 11) and non-pregnant females (n = 11). One cow appeared to have recently lost a pregnancy and was not used in either group. Samples were snap frozen in liquid nitrogen until analysis.

RNA Isolation

Total RNA was isolated from approximately 300 μg of each ipsilateral uterine horn sample using the mirVANA RNA isolation kit (Thermoscientific, Waltham, MA, USA) following manufacturer's recommendations. Quantity and purity of total RNA was determined on a Nanodrop 1000 Spectrometer (Thermoscientific, Waltham, MA, USA). Quality of total RNA was determined on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA quality is measured in terms of A260/A280 and A260/A230 ratios. Four randomly chosen samples from pregnant and non-pregnant uteri samples measuring a minimum quality of A260/A280 ratio of 1.8 and A260/A230 ratio of approximately 1, a minimum concentration of 5 μg/μL or greater, and an RNA integrity number (RIN) of 7.0 or greater were sent for further analysis.

RNA Sequencing

Eight libraries were generated (4 biological replicates per group) and subjected to RNAseq procedures and bioinformatic analysis by LC Sciences (Houston, TX, USA). The RNAseq procedure at LC Sciences (Houston, TX, USA) includes total RNA extraction via the Trizol reagent (Invitrogen, CA, USA), and analyizing total RNA quality and quantity by the usage of Bioanalyzer 2100 (Agilent, CA, USA). Using approximately 1 μg of total RNA, small RNA library was prepared according to the TruSeq Small RNA Sample Prep Kits Protool (Illumina, San Diego, USA). Following preparation of the small RNA library, single-end sequencing 50bp on an Illumina Hiseq 2500 was performed at LC Sciences (Hangzhou, China), following the recommended protocol by the vendor. Raw sequence data were analyzed using the ACGT101-miR program (LC Sciences, Houston, TX) removing extraneous sequence information. The

remaining sequences ranging in size for 18 to 26 nucleotides were mapped to known bovine miRNA sequences (miRbase 22.1) and the bovine genome. The sequence data was normalized by dividing the counts by library size for each sample (calculated at the median value of the ratio between the counts of a specific sample and a pseudo-reference sample (the geometric mean across all samples).

Target Prediction and Enrichment Analysis

Target prediction was performed using TargetScan (Lewis et al., 2005) for differentially abundant miRNA. MicroRNAs were then screened to confirm all miRNA targeted multiple genes and that each gene was targeted by multiple miRNA. Functional enrichment analysis of the miRNA target genes was conducted using the Database for Annotation, Visualization and Integrated Discovery (DAVID 2021; http://david.abcc.ncifcrf.gov). Gene targets were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000).

Statistical Analysis

False discovery was corrected by filtration of non-miRNA sized data and confirmation of exact matches in the miRbase 22.1 database, the bovine genome, or both. The sequence data were normalized by dividing the counts by a library size parameter of the corresponding sample, which is the median value of the ratio between the counts of a specific sample and a pseudo-reference sample (LC Sciences). The count number in the pseudo-reference sample is the count geometric mean across all samples. Differentially expressed miRNAs based on using normalized deep sequencing counts were then subjected to ANOVA ($P < 0.05$).

RESULTS

The differential expression of miRNAs (n = 55) found in pregnant (P) and non-pregnant (NP) ipsilateral uterine horn samples at d 30 of gestation are reported in Table 1. Of these 55 miRNAs, 36 were upregulated in NP uteri and 20 were upregulated in P uteri.

A total of 3395 unique target genes were found from target prediction. The top 10 target genes were AKT3, PIK3CA, PIK3CB, PIK3R3, PIK3R1, GSK3B, MAPK1, MAPK8, PRKCA, and IGF1, and are reported in Table 2. The miRNA target genes were found to be associated with 250 KEGG pathways. The top 10 KEGG pathways across all significant and differentially abundant miRNAs are reported in Table 3. These top KEGG pathways were pathways in cancer, PI3K-Akt signaling pathway, MAPK signaling pathway, human papillomavirus infection, metabolic pathways, endocytosis, focal adhesion, proteoglycans in cancer, Ras signaling pathway, and axon guidance, respectively.

DISCUSSION

In the current study, the differentially abundant miRNAs identified in the ipsilateral uterine horn tissue of both pregnant and non-pregnant cows have been previously identified in other research. Ioannidis and Donadeu, (2017) identified 77 differentially expressed miRNAs in bovine plasma of dairy heifers at d 60 of gestation compared to d 0. Of miRNAs from d0 to d 60, bta-miR-324 and bta-miR-101was found

have greater expression at d 0 compared to d 60 of gestation (Ioannidis and Donadeu 2017). In the current study, bta-miR-324 and bta-miR-101 were found to be upregulated in non-pregnant cows at d 30 in ipsilateral uterine horn samples. Ioannidis and Donadeu, 2016b) also identified bta-miR-101 in plasma with a fold change between non-pregnant and pregnant of 0.78 at d 16 of gestation. Both of these miRNA may be associated with non-pregnant cows and possibly pregnancy loss, and further studies determining origin of circulating miRNAs and their relationship to those in uterine tissue are necessary. One of these miRNA, bta-miR-324 was not identified in plasma extracellular vesicles in cows that had experienced embryonic mortality and those at d 17 of gestation, however bta-miR-101 was but there was no difference in pregnant cows and cows in the embryonic mortality group (Pohler et al., 2017). From that study, bta-miR-127, bta-miR-409b, and bta-miR-652 were also identified in extracellular vesicles in plasma, and in ipsilateral uterine horn samples from the current study (Pohler et al., 2017). Both bta-miR-127 and bta-miR-409b has increased abundance in the extracellular vesicles of cows in the embryonic mortality group in the previous study mentioned and were upregulated in the uterine horn tissue of the current study (Pohler et al., 2017). The third miRNA with increased abundance in the embryonic mortality group at day 17, bta-miR-652, was found to be upregulated in pregnant ipsilateral uterine horn samples at d 30 of gestation in our study (Pohler et al., 2017)

Another miRNA that could be associated with pregnancy loss is bta-miR-378. In previous studies, miR-378 has been found to decrease aromatase protein expression and estradiol production in the granulosa cells of porcine ovaries (Xu et al., 2011). One study

found that bta-miR-378 was upregulated in the non-regressed corpus luteum (CL) (Ma et al., 2011). Ma et al., (2011) also found that bta-miR-378 decreased the expression of the interferon gamma receptor 1 (IFNGR1) gene at different stages of CL development, which potentially suggests the role of bta-miR-378 in apoptosis of the bovine CL. The miR-212-132 miRNA cluster was also found to be associated with CL development, as this cluster was increased in luteal relative to follicular tissues (Mohammed et al., 2017)

Some of the miRNAs identified in the current study that were upregulated in pregnant cows at d 30 were bta-miR-146b and bta-miR-7. Both bta-miR-146b and bta-miR-7 were found to be differentially expressed between non-pregnant and pregnant dairy cows at d 30 (Markkandan et al., 2018). Taylor et al., (2022). identified differential abundances of miRNAs in the cotyledons of day 110 cows and d 30 to d 190 cows. Four of those miRNA that had increased abundance between d 30 and d 190, bta-miR-146b, bta-miR-340, bta-miR-378c, and bta-miR-432, were identified in the current study at d 30 in pregnant cows (Taylor et al., 2022). Of the four identified in the current study, bta-miR-146b was upregulated in pregnant cows, while bta-miR-340, bta-miR-378c, and bta-miR-432 were downregulated in pregnant cows. In the same study, bta-miR-146b, bta-miR-186, bta-miR-191, were differentially abundant in d 110 cotyledons from nutrient restricted (NR) cows compared to control animals, these miRNAs were upregulated at d 30 for pregnant cows in the current study (Taylor et al., 2022).

Pathways in cancer was the top KEGG pathway associated with 2435 target genes and is associated with 42 of the significant and differentially expressed miRNAs in the

current study. The genes associated with pathways in cancer have been found to regulate a variety of biological processes, with most being related to human disease and cancer, including disease related to the reproductive system including endometrial hyperplasia and cancer (Matias-guiu et al., 2001; Hecht and Mutter, 2006; Bidarimath et al., 2014).

Another top enriched KEGG pathway in this study was PI3K-Akt signaling pathway, which was associated with 27 of the significant and differentially expressed miRNAs in the current study. The Phosphoinositide-3-kinase signaling pathway is mainly mediated by the phosphoinositide-3-kinase-protein kinase B/AKT (Vara et al., 2004). The PI3K-Akt signaling pathway is strongly associated with various cancers and diseases (Vara et al., 2004; Martini et al., 2014; Li et al., 2019). The PI3k-Akt signaling pathway has been found to be associated with mouse embryo implantation by regulating the expression of the protein RhoA (Liu et al., 2014). In humans, the activation of the PI3K-Akt signaling pathway is associated with chemokine-mediated endometrial epithelium function in the first trimester of pregnancy (Zheng et al., 2020). In in-vitro studies in humans, PI3K-Akt signaling was found to promote apoptosis and inhibit proliferation of trophoblast cells with the downregulation of storkhead box 1 (STOX1) transcription factor (Li et al., 2018)

Mitogen-activated protein kinases (MAPK) signaling pathway was also enriched for 32 of the significant and differentially expressed miRNAs in the current study. Studies have reported the significance of MAPK signaling pathway in the regulation of many biological processes such as apoptosis, cell proliferation, and development (Seger and Krebs, 1995; Pearson et al., 2001; ZHANG and LIU, 2002; Yue and López, 2020).

The MAPK signaling pathway is activated in the inflammatory response, particularly in the release of cytokines (Sheldon 2014). Suppression of the MAPK signaling pathway may cause the decrease of proinflammatory cytokine expression and the inflammatory response in bovine endometrial epithelial cells by cortisol (Dong 2018). The MAPK signaling pathway has also been found to be associated with the secretion of prostaglandin-F-2α, which suggests the role of corpus luteum maintenance (Thatcher 2001).

Another enriched KEGG pathway associated with 34 of the significant and differentially expressed miRNAs in the current study was human papillomavirus (HPV) infection, which is similarto bovine papillomavirus (BPV) Human papillomavirus infection is one of the main causes of cervical cancer in humans, thus this pathway is strongly associated with immune response and carcinogenesis (Karim et al., 2011; Zhang et al., 2019). In humans, HPV infection during gestation has been associated with preterm birth and spontaneous abortion (Niyibizi et al., 2021; Xiong et al., 2018). In cows, it has been found that papillomavirus infections can occur in the placenta of gestating cows, posing a threat to pregnancy and the overall health of the reproductive tissue (Roperto et al., 2012).

Metabolic pathways were also an enriched KEGG pathway associated with the significant and differentially expressed miRNAs in the current study. The metabolic pathways associated genes are significant in regulating biological processes, most importantly, energy production.

The majority of target genes and KEGG pathways enriched in the significant and differentially expressed miRNA in this study relate to immunology, carcinogenesis, cell proliferation, development, apoptosis, and metabolism, all of which are crucial in the production of various reproductive hormones and maintenance of pregnancy (Seger and Krebs, 1995; ZHANG and LIU, 2002; Miura et al., 2010; Ma et al., 2011; Bidarimath et al., 2014; Martini et al., 2014; Ioannidis and Donadeu, 2017; Gebremedhn et al., 2018; Markkandan et al., 2018; Zhao et al., 2019; Yue and López, 2020; Taylor et al., 2022)

In conclusion, our results suggest the variation of miRNA expression in ipsilateral uterine horn tissue of pregnant and non-pregnant beef cows up to d 30 of gestation. Target prediction and functional enrichment analysis has shown that the KEGG pathways and genes associated with these significant and differentially expressed miRNAs are important in pregnancy. Further research in the miRNA expression during various stages and statuses of bovine pregnancy are crucial to understanding the use of miRNAs as a potential biomarker of pregnancy loss in cattle.

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Tables and Figures

Table 2.1 Significant differential expression of d 30 ipsilateral uterine horn tissue from pregnant (P) and non-pregnant (NP) cows.

	Mean		*P*-value
	P	NP	miRNA
n	4	4	-
bta-miR-154c	618.88	1035.89	0.000
bta-mir-491	131.56	180.10	0.001
bta-miR-324	187.52	291.36	0.002
bta-miR-378c	74.26	123.33	0.002
bta-miR-382	290.28	439.47	0.003
bta-miR-7858	40.20	28.04	0.003
bta-miR-340	367.72	670.64	0.004
bta-miR-378	852.86	1289.12	0.004
bta-miR-127	16870.12	23720.48	0.006
bta-miR-2285aa	8.63	4.41	0.006
bta-miR-329b	4.92	15.91	0.006
bta-miR-432	175.28	251.31	0.007
bta-miR-212	35.21	66.60	0.009
bta-miR-29d-5p	211.90	110.86	0.009
bta-miR-485	18.41	35.31	0.010
bta-miR-1721	954.95	513.45	0.010
bta-miR-409b	765.42	1116.16	0.010
bta-miR-29c	3710.11	2005.71	0.010
bta-miR-330	32.97	64.44	0.010
bta-miR-216a	1.10	7.00	0.011
bta-miR-136	65.03	93.16	0.011
bta-miR-146b	382.92	249.19	0.015
bta-miR-652	53.28	37.19	0.019
bta-miR-101	42475.89	26826.89	0.020
bta-miR-2451	27.48	50.88	0.021
bta-miR-199a-5p	41832.63	55972.37	0.021
bta-miR-31	1.54	17.96	0.021
bta-miR-191	18135.82	14377.79	0.022

bta-miR-1260b	364.99	279.59	0.022
bta-miR-24-3p	8.98	4.11	0.022
bta-miR-2285o	6.84	3.87	0.024
bta-miR-369-5p	46.86	81.38	0.025
bta-miR-378b_R+2_1ss4TG	0.93	3.89	0.025
bta-miR-6518	99.98	194.90	0.026
bta-miR-433	46.36	88.20	0.026
bta-miR-2285b	5.48	2.54	0.027
bta-miR-154b	54.62	124.29	0.028
bta-miR-149-5p	73.94	129.98	0.029
bta-miR-139	1859.49	2997.51	0.030
bta-miR-493	268.72	376.04	0.030
bta-miR-380-3p	776.18	1236.51	0.035
bta-miR-130b	19.93	26.83	0.035
bta-miR-186	12704.26	10675.03	0.037
bta-miR-7	2.43	0.98	0.039
bta-miR-378d	8.60	12.06	0.039
bta-miR-431	2.27	12.50	0.039
bta-miR-378-1-p5	16.51	33.58	0.039
bta-miR-502a_L-1R-1	15.66	8.92	0.040
bta-miR-500-p3	235.58	314.81	0.041
bta-miR-2284aa	1.49	0.77	0.041
bta-miR-2387	94.86	154.52	0.041
bta-miR-29b	102.29	53.89	0.045
bta-miR-132	143.93	241.23	0.049
bta-miR-323	5.04	9.80	0.050
bta-miR-423-5p	14.18	7.17	0.050

Table 2.2 Top 10 target genes of significant ($P < 0.05$) and differentially abundant microRNAs in pregnant and non-pregnant ipsilateral uterine horn at d 30 post-AI.

AKT3	1025
PIK3CA	734
PIK3CB	698
PIK3R3	675
PIK3R1	623
GSK3B	503
MAPK1	466
MAPK8	455
PRKCA	422
IGF1	422

Table 2.3 Top 10 enriched KEGG pathways of significant ($P < 0.05$) and differentially abundant microRNAs in pregnant and non-pregnant ipsilateral uterine horn at d 30 post-AI.

KEGG pathway	Gene count
Pathways in cancer	2435
PI3K-Akt signaling pathway	1371
MAPK signaling pathway	1277
Human papillomavirus infection	1220
Metabolic pathways	1124
Endocytosis	1075
Focal adhesion	1045
Proteoglycans in cancer	1024
Ras signaling pathway	1014
Axon guidance	1010