Detection and Management of Pregnancy Outcomes in Cattle

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DEDICATION

To my family who have always encouraged me to dream big and to shoot for the stars.

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ABSTRACT

Pregnancy success is a key factor in order for any beef producer to have a profitable operation. Subsequently, the ability to detect compromised pregnancies is of upmost importance. Currently, pregnancy-associated glycoproteins [PAG], which are secreted by the ruminant placenta, remain as the only chemical-based, pregnancy-specific detection method available on a commercial scale for cattle pregnancy diagnosis. Furthermore, PAG cannot be used for diagnosis until day 28 of gestation. Small noncoding RNAs, microRNAs [miRNAs], have been successfully used as biomarkers for certain human diseases and disorders. It is possible that pregnancy-associated miRNAs located in bovine serum and uterine flush fluid can be detectable prior to PAG. Additionally, management efforts, such as evaluating reproductive tracts and estrus expression, can be performed prior to breeding to potentially identify and select females with optimal fertility. The aim of the two studies is to determine if pregnancy-related miRNAs can be identified in bovine serum and uterine flush fluid on day 18 of gestation, and to determine if reproductive tract size and position scores [SPS] and estrus are useful predictors of fertility in *Bos indicus* and *Bos taurus* cows. The first study was able to identify two novel extracellular vesicle-miRNAs as being more abundant in pregnant heifers compared with non-pregnant heifers on day 18 of gestation in both serum and uterine flush fluid. The second study identified that

pregnancy rate, but not pregnancy loss, is interactively influenced by reproductive tract SPS and estrus activity.

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LIST OF ABBREVIATIONS

CADM1	Cell Adhesion Molecule 1
CIDR	Controlled Internal Drug Release
CD81	Cluster of Differential 81
CL	Corpus Luteum
CPM	Counts Per Million
EB	Estradiol Benzoate
eCG	Equine Chorionic Gonadotropin
ECP	Estradiol Cypionate
EM	Embryonic Mortality
EV	Extracellular Vesicle
E2	Estradiol
GnRH	Gonadotropin Releasing Hormone
FTAI	Fixed Time Artificial Insemination
IFI6	Interferon-Inducible Protein 6
IFNT	Interferon Tau
ISG	Interferon-Stimulated Gene
ISG15	Interferon-Stimulated Gene 15
LEM	Late Embryonic Mortality
miRNA	MicroRNA
miscRNA	Miscellaneous RNA
mRNA	Messenger RNA
Mt-rRNA	Mitochondrial Ribosomal RNA
Mt-tRNA	Mitochondrial Transfer RNA
MX1	Myxovirus-Resistance Protein 1
MX2	Myxovirus-Resistance Protein 2
ncRNA	Non-Coding RNA
NFIB	Nuclear Factor I/B
OAS-1	2’-5’-Oligoadenylate Synthetase
PAG	Pregnancy-Associated Glycoprotein
PBS	Phosphate Buffered Saline
PGF	Prostaglandin F2 Alpha
PMBC	Peripheral Mononuclear Blood Cells
P4	Progesterone
RPL7	Ribosome Protein L7
rRNA	Ribosomal RNA
snRNA	Small Nuclear RNA
snoRNA	Small Nucleolar RNA
SPS	Size and Position Score
THBS2	Thrombospondin-2
US	Ultrasonography
3D	Three-Dimensional

CHAPTER I: LITERATURE REVIEW

Publication Statement

A version of this chapter was originally published as:

S.L. Speckhart, S.T. Reese, G.A. Franco, T.B. Ault, R.V. Oliveira Filho, A.P. Oliveira, J.A. Green, J.L.M. Vasconcelos, and K.G. Pohler. “INVITED REVIEW: Detection and management of pregnancy loss in a cow herd.” *Professional Animal Scientist* 34 (2018): 544-557.

This review highlights various research areas of interest to the corresponding author, K.G. Pohler, in addition to his collaborators. S.L. Speckhart was the main author and composed a majority of the writing. S.T. Reese provided information on chemical methods of pregnancy diagnosis. G.A. Franco provided information on manual methods to assess pregnancy status as well as information regarding sire selection for management of pregnancy loss. R.V. Oliveira Filho and A.P. Oliveira provided information on reproductive tract scoring system for management of pregnancy loss. Research from the labs of Green, Vasconcelos, and Pohler were included in the manuscript. K.G. Pohler and T.B. Ault provided assistance with the editing process.

Abstract

Various methods and tools have been developed to detect and manage pregnancy loss in cattle to maximize reproductive efficiency by increasing pregnancy rates and decreasing pregnancy loss. Embryonic mortality constitutes the majority of pregnancy loss in cattle and can be divided into 2 periods: early embryonic mortality (<28 d of gestation) and late embryonic mortality/early fetal mortality (≥28 d of

gestation). Much research has revolved around elucidating causes of early embryonic mortality; although little is known about the mechanisms contributing to late embryonic mortality/early fetal mortality, its effects can have significant economic consequences. Current pregnancy diagnostic tools in cattle vary in accuracy until about d 28 of gestation. Refinement of current pregnancy diagnostic tests, with the ability to be accurate at approximately the third week of gestation, or the development of new methods that are able to assess embryonic viability would both decrease the financial ramifications linked to embryonic mortality and increase the reproductive efficiency of the herd. The following review will highlight some techniques that have been reported to detect and predict pregnancy loss and some of the potential management strategies that might mitigate these losses.

Introduction

Suboptimal reproductive efficiency plagues beef and dairy cow herds, with pregnancy loss being the main contributing factor. Negative economic effects stem from an increased number of nonpregnant cows accruing maintenance costs, fewer total pounds at weaning, and increased culling rates in dairies. Despite these incentives to perform pregnancy diagnosis, only 20% of beef producers in the United States do so, and most are unaware of the exact pregnancy status of their herd (NAHMS, 2009). Even though most dairies perform one or more pregnancy evaluations per female,

identifying cows that will ultimately fail to produce milk due to pregnancy loss remains an elusive task (NAHMS, 2014).

The rate of pregnancy loss peaks during embryonic development and decreases after d 45 as gestation progresses and active placentation is complete (Diskin and Sreenan, 1980; Santos et al., 2004). Embryonic mortality (EM) can be classified as early embryonic mortality, occurring before d 28 of gestation, or late embryonic mortality (LEM), occurring after 28 d of gestation (Silke et al., 2002). Most pregnancy loss in both beef (21.8–35.6%; Maurer and Chenault, 1983; Breuel et al., 1993; Ahmad et al., 1995; Dunne et al., 2000; Santos et al., 2004) and dairy (28.9–46.3%; DeJarnette et al., 1992; Dalton et al., 2001; Sartori et al., 2002; Santos et al., 2004) cattle occurs during early embryonic development. However, LEM between d 28 and 45 remains a significant problem resulting in termination of 5 to 17.5% of all cattle pregnancies, with an increased negative effect in high producing dairy cattle (Diskin and Sreenan, 1980; Vasconcelos et al., 1997; Silke et al., 2002; Galvão et al., 2004; Santos et al., 2004; Grimard et al., 2006; Pohler et al., 2016a). Pregnancy loss can be attributed to infectious and noninfectious causes. Management and prevention of diseases with reproductive ramifications such as, infectious bovine rhinotracheitis, bovine viral diarrhea, and leptospirosis, reduce pregnancy losses associated with infectious disease (Aono et al., 2013; Pereira et al., 2013). However, this review will focus on detection and management of noninfectious, physiological causes of pregnancy loss. Physiological mechanisms contributing to pregnancy failure differ between periods as

does the effect on production and economic losses. Detecting cows that undergo EM or those failing to establish a pregnancy is critical to decrease calving and interbreeding intervals. The objectives of this review are to (1) describe methods to detect pregnancy loss in cattle (summarized in Table 1 in Appendix I) and (2) discuss management tools to decrease the amount of pregnancy losses within a herd (summarized in Table 2 in Appendix I).

Review and Discussion: Detection Methods of Pregnancy Loss

Manual Methods

Return to Estrus

Return to estrus after insemination or a positive pregnancy diagnosis is an important aspect of reproductive management in dairy and beef operations. A cow's estrous cycle can range from 17 to 24 d but averages 21 d in duration for both Bos taurus and Bos indicus cattle (Odde et al., 1980; Bó et al., 2003; Sartori et al., 2004); however, lactating dairy cows tend to have a slightly longer estrous cycle of 23 d (Sartori et al., 2004). If a cow does not achieve pregnancy after insemination, a return to estrus can be observed if she is cycling. If there is no indication of estrus beyond 24 d after insemination, it is suggested that conception may have occurred (Youngquist, 2006). However, this method of determining pregnancy status should be limited because there is a high risk of false positives that can be attributed to the following: embryonic loss resulting in delayed luteal regression (Wijma et al., 2016), number of follicular waves resulting in a longer interestrus interval (Santos et al., 2004; Remnant et

al., 2015), resynchronization techniques (Chebel et al., 2006; Giordano et al., 2015), or anestrus cows, which can occur due to several factors including but not limited to nutritional status, lactation, season, and stress (Short et al., 1990). Regarding detection of pregnancy loss, return to estrus may be used effectively after an initial pregnancy confirmation to identify potential cows standing in estrus. With the use of automated estrus detection systems, these cows could simply be rebred, or a confirmation pregnancy test could be conducted.

Transrectal Palpation

During early to mid-gestation, anatomical structures continue to grow and develop making rectal palpation a common method of pregnancy diagnosis in cattle. The earliest time for accurate pregnancy diagnosis via rectal palpation is between 30 and 35 d of gestation by detecting a membrane slip or palpation of the amniotic vesicle (Roberts, 1986; Momont, 1990). However, there has been a debate as to the use of early gestation palpation due to the fragile nature of the amniotic vesicle. Ball and Carroll (1963) recommended not conducting early gestation palpation (<35 d) to prevent puncturing of the amniotic vesicle resulting in termination of pregnancy. A 1978 survey reported rectal palpation, specifically the slipping of chorioallantoic membranes, led to an incidence of embryonic loss of 5.8, 6.03, and 0.82% when palpated at <35, 35 to 45, and >45 d after insemination, respectively (Paisley et al., 1978); however, it is difficult to determine whether any proportion of these losses were due to idiopathic origins or a

direct result from palpation. Conversely, a more recent study concluded no difference in embryonic loss due to rectal palpation by performing a membrane slip between d 34 and 41 of gestation compared with females that were not rectally palpated (Romano et al., 2007). Nevertheless, rectal palpation exams generally take place between d 45 and 60 of gestation, which decreases the chance to cause embryonic loss and increases the accuracy of pregnancy diagnosis (Santos et al., 2004). Although transrectal palpation is reliable and one of the most widely accepted industry methods for pregnancy diagnosis, it provides only a static assessment of pregnancy status and cannot be used to assess embryonic viability or the absence or presence of a heartbeat.

Transrectal Ultrasonography

Transrectal B-mode ultrasonography (US) is currently regarded as the gold standard for determining pregnancy status and embryonic and fetal viability in cattle because it provides potential for visual and morphological assessment of the uterus, ovaries, and embryo and fetus. Even though the embryonic vesicle can be visualized in the uterine horn ipsilateral to the corpus luteum (CL) as early as d 10 to 17 of gestation and the embryo itself from d 19 to 24 of gestation, accurate pregnancy diagnosis using B-mode US is not recommended before d 26 to 29 of gestation (Pierson and Ginther, 1984; Curran et al., 1986a,b; Kastelic et al., 1988; Pieterse et al., 1990). During the d 26 to 29 window, one can expect to observe an embryo in a prominent “C” shape, followed by straightening of the neck and head generating an “L” shaped embryo until d 39 of gestation (Curran et al., 1986b). Real-time US has greatly influenced the standard for

future pregnancy diagnostic tests or exams because it allows for the visualization of the embryo and heartbeat, which is detectable as early as d 21 to 25 of gestation to assess immediate viability (Curran et al., 1986b; Ginther, 1998). Currently, real-time US is most often performed between d 28 and 32 as a method of early pregnancy detection in cattle.

The incorporation of new ultrasound technologies, such as Doppler US, enables a more detailed assessment of the uterus, ovarian follicles, and CL. Doppler US uses blood flow to generate images, which are dependent on the type of Doppler setting being applied (i.e., color, power, or spectral). Color Doppler US displays blood flow traveling toward, indicated by a red color, and away, indicated by a blue color, from the transducer (Herzog and Bollwein, 2007; Matsui and Miyamoto, 2009). Spectral Doppler (pulse wave and continuous wave) is unique compared with the other Doppler modes because it presents blood flow in wave form instead of a color map. More importantly, pulse-wave Doppler uses a gate, which allows for a precise measurement of blood flow within the region of the vessel of interest. The use of transrectal color Doppler US for reproductive studies in large animals was first described in 1998, and thereafter, several studies described the changes in ovarian and uterine blood flow throughout the estrous cycle and early gestation in the mare and cow (Bollwein et al., 1998; Acosta et al., 2005; Siddiqui et al., 2009; Silva and Ginther, 2010; Herzog et al., 2011; Bollwein et al., 2000, Bollwein et al., 2016). Although the technique is predominately used for research purposes, incorporation of Doppler US strategies into commercial reproductive

programs may aid in increasing reproductive efficiency. In pregnant cows, changes in uterine blood flow can be observed beginning in the third week of gestation (Silva and Ginter, 2010; Bollwein et al., 2016). Due to the large variation in blood flow between animals, a single measurement of uterine blood flow is not accurate enough for early pregnancy detection (Pugliesi et al., 2014a). Advancements in US technology and quality may provide opportunity to use a combination of color and pulse-wave Doppler US simultaneously to assess umbilical artery blood flow using pulsatility index values as used in humans. These indices have been able to predict unfavorable pregnancy outcomes such as intrauterine growth restriction in humans (Alfirevic et al., 2017; Gudmundsson et al., 2017), and there is potential to adapt these concepts to predict likelihood of pregnancy loss in cattle.

In nonpregnant cows that regressed their CL 15 to 18 d after insemination, a decrease in blood flow was detected through color Doppler US at d 19 after ovulation (Matsui and Miyamoto, 2009). Several studies have shown that evaluating CL blood flow during luteolysis or maternal recognition of pregnancy could be an accurate method for early detection of pregnancy (Utt et al., 2009; Herzog et al., 2011; Pugliesi et al., 2014b; Scully et al., 2015). Pugliesi et al. (2012) established criteria to differentiate a functional CL from a regressing CL by detecting both reduction in size and blood flow. When early pregnancy diagnosis by color Doppler US, evaluating CL area and blood flow, was compared with transrectal ultrasound later on in gestation, satisfactory sensitivity and accuracy was achieved for pregnancy diagnosis 20 d after insemination

in beef (Pugliesi et al., 2014a) and dairy cows (Siqueira et al., 2013). Even though this technique has high accuracy in detecting nonpregnant animals, there is a considerable proportion of false positives or cows diagnosed pregnant at d 20 but not pregnant at d 30 by US. Presence of a functional CL in nonpregnant animals could be related to several factors including the delay in ovulation during synchronization protocols or lengthened estrus cycles or may be due to pregnancy loss that occurs between the early (d-20) diagnosis and conventional diagnosis. Data discussed later in this review highlight potential methods of early pregnancy diagnosis that consider these pregnancy losses during this time period. In dairy cows, the occurrence of false positives may be disproportionately greater due to a higher incidence of EM during this interval (Pohler et al., 2016a; Reese et al., 2018).

Three-dimensional (3D) US to visualize pregnancy and assess viability of the developing fetus has been well characterized in humans for the last 2 decades (Hata et al., 1997; Leijnse et al., 2018). However, this type of research in cattle has been hindered by technological issues and the time course of development for the bovine embryo. Three-dimensional US requires that the embryo or fetus remain immobile and properly oriented within the uterus to capture an image, which requires significant skills by the technician. During early development, the bovine embryo lacks definition making it difficult to capture a 3D image during the embryonic stage. However, by d 45 of gestation, an image of the bovine fetus using the surface feature on 3D US is more easily acquired when development of anatomical structures such as the head, body,

and legs can be visualized (Kähn, 1989). Because the percentage of fetal loss is lower than embryonic loss, 3D US past d 45 of gestation may not provide enough beneficial information to be useful for determining pregnancy status or pregnancy loss compared with other currently available methods.

Chemical Methods

Pregnancy diagnosis using chemical-based methods earlier in gestation may be more beneficial compared with rectal palpation and US due to the potential ability to detect pregnancy loss. These chemical-based methods fall into 2 categories: markers that are pregnancy specific (expressed or secreted by the conceptus) and those that are not pregnancy specific (produced under other physiological conditions). Some of the most common methods explored in these areas are progesterone, interferon-stimulated genes (ISG), small noncoding RNA such as microRNA (miRNA), and pregnancy-associated glycoproteins (PAG).

Markers that Are Not Pregnancy Specific: Progesterone

One of the most common, non-pregnancy-specific tests uses progesterone, a steroid hormone that is produced by the CL to maintain pregnancy (Lukaszewska and Hansel, 1980). In a cycling cow, concentrations of progesterone peak during the luteal phase and reach nadir during the follicular phase due to CL regression. Alternatively, in a pregnant cow, the CL will not regress, and elevated progesterone concentrations will be maintained (Niswender et al., 2000). Progesterone assays for pregnancy diagnosis can be performed between d 20 and 21 of gestation (Sasser, 1987; Nebel, 1988). In

regard to progesterone and pregnancy loss, measurement of progesterone early in gestation or during the critical embryonic loss period could be useful in determining which females are at risk for experiencing embryonic loss or to diagnosis embryonic loss itself, based on the observation of a decrease in progesterone concentration over time (Darwash and Lamming, 1998; Starbuck et al., 2001; Diskin et al., 2002; Mann et al., 2005; McNeill et al., 2006). There are conflicting reports in the published literature between the relationship of progesterone and cows undergoing pregnancy loss. Starbuck et al. (2004) reported cows undergoing LEM had decreased circulating concentrations of progesterone compared with cows having a successful pregnancy; however, Pohler et al. (2013, 2016a,b), reported no difference. Some of these conflicting results could be the result of comparing different types of cattle in different environments. Nevertheless, in terms of pregnancy diagnosis, the consequence of using a non-pregnancy-specific chemical-based test such as progesterone is that there is a risk for yielding false-positive results in cows that have longer luteal phases (3 versus 2 follicular waves), ovarian cysts, a prolonged CL, or EM similar to the challenges of nonreturn rates discussed earlier (Roberts, 1986; Pohler et al., 2015). A single progesterone concentration as a form of pregnancy diagnosis should be avoided because false-positive results are likely; however, the potential may exist for daily monitoring of progesterone fluctuations via in-line milk testing or other robotic approaches. In-line milk progesterone sampling machines (Herd Navigator, DeLaval International, Tumba, Sweden) could drastically improve progesterone's overall value

as a means of detecting both pregnancy success and loss by the machine's ability to collect and analyze samples automatically. To provide a more accurate diagnosis, this technology could easily be applied throughout the length of the entire embryonic period in dairy cattle, which is currently limited due to manual, labor-intensive sampling methods.

Markers that Are Not Pregnancy Specific: ISG

Corpus luteum rescue enabled by maternal recognition of pregnancy in ruminants is established by a Type 1 interferon, interferon tau (IFNT; Roberts et al., 1992; Bazer et al., 1997). Interferon tau is secreted by the elongating conceptus from d 14 to 16 of gestation (Bazer, 1992; Roberts et al., 1999). During this period, IFNT prevents expression of endometrial estrogen receptors, which in turn leads to the inhibition of oxytocin receptor formation, prohibiting large, pulsatile secretions of prostaglandin F2α that are capable of CL lysis (Wathes and Lamming, 1995). Along with its antiluteolytic role at the level of the endometrium, IFNT has more recently been identified to act on both the CL and circulating peripheral mononuclear blood cells (PMBC; Oliveira et al., 2008; Bott et al., 2010; Hansen et al., 2010; Pohler et al., 2015). Due to limitations of current IFNT detection assays, ISG [interferon-stimulated protein 15 kDa (ISG15), myxovirus-resistance protein 1 (MX1), myxovirus-resistance protein 2 (MX2), and 2′-5′-oligoadenylate synthetase (OAS-1)] have been explored, specifically in PMBC, to diagnosis pregnancy success and loss in cattle and sheep (Yankey et al., 2001; Han et al., 2006; Gifford et al., 2007; Stevenson et al., 2007; Green et al., 2010;

Matsuyama et al., 2012; Pugliesi et al., 2014a; Pohler et al., 2015, 2017). In cattle, pregnant animals tend to have increased expression of ISG15, MX1, MX2, and OAS-1 in PMBC (Han et al., 2006; Gifford et al., 2007; Green et al., 2010). However, because interferons are released in response to viral infections, this can also initiate expression of ISG regardless of pregnancy status (Nakaya et al., 2001; Lenschow et al., 2005). Consequently, when considering the use of ISG as a detection method, it should either be limited for detection of nonpregnant heifers or cows or a baseline sample taken to declare all cattle void of infection or increased ISG. Stevenson et al. (2007) used a combination of progesterone and ISG in dairy heifers to determine pregnancy loss between d 21 and 30, d 30 and 60, and d 21 and 60. Heifers were characterized to have undergone pregnancy loss if d-21 progesterone concentration was >2.9 ng/mL but no embryo was present at d 30. Myxovirus-resistance protein 2 messenger RNA from PMBC was evaluated on d 0 (day of estrus) and d 18 after insemination to classify heifer pregnancy status as initially determined by progesterone as being a true positive, true negative, false positive, or false negative (Stevenson et al., 2007). Although expression of MX2 tended to correlate with pregnancy loss from d 21 to 30 as well as d 21 to 60, it still was not accurate in predicting reproductive outcome due to both low sensitivity and negative predictive values (Stevenson et al., 2007). Additionally, Wijma et al. (2016) used ISG in conjunction with PAG to determine EM among lactating dairy cows. There was an observed increase in ISG (ISG15 and MX2) from PMBC on d 18 and 20 after insemination in pregnant and EM cows compared with both sham-

inseminated and inseminated, nonpregnant cows. The authors concluded that a combination of ISG and PAG served as an acceptable experimental approach to identify the presence or absence of an embryo. Therefore, ISG may be a promising method to detect pregnancy loss between the second and third week of gestation in a research setting when combined with an additional pregnancy detection method such as progesterone or PAG. Over the last decade there has been numerous attempts to generate a commercially available ISG test; however, to date there are none on the market.

Markers that Are Not Pregnancy Specific: miRNA Biomarkers

MicroRNA are a class of small (~22 nucleotides), noncoding RNA that are posttranscriptional regulators of gene expression and have been used for identification of several human diseases and disorders (Bartel, 2004; Reid et al., 2011). They can be noninvasively measured and maintain their stability in biological substances such as blood, making them ideal biomarkers (Chen et al., 2008). Research indicates that miRNA may play important roles in reproductive function including but not limited to folliculogenesis (Ro et al., 2007), CL function (Otsuka et al., 2008), implantation (Chakrabarty et al., 2007), early embryonic development (Wienholds et al., 2003, 2005), and placentation (Luo et al., 2009; Miura et al., 2010; Donker et al., 2012; Zhao et al., 2012). Currently, bovine miRNA biomarkers are being investigated in a research setting and are not available for commercial use to diagnose pregnancy in cattle.

To date, pregnancy-associated miRNA candidates have been located in milk, plasma, serum, and whole blood (Ioannidis and Donadeu, 2016, 2017; De Bem et al., 2017; Pohler et al., 2017; Schanzenbach et al., 2017; Markkandan et al., 2018). However, there has been vast inconsistency in specific miRNA that have been identified as potential candidates due to varying origin of miRNA that were extracted, isolation material, and wide variation in research techniques and protocols. Our group has recently demonstrated that cows undergoing EM between d 17 and 31 of gestation have a significant number of differentially abundant miRNA compared with cows that successfully establish a pregnancy (Pohler et al., 2017). Specifically, miR-25, −16b, and −3596 were identified as potential EM-associated candidates based on additional validation methods. Interestingly, these miRNA were also shown to be involved in several critical pathways, one of which was prostaglandin synthase 2, the rate-limiting enzyme for prostaglandin production (Bazer, 2013). This pathway provides a potential connection between specific miRNA and pregnancy loss.

Identification of a pregnancy-associated miRNA that is able to assess embryonic viability or an EM-specific miRNA has the potential to aid pregnancy diagnosis in cattle by offering additional information not available through current commercial pregnancy detection methods; however, many technological aspects, along with validation must be completed before on-farm application. Furthermore, a miRNA biomarker that is able to decipher embryonic viability in cattle would greatly reduce days a heifer or cow spends open, which in turn would allow for earlier rebreeding.

Markers that Are Pregnancy Specific: PAG

A search for a pregnancy-specific marker that could be easily detectable in blood led to the identification of PAG. First identified in the 1980s with the discovery of PAG1, also known as pregnancy-specific protein B (Butler et al., 1982), over 2 dozen PAG genes have since been annotated to the bovine genome (Green et al., 2000; Telugu et al., 2009). Pregnancy-associated glycoproteins are pregnancy-specific proteins secreted by binucleated trophoblast cells in the placenta of many ruminants and are detectable in maternal circulation around d 24 of gestation (Szafranska et al., 1995; Xie et al., 1997; Garbayo et al., 2000; Green et al., 2000; Egen et al., 2009, Pohler et al., 2013). Phylogenetic analysis has identified 2 main groups of PAG, “ancient” and “modern,” which differ in trophoblast expression patterns in the bovine conceptus (Green et al., 2000; Hughes et al., 2000). Although the exact function of PAG remains elusive, it has been hypothesized that they play a role in immune function or may possess luteoprotective action (Wallace et al., 2015). Many factors can affect circulating concentration of PAG: subspecies, parity of dam, sire, fetal sex, twinning, birth weight, and day of gestation (Zoli et al., 1992; Patel et al., 1997; Echternkamp et al., 2006; Lobago et al., 2009; Mercadante et al., 2013; Lawson et al., 2014; Ricci et al., 2015; Pohler et al., 2016b; Franco et al., 2018b). Commercial tests currently available through BioTracking (BioPRYN; BioTracking LLC, Moscow, ID), IDEXX (IDEXX Bovine Pregnancy Test; IDEXX Laboratories Inc., Westbrook, ME), and Genex (D29 Blood Pregnancy Test; Genex Cooperative Inc., Shawano, WI) use PAG in blood or milk

samples as early as d 28 after insemination to detect pregnancy in cattle. Accuracy of PAG testing is comparable to US for pregnancy diagnosis, and false positive risk requires a minimum waiting period following parturition (60–75 d) to clear residual circulating PAG (Silva et al., 2007; Romano and Larson, 2010). Although the testing of PAG concentrations is 98 to 99% accurate in diagnosing true pregnancies with low occurrences of false positives (<5%) at the recommended day of sampling, use of commercial tests to assess embryonic or fetal viability has been less explored.

In addition to pregnancy diagnosis, recent research indicates circulating concentration of PAG can be used to determine likelihood of pregnancy success or failure in cattle. Increased PAG concentrations are directly correlated with an increased probability of pregnancy success, whereas decreased circulating PAG concentrations are related to increased pregnancy loss (Thompson et al., 2010; Breukelman et al., 2012; Pohler et al., 2013; Engelke et al., 2015; Pohler et al., 2016a,b; Wijma et al., 2016; Gatea et al., 2018; Reese et al., 2018). Specifically, circulating PAG concentrations between d 28 and 31 were significantly increased following fixed-time AI (FTAI) and fixed-time embryo transfer in beef and dairy cows that maintained pregnancy compared with cows that underwent LEM (Thompson et al., 2010; Breukelman et al., 2012; Pohler et al., 2013; Pohler et al., 2016a,b). Although circulating concentrations of PAG differ between subspecies throughout gestation, studies using predictive value analysis identified cutoff concentrations in Bos indicus and Bos taurus cows with the greatest likelihood of undergoing EM, further supporting PAG biomarker potential

(Pohler et al., 2016a,b). More recent studies found early gestation circulating PAG concentrations at d 24 to yield similar results in dairy and beef cattle of both subspecies; however, assay refinement and larger sample sizes are needed to identify suitable predictive cutoff values (Guirado Dantas et al., 2017; Reese et al., 2018). Because considerable pregnancy loss occurs during the period of maternal recognition of pregnancy (d 16 to 25), there is hesitation to develop pregnancy diagnosis methods that are unable to detect early loss. Estimated loss between d 19 to 24 and d 31 based on both ISG expression and early circulating PAG concentrations range from 17 to 31% (Matsuyama et al., 2012; Monteiro et al., 2014; Pohler et al., 2016a; Wiltbank et al., 2016; Reese et al., 2018). In a model using a d-24 PAG increase to predict likely EM, cows that were not pregnant at d 31 but had elevated PAG at d 24 had numerically lower PAG concentration compared with cows that successfully maintained pregnancy (Reese et al., 2018). Unlike other static detection methods, circulating PAG concentration presents evidence as a pregnancy loss detection tool that may be used earlier in gestation to avoid diagnosis of cows that will undergo early embryonic mortality or LEM as pregnant. Considering the prevalence of LEM in cattle, especially lactating dairy cows, commercial development of a pregnancy diagnosis test that is able to detect LEM would be an innovative tool for the dairy and beef industries.

Review and Discussion: Risk Factors and Management of Pregnancy Loss

Estrus Activity

Estrus activity at time of AI or preceding embryo transfer has a direct relationship with increased pregnancy rates and decreased EM (Pursley et al., 1998; Souza et al., 2007; Pereira et al., 2016). Heat detection patches (Estrotect, Rockway Inc., Spring Valley, WI), automated estrous detection systems, and many other methods have been proven to be reliable tools for identifying females that exhibit estrus activity (Rorie et al., 2002; Saint-Dizier and Chastant-Maillard, 2012; Pereira et al., 2016; Pohler et al., 2016b; Colazo et al., 2018; Speckhart et al., 2018) and may hold more value for certain breeds or types of cattle compared with others. For instance, Bos indicus females have a shorter estrus period (10.9–12.9 h vs. 16.3 h) compared with Bos taurus females (Mizuta, 2003). Furthermore, most (~80%) Bos indicus cows exhibit estrus during the evening, creating an issue for manual, visual estrus observation (Pinheiro et al., 1998). Additionally, short estrus periods and intensity have been observed in high producing Bos taurus dairy cows (Lopez et al., 2004).

Pereira et al. (2016) demonstrated that dairy cows exhibiting increased estrus activity and that underwent FTAI had greater pregnancy rates on d 32 and 60 of gestation compared with cows that did not exhibit estrus. Additionally, LEM significantly decreased in FTAI cows that exhibited estrus (14.4%) compared with cows that did not exhibit estrus (20.1%). Similarly, cows that were subject to fixed-time embryo transfer demonstrated parallel results to those that were subject to FTAI. Studies using activity-

based monitoring systems in dairy cattle have reported similar results (Madureira et al., 2015). Collectively, these results indicate estrus expression at time of FTAI and before fixed-time embryo transfer is positively associated with an increase in pregnancy rates and a decrease in LEM.

In a study by Pohler et al. (2016b) using postpartum Bos indicus beef cows, an Estrotect heat detector patch scoring system was established to determine a relationship of estrus at time of FTAI with concentration of PAG secreted on d 28 of gestation. Patch scores ranged from 0 to 4, where a patch score of 0 signified a lost patch; a score of 1 signified <25% activation; a score of 2 signified <50% activation; a score of 3 signified <75% activation; and a score of 4 signified >75% activation. Cows having either patch scores of 3 or 4 were considered to have exhibited estrus activity. Results indicated that as patch scores increased, the concentration of PAG at d 28 also significantly increased (Figure 1 in Appendix I). Speckhart et al. (2018) observed similar results in Bos taurus females using the same parameters described earlier (Figure 1). Additionally, d-30 pregnancy rates and AI calving rates were greater in females that exhibited estrus compared with those that did not express estrus (Figure 2 in Appendix I). Therefore, using Estrotect heat detector patches in a herd can serve as an inexpensive identifier of cows with increased likelihood of maintaining their pregnancy. Beef and dairy producers could use estrus activity or potential estrus intensity at or before timed AI or 7 d before timed embryo transfer as measured via methods described above as a management tool to decide which cows should be inseminated or

have embryos transferred into, respectively, to decrease the chance of pregnancy loss. Specifically, a cow with decreased estrus activity before timed AI or timed embryo transfer that has an increased risk for pregnancy loss, would be a candidate for a high fertility sire or embryo with decreased genetic merit (decreased price) to decrease production cost or loss or skip insemination or embryo transfer completely. These decisions would be farm and type of cow dependent.

Reproductive Tract Scoring System

It has been reported in published literature regarding lactating dairy cows that increased uterine size measured using 2-dimentional US is related to decreased fertility (Baez et al., 2016). The physiological mechanism relating uterine size and fertility is not well understood but may be due to sperm transport failure in the female reproductive tract or reduced exposure to interferon-tau in larger reproductive tracts, which leads to erroneous maternal recognition of pregnancy and premature CL regression during early pregnancy (Baez et al., 2016).

Young et al. (2017) developed a reproductive tract size and position score (SPS) system, which can easily identify, through rectal palpation, potentially less fertile females within the herd. There are 3 reproductive tract classifications: SPS1, SPS2, and SPS3. Cows designated SPS1 had small and compact uterine horns resting within the pelvic cavity; cows designated SPS2 had intermediate size uterine horns resting partially outside the pelvic cavity; and cows designated SPS3 had large uterine horns resting mainly outside of the pelvic cavity. Authors found that parity influences tract size

and position scores; as parity increased, the percentage of SPS1 cows decreased, whereas the number of SPS2 and SPS3 cows increased. However, multiparous SPS1 cows had similar pregnancy rates to first and second parity SPS1 cows, indicating tract size is influential beyond parity alone. In lactating dairy cows, there was a significant increase in pregnancy rates among cows as reproductive tract scores decreased (Young et al., 2017; Oliveira et al., 2018). Additionally, Madureira et al. (2017) reported pregnancy loss between d 31 and 120 to be increased in lactating Holstein cows with SPS3 compared with SPS2 and SPS1 (14.9% vs. 9.6% vs. 2.9% for SPS3, SPS2, and SPS1, respectively), whereas pregnancy rates were greater among SPS1 females compared with SPS2 and SPS3 (42.4% vs. 32.6% vs. 23.9%). Adapting a reproductive tract size and position scoring system in both dairy and beef operations can be a useful tool to help producers in managing the fertility of their females by offering an opportunity to selectively decide which cows are to be bred based on their respective reproductive tract and position scores.

Sire Selection

Research regarding pregnancy loss in cattle has mainly focused on the maternal side; however, the paternal contributions cannot be overlooked considering the substantial variation in the amount of pregnancy loss reported between sires (Markusfeld-Nir, 1997; López-Gatius et al., 2002; Pegorer et al., 2007) and the large influence paternal genetics have on placental formation (Barton et al., 1985; Surani et al., 1987). In a study by Franco et al. (2018b), postpartum, multiparous Nelore cows

were subjected to FTAI by Angus sires that were categorized as either high LEM (mean 7.25%) or low LEM (mean 3.93%), which is equivalent to a 1.9× greater rate of pregnancy loss. Circulating concentrations of PAG at d 30 of gestation were significantly decreased in cows inseminated with high embryonic loss sires. Preliminary data from our laboratory has indicated variation in the amount of PAG gene copy number in semen from sires of different breeds and fertility. More interestingly, we observed variation among phylogenetic groups, specifically ancient PAG genes were greater in high pregnancy loss samples, whereas modern PAG genes were increased in low pregnancy loss samples (Franco et al., 2018a). These data are preliminary and limited to a small group of sires; however, exploring this relationship may elucidate some of the variation observed in fertility and pregnancy loss that exists among sires. Using specific, yet to be identified, genomic characteristics to identify bulls with higher fertility (i.e., low embryonic loss) can improve sire selection and decrease pregnancy losses.

Additionally, the incorporation of Bos indicus genetics has been used to increase the embryo's ability to tolerate heat stress in tropical environments, specifically in dairy herds, whereas other studies used Bos taurus genetics in Bos indicus herds to increase heterosis and improve embryo development. Pegorer et al. (2007) observed higher pregnancy rates ($P < 0.05$) and lower incidence of pregnancy loss ($P < 0.05$) in Holstein cows that were artificially inseminated to a Bos indicus sire. In addition, our group has seen similar results: Nelore cows inseminated with Bos indicus sires had greater ($P < 0.001$) pregnancy rate at d 30 of gestation but greater ($P = 0.014$) pregnancy loss

between d 30 and 100 of gestation, when compared with cows inseminated with Bos taurus sires (Franco et al., 2018b). Even though the exact genetic characteristics and mechanisms regarding sire contribution to pregnancy loss have yet to be elucidated, this research is ongoing and holds promise to increase reproductive efficiency in cattle. The future implication of this research would aid in the selection process of both beef and dairy sires by genetically identifying high pregnancy loss from low pregnancy loss sires.

Conclusion

There are several ways to detect and manage pregnancy loss in cattle (Table 1 and 2 in Appendix I), and some provide greater accuracy and specificity compared with others. Blood and milk tests that use pregnancy or non-pregnancy-specific methods as their basis for testing could become commercially available for cattle producers and may have the potential to predict pregnancy loss or maintenance. This prediction is of interest to ultimately limit days a female spends open, which in turn will reduce costs associated with maintaining a nonpregnant female and will allow for earlier rebreeding.

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Appendix I

Table 1. Summary of detection methods of pregnancy loss in cattle with respective advantages and disadvantages of each.

Item for Manual Methods	Accuracy	Assess Embryonic Viability	Earliest Day for Accurate Use
Return to Estrus	Low	No	24 days
Transrectal Palpation	High	No	30 – 35 days
Transrectal Ultrasonography: Two-Dimensional	High	Yes	26 – 29 days
Transrectal Ultrasonography: Color Doppler	Medium	Yes	19 – 20 days
Transrectal Ultrasonography: Three-Dimensional	High	No	45 days
Item for Chemical Methods	**Accuracy**	**Assess Embryonic Viability**	**Earliest Day for Accurate Use**
Progesterone (Commercial Test Available)	Low	No	19 – 20 days
Interferon-Stimulated Genes (No Commercial Test Available)	Medium	Yes	18 – 20 days
MicroRNA Biomarkers (No Commercial Test Available)	Unknown	Potentially	8 – 30 days
Pregnancy-Associated Glycoproteins (Commercial Test Available)	High	Yes	28 days

Table 2. Summary of risk factors and management variables that can affect pregnancy loss in cattle.

Pregnancy loss (%)		Reference
Sire		
High fertility	*Low fertility*	
3.93	7.25	Franco et al., 2018
3.2	17.6	Lopez gatius et al., 2002
5.9	10.6-17.9	Markusfeld-Nir, 1997
Estrus		
Yes	*No*	
7.9	7.8	Speckhart et al., 2018
14.4	20.1	Pereira et al., 2016 *FTAI
18.6	22.7	Pereira et al., 2016 *FTET
Uterine size		
Small (SPS1)	*Large (SPS3)*	
2.9	14.9	Madureira et al., 2017
13.5	50	Oliveira et al., 2018
4.0	4.8	Baez et al., 2016 *Primiparous
7.9	9.1	Baez et al., 2016 *Multiparous

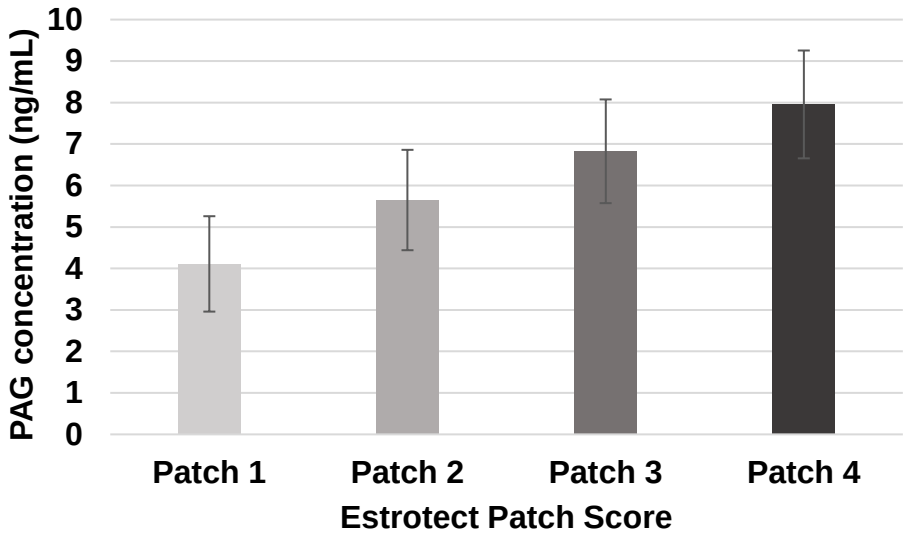

Figure 1. Pregnancy-associated glycoprotein (PAG) concentration by Estrotect patch score. Adapted from Pohler et al. (2016b) and Speckhart et al. (2018). Combined serum concentration of day 28 and day 30 PAGs (mean ± SEM) with different levels of Estrotect patch activation (1, <25% activated; 2, <50% activated; 3, <75% activated; and 4, >75% activated) at TAI in postpartum primiparous Nelore beef cows and Angus females, respectively. As estrus intensity increased, as determined by Estrotect patch scores, there was a numerical increase ($P > 0.05$) in circulating PAGs.

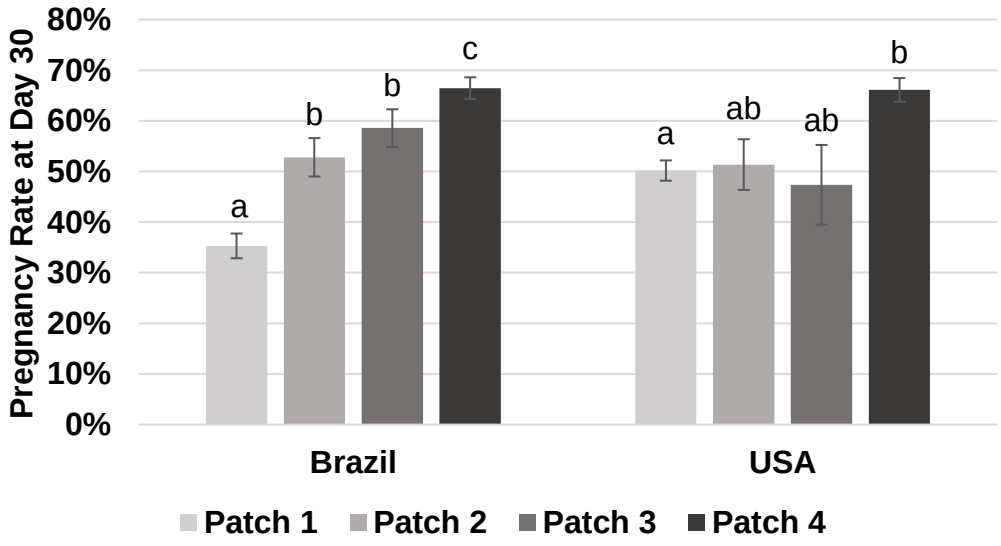

Figure 2. Pregnancy rates at day 30 by Estrotect patch scores. Adapted from Franco et al. (2018a) and Speckhart et al. (2018). Multiparous, Nelore cows (*n* = 1228) in a Brazil study observed increased pregnancy rates at day 30 of gestation as Estrotect heat detector patch scores increased in number (Patch 1, 35.3% vs. Patch 2, 52.8% vs. Patch 3, 58.6% vs. Patch 4, 66.5%; patch score 1 vs. 2 and 3, $P = 0.0006$, patch score 2 and 3 vs. 4, $P < 0.0001$). Angus females (*n* = 1304) in a study from the USA observed similar results to the Brazil data, pregnancy rates demonstrated an increase as Estrotect heat detector patch scores increased in number (Patch 1, 50.2% vs. Patch 2, 51.4% vs. Patch 3, 47.4% vs. Patch 4, 66.1%; patch score 1 vs. 4, $P < 0.0001$).

CHAPTER II: COMPARISON OF CIRCULATING AND UTERINE MICRORNAS IN PREGNANT AND NON-PREGNANT BEEF HEIFERS

Abstract

MicroRNAs (miRNAs) isolated from milk and blood have recently been used as pregnancy biomarkers in cattle; however, miRNAs located in uterine flush fluid may harbor more accurate candidates. The objective of the current study was to identify pregnancy-related, extracellular vesicle (EV) derived miRNAs from serum and uterine flush fluid on day 18 post-insemination in cattle. We hypothesized EV-derived miRNAs from pregnant and non-pregnant heifers would differ in serum and uterine flush fluid. Beef heifers were randomly allocated into one of two groups (14 heifers received live semen; 6 heifers received dead semen, control heifers) for artificial insemination at day 0. Sacrifice was performed on day 18 where presence or absence of a conceptus was reported, and samples were collected. Extracellular vesicles were isolated by ultracentrifugation for western blot analysis (for CD81) and transmission electron microscopy prior to small RNA extractions. After confirmation of successful EV isolation, small RNAs were extracted using TRIzol. Next generation sequencing of small RNAs was performed on 12 samples. Each heifer (pregnant, n = 3; non-pregnant, n = 3) had two samples that were sequenced; one serum and one uterine flush fluid sample. Various differential abundance parameters identified two novel miRNAs at different loci that were located in both serum and uterine flush fluid from pregnant and non-pregnant heifers. These novel miRNAs were more abundant in pregnant heifers compared with non-pregnant heifers. In summary, EV-derived miRNAs were located in serum and

uterine flush fluid from pregnant and non-pregnant beef heifers at day 18 post-insemination.

Introduction

Reproductive failures in the United States beef industry accounts for more than $1 billion in losses annually (Geary, 2005). The majority of these losses occur within the first 28 days of gestation which is termed early embryonic loss, and a smaller portion of losses occur between days 28 and 42 of gestation termed as late embryonic loss, followed by even smaller losses after day 42, fetal loss (Santos, Thatcher, Chebel, Cerri, & Galvao, 2004; Silke et al., 2002; Sreenan, Diskin, & Morris, 2001). A pregnancy-specific marker called pregnancy-associated glycoprotein (PAG), secreted from binucleated trophoblast cells of the placenta, was identified almost four decades ago and has remained as the only pregnancy-specific, chemical-based method for pregnancy detection in cattle (Butler et al., 1982; Szafranska, Xie, Green, & Michael Roberts, 1995; Xie et al., 1997). Pregnancy-associated glycoproteins can be detected in blood as early as day 24 of gestation (Green et al., 2000; Sasser, Ruder, Ivani, Butler, & Hamilton, 1986; Zoli, Guilbault, Delahaut, Ortiz, & Beckers, 1992); however, current commercial PAG pregnancy tests are not accurate at diagnosing cattle pregnancies until day 28 of gestation. Consequently, there is interest to develop a different pregnancy detection method that can be used earlier in gestation than PAG. Small (~22 nucleotides) molecules called microRNAs (miRNAs) have been used as biomarkers for the detection of several physiological conditions and diseases in humans due to their

ability to be non-invasively sampled (Bartel, 2004; Reid, Kirschner, & van Zandwijk, 2011; Velu, Ramesh, & Srinivasan, 2012). More recently in the field of cattle reproductive physiology, miRNAs are being examined for their potential to serve as a pregnancy-specific biomarker in order to determine cattle pregnancy status before PAG.

MicroRNAs are small, non-coding RNAs (ncRNAs), which most often act at the level of posttranscription by targeting messenger RNA (mRNA) for degradation or translational repression (Bartel, 2004; Valencia-Sanchez, Liu, Hannon, & Parker, 2006). There are many other ncRNAs that possess the potential for biomarker discovery in the future; however, miRNAs are currently the best characterized and have been strongly correlated with diseases including the expression pattern and development (Etheridge, Lee, Hood, Galas, & Wang, 2011; Russo et al., 2016). MicroRNAs have been proposed as optimal biomarkers because they possess key features including stability, non-invasiveness, tissue specificity, and accuracy and rapidity regarding detection methods (Turchinovich, Weiz, Langheinz, & Burwinkel, 2011; Velu et al., 2012). They have been identified to exist in different forms including those located within extracellular vesicles (EVs) (Valadi et al., 2007). Extracellular-derived miRNAs are of particular interest as this form of miRNAs remains the most extensively verified, and have been documented to be released by presumably every cell type as a method of cell to cell communication to elicit various biological effects (Liang, Wang, & Wang, 2017; Turchinovich, Weiz, & Burwinkel, 2012). There are two main groups of EVs, microvesicles or exosomes, differing in their size and how they exit their secretory cells (Al-Nedawi, Meehan, & Rak,

2009; Stoorvogel, Kleijmeer, Geuze, & Raposo, 2002; Théry, Zitvogel, & Amigorena, 2002; van Niel, D'Angelo, & Raposo, 2018). Once microvesicles and exosomes are released extracellularly, it is difficult to determine their original origin, so they are referred collectively as EVs as both classes are known to contain miRNAs as well as other nucleic acids, lipids, and proteins (Raposo & Stoorvogel, 2013; van Niel et al., 2018).

Previous bovine pregnancy-related miRNA research has resulted in several candidates that were isolated from milk, plasma, serum, or whole blood (Bem et al., 2017; Gebremedhn et al., 2018; Ioannidis & Donadeu, 2016, 2017; Markkandan et al., 2018; Pohler et al., 2017; Schanzenbach, Kirchner, Ulbrich, & Pfaffl, 2017). A significant limitation in this area of research is that there is no standardized approach in regard to extraction and isolation techniques, which lead to differing results regarding abundance of diverse miRNAs. As we attempt to progress the identification of pregnancy-related biomarkers using miRNAs, we are exploring EV-derived miRNAs located in bovine uterine flush fluid. It is plausible that the uterus will directly release pregnancy-related EV-derived miRNAs into the lumen of the uterus, which could migrate into maternal circulation. Therefore, we hypothesized that EV-derived miRNAs from pregnant and non-pregnant heifers in serum will be different than EV-derived miRNAs found in uterine flush fluid from the same heifers; even though, some uterine flush fluid EV-derived miRNAs may still be conserved in circulation. To test this, EV-derived miRNAs from

serum and uterine flush fluid samples of both pregnant and non-pregnant heifers on day 18 post-insemination were examined.

Materials and Methods

All animal experimental procedures were performed after approval of the University of Missouri Institutional Animal and Care and Use Committee. The following methods for experimental design and sample collection are as described in Wallace et al. (2019); methods for sequencing and bioinformatics are as described in Pohler et al. (2017).

Animals and Sample Collection

Angus crossbred heifers (*n* = 20), from the University of Missouri Beef Research and Teaching Farm, were synchronized to ovulate using a modified 5-day CO-synch plus controlled intravaginal drug release (CIDR; Zoetis) protocol (Figure 3 in Appendix II): 100 µg of gonadotropin releasing hormone (GnRH; Cystorelin) (Merial) in 2 mL was administered intramuscularly (IM) and an EAZI-breed CIDR containing 1.38 g progesterone was inserted on day -7; prostaglandin F2α (PGF2α; Estrumate) (Merck) was administered IM, equivalent to 1 mg of cloprostenol, and the CIDR was removed on day -2; second injection of 100 µg GnRH (Cystorelin) (Merck) in 2 mL was administered IM 60 hr following PGF2α; artificial insemination on day 0.

Insemination using semen from a high fertility Jersey bull was performed in all heifers (14 heifers received live semen; 6 heifers received dead semen, control heifers)

regardless of estrus expression. Control heifers were inseminated with dead semen that had been exposed to room temperature for 20 to 24 hours. Dead semen was used in the control heifers because sperm and seminal plasma are known to contain miRNAs. Motility of the dead sperm was confirmed using microscopy prior to insemination.

A second CIDR was inserted in all heifers from day 16 to day 18 post-insemination. This was done to ensure comparable progesterone levels between pregnant and non-pregnant heifers because the corpus luteum of non-pregnant heifers would be regressing around this time, resulting in decreasing progesterone. Therefore, any changes observed in miRNAs across heifers would not be due to differences in progesterone levels. All heifers were sacrificed on day 18 post-insemination at a USDA inspected abattoir where reproductive tracts were excised and blood, endometrial explants, and uterine flush fluid was collected.

Presence or absence of a conceptus was reported at time of sacrifice on day 18. Pregnancy was confirmed by presence of conceptus and increased expression of at least 2 of 3 interferon-stimulated genes (IFI6 [Interferon-Inducible protein 6], ISG15 [Interferon-stimulated protein, 15 kDa], and 2’-5’-Oligoadenylate Synthetase 1]). Non-pregnant heifers were determined as having absence of a conceptus and no elevation in ISGs at day 18 post-insemination.

Blood Collection

Blood serum samples were taken at day 18 post-insemination for small RNA sequencing. Blood was collected by venipuncture into a 10 mL vacutainer tube. Serum tubes were allowed to incubate for 1 hour at room temperature, and then incubated for 24 hours at 4°C followed by centrifugation. After centrifugation, serum was stored at -80°C until needed for progesterone assays and EV-isolation.

Uterine Flush Fluid Collection

Reproductive tracts were removed and transported to a laboratory biological safety cabinet within 30 minutes following sacrifice. Tracts were sprayed with 70% ethanol before the lumen of uterine horns were individually flushed from the oviduct using 60 mL sterile Dulbecco's Phosphate-Buffered Saline (PBS). Samples were then put on ice until able to be frozen at -80°C where they were kept until needed for EV isolation.

Endometrial Explant Collection

Following uterine flush collection, small endometrial explant samples (5 – 6 mm in diameter) were collected from each heifer for determination of interferon-stimulated genes (ISGs) including Interferon-Inducible protein 6 (IFI6), Interferon-Stimulated Gene 15 (ISG15), and 2′-5′-Oligoadenylate Synthetase 1 (OAS1) (Wallace et al., 2019).

Progesterone Radioimmunoassay (RIA)

A Coat-a-Count RIA kit (Diagnostic Products Corporation, Los Angeles, CA) was used to quantify serum progesterone in all heifers as described by Kirby, Wilson, and Lucy (1997). Assay sensitivity was 0.08 ng/mL and intra-assay coefficients of variations were <10%. The GLM (SAS 9.4, Cary, NC) procedure was used for statistical analysis of serum progesterone concentrations.

RNA Isolation and PCR for Interferon-Stimulated Gene Expression

For RNA isolation, endometrial explant samples were minced and total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacture instructions (Wallace et al., 2019). Real-time quantitative PCR was performed as described by Wallace et al. (2019). Expression of interferon-stimulated genes was determined by the presence or absence of product (IFI6 [Interferon-Inducible protein 6], ISG15 [Interferon-stimulated protein, 15 kDa], and 2'-5'-Oligoadenylate Synthetase 1]). Ribosome protein L7 (RPL7) served as the positive control for all samples.

Extracellular Vesicle Isolation

Extracellular vesicle isolation was completed as described by Pohler et al. (2017). Serum (1250 µL) and uterine flush fluid (4 mL) samples can contain cellular debris; therefore, the samples from day 18 post-insemination were initially centrifuged at a low speed (300 g) for 10 minutes in order to rid them of debris. The supernatant, each time making sure to change ultra-centrifuge tubes, resulting from each

ultracentrifugation step was kept for further centrifugation. The series of ultracentrifugation steps were all performed at 4°C as follows: supernatant with the addition of 2.5 mL of PBS centrifuged at 2000 g for 20 minutes; 18,000 g for 45 minutes, and the supernatant from this spin was filtered (Millex GP 0.22-µm filter) into a new ultra-centrifuge tube; 110,000 g for 3 hours, and white pellets were rinsed with PBS; 110,000 g for 90 minutes. The end result of ultracentrifugation was a small, enriched EV pellet, which was resuspended in 1 mL or 400 µL of TRIzol then frozen at -80°C for serum and uterine flush fluid samples, respectively (see Small RNA Extraction section for complete details).

Western Blot Analysis

Western blot analysis will be executed as described by Pohler et al. (2017). Once purified EVs were obtained by ultracentrifugation, an aliquot was taken for EV suspension in 40 µL of M-PER with HALT protease inhibitor cocktail and incubated for 15 minutes. Lysates were vortexed with Laemmli sample buffer and allowed to denature for 5 minutes at 95°C. Gel electrophoresis (12% polyacrylamide) was used to separate denatured lysates. Running buffer was placed over the gel, and the voltage was set to 150V for 60 minutes. When the time expired, protein from the gel was transferred to 0.45 µm Protran BA 85 nitrocellulose membrane in Towbin transfer buffer. Membranes were allowed to incubate for 1 hour in blocking buffer (5% non-fat dairy milk based buffer). Next, the primary antibody (CD81) diluted 1:20,000 in blocking buffer, was

introduced to the membranes and allowed to incubate at 4°C for 1 hour. Afterwards, membranes were washed with TBS before the addition of the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit) diluted 1:10,000 and allowed to incubate for 1 hour. Following incubation, the membranes were washed with TBST. Once washed, they incubated with SuperSignal West Pico Chemiluminescent Substrate for 3 minutes prior to imagining.

Transmission Electron Microscopy

Transmission electron microscopy was performed as described by Navakanitworakul et al. (2016). An aliquot of purified EVs resulting from ultracentrifugation was used and fixated in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight. After, the fixative was removed and 0.1 M sodium cacodylate buffer was added twice for 5 minutes each. The EV fraction was post-fixed for 1 hour in a solution containing 1% osmium tetroxide and 1% potassium ferric cyanide buffered in 0.1 M cacodylate buffer. The resulting EV fraction was dehydrated and then embedded in Embed 812 resin for curation overnight in a 60ºC oven. A Leica UC-7 ultramicrotome was used to cut 80 nm sections. The cut sections were mounted on copper thin bar 300 mesh grids where they were contrasted using 4% uranyl acetate and Sato's lead stain. All samples were imaged using a JEOL-JEM-1400 transmission electron microscope at 80kV with 25,000x magnification.

Small RNA Extraction

Small RNA was extracted from each EV-enriched pellet that resulted from ultracentrifugation. One mL of TRIzol reagent was added to EV-enriched serum fractions from each heifer (pregnant heifers, *n* = 3; non-pregnant heifers, *n* = 3) according to manufacturer's instructions. It was followed entirely for the duration of serum small RNA extractions. Two ultracentrifuged uterine flush fluid samples (right and left uterine horns) were pooled from each heifer (pregnant heifers, *n* = 3; non-pregnant heifers, *n* = 3) separately. The combination accounted for a total of 800 µL (400 µL from the right uterine horn and 400 µL from the left uterine horn), which was the solution of extracellular vesicles resuspended in TRIzol after ultracentrifugation. Additional TRIzol was added (200 µL) to the uterine flush fluid samples in accordance with manufacturer's instructions. It was followed entirely for completion of the extractions.

Small RNA Sequencing

At the University of Kansas Medical Center Genomics Core (Kansas City, KS), all 12 samples from day 18 pregnant (serum, *n* = 3; uterine flush fluid, *n* = 3) and non-pregnant heifers (serum, *n* = 3; uterine flush fluid, *n* = 3) were sequencing using the Illumina HiSeq2500 system. The TruSeq Small RNA library preparation protocol (Illumina, San Diego, CA) used EV RNA ranging from 1.8 to 100 ng, which was ligated with 3' and 5' RNA adapters. Next, EV RNA was subjected to a modified reverse-transcription reaction and PCR amplification as described by Pohler et al. (2017).

Complementary DNA library purification and size selection was performed using 3% marker H gel cassettes on the Pippin Prep size fractionation system (Sage Science). Purified libraries were validated by an Agilent 2100 Bioanalyzer using a High Sensitivity DNA Kit or DNA1000 kit (Agilent), and quantified using the Illumina ECO Real-Time PCR System using KAPA SYBR Universal Library Quant kit (KAPA Biosystems, Wilmington, MA). Once quantified, libraries were adjusted to a 2 Nm concentration and then pooled for multiplex sequencing. After libraries were denatured and diluted according to the correct picomolar concentration, clonal clustering onto the sequencing flow cell was performed using the TruSeq Rapid Single Read Cluster Kit-HS (Illumina) and the automated Illumina cBOT Cluster Station. Illumina HiSeq 2500 system in Rapid Read mode was used to sequence the clustered flow cell with 1 x 50 cycle read and index read using the TruSeq Rapid SBS kit-HS (Illumina). High-throughput sequencing was conducted at 50 base pair, single-end resolution. Sequencing data was transformed from .bcl file format to FASTQ files following collection, and then sorted for additional downstream analysis based on the particular index sequence present. All generated sequencing data are available for public access through Gene Expression Omnibus.

Processing of Small RNA Data

All sequencing reads were mapped to the bovine genome using Bowtie2 software (Langmead & Salzberg, 2012) in the local-sensitive mode after 3’ adaptor

removal. Mapped reads were then processed according to Pohler et al. (2017). The 12 samples' reads were merged and scanned for high-density regions, which is a continuous region whose read count at each base is greater than 20% of the highest base read count for the locus. Loci with an effective length ≥18 nucleotides were retained. The effective lengths are high-density regions that form the effective region of the locus. Effective read counts are formed from each samples' number of reads mapped to the effective region. Loci were filtered on their normalized effective read counts (normalized to the number of counts per million reads [cpm]), and loci with ≥10 cpm were retained.

The Ensemble gene annotation file for bovine (release 70) and miRBase (release 21) were used to annotate genomics features of the effective regions. Initially, effective regions that were able to map to annotated bovine mature miRNAs were identified, whereas the remaining effective regions were compared to known miRNAs from bovine and other species in the miRBase (release 21). The following criteria was required for a region to be labeled as a microRNA by homology: "a gapless alignment of the effective region to the mature reference miRNA with at most two mismatches in the core, at most one gap per mismatch at the 5' and 3' ends, and less than 10% mismatches in the alignment of the reference hair-pin sequence to the extended locus region in the genome" (Pohler et al., 2017). For miRNA to be considered novel, their extended effective region should have a predicted miRNA-like hairpin structure (Jiang et al.,

2007), and the effective region should have greater than 80% pairing while falling within the stem region (Navakanitworakul et al., 2016).

Generalized linear models (GLM) were utilized to determine statistical differences in miRNA abundance between groups using edgeR software package (Robinson, McCarthy, & Smyth, 2010). To determine the total number of biologically relevant miRNAs contained within a given group, each replicate had to contain ≥10 cpm for one of the group(s) being compared. For example, when determining total miRNAs present in non-pregnant serum only, all 3 replicates (heifers) that compose this given group were filtered to contain reads only with ≥10 cpm while the other 3 groups (non-pregnant heifer uterine flush fluid, pregnant heifer serum, and pregnant heifer uterine flush fluid), each containing 3 replicates themselves, did not need to be filtered. However, to determine which miRNAs were unique to a given group(s), the replicates of the group(s) of interest were filtered to contain reads ≥10 cpm, while all remaining replicates were filtered to 0 cpm. In edgeR, RNA composition of each sample was able to be normalized using trimmed mean of M-values method. The Benjamini and Hochberg method was utilized to correct the associated *P*-values for multiple hypothesis testing (Benjamini & Hochberg, 1995). Significance was determined as having absolute expression differences ≥1.5 with a false-discovery rate ≤0.1.

Results

Progesterone Analysis and Interferon-Stimulated Gene Expression

At day 18 post-insemination, all heifers had a combined average progesterone blood concentration of 4.8 ng/mL. Mean progesterone concentration at day 18 for pregnant and non-pregnant heifers was 5.7 ng/mL and 3.8 ng/mL, respectively ($P > 0.05$). Pregnant heifers had increased transcript expression of all ISGs ($P < 0.001$ for IFI6, ISG15, and OAS1) when compared with non-pregnant heifers at day 18 post-insemination.

Western Blot Analysis and Transmission Electron Microscopy

For day 18 post-insemination serum and uterine flush samples, each heifer had positive EV immunoreactivity for the tetraspanin, Cluster of Differential 81 (CD81) (Figure 4a in Appendix II). Transmission electron microscopy revealed EVs with diameters representative of exosomes and microvesicles (Figure 4b in Appendix II).

Small RNA Profiles and Sequencing

Small RNA profiles were evaluated using an Agilent small RNA chip ran on an Agilent 2100 Bioanalyzer. Peaks ranged from ~18 nucleotides and up to ~200 nucleotides indicating a diverse collection of ncRNAs other than miRNAs (Figure 5), which was also verified by small RNA sequencing. The majority of ncRNAs corresponded to miRNAs (54%), followed by small nucleolar RNA (snoRNA) (20%), ribosomal RNA (8%) and small nuclear RNA (snRNA) (8%) (Figure 6 in Appendix II). A total of 334 known and 564 novel *Bos taurus* miRNAs on day 18 post-insemination were

confirmed by small RNA sequencing. Each sample resulted in an average of 7.5 and 11.1 million reads, and of which, 6 to 8 million reads mapped to the genome. The majority of both known and novel miRNAs were similar across groups (Supplementary Figures) with only a few unique miRNAs specific to a singular group (Supplementary Figures).

Differential Expression of MiRNAs

Values for fold change (≥ 1.5), *P*-value (≤ 0.05), and FDR (≤ 0.1) enabled the identification of several known and novel miRNAs to be significantly and differentially abundant according to each comparison group (Table 3 in Appendix II; Supplementary File). Interestingly, no miRNAs were differentially expressed in the pregnant heifer serum vs. non-pregnant heifer serum group, which was due to all miRNAs within this group having ≥ 1.68 FDR (data not shown). Nonetheless, the different in pregnant vs. non-pregnant heifers in both serum and uterine flush fluid group holds the most promise for an ideal biomarker, and MiPred software identified 2 novel miRNAs located at different loci (19:43923840-43923864 and 3:107486424-107486448) in this group. Both novel miRNAs had significantly greater abundance in pregnant heifers compared with non-pregnant heifers (Tables 4 and 5 in Appendix II).

Discussion

In an attempt to narrow down and identify candidates that are more likely to be related to pregnancy, we examined EV-derived miRNAs located in uterine flush fluid in

addition to serum. In the present study, western blot analysis and transmission electron microscopy confirmed successful isolation of EVs indicated by the presence of CD81 (Figure 4a in Appendix II) and size consistent with EVs (Figure 4b in Appendix II) (Al-Nedawi et al., 2009; Stoorvogel et al., 2002; Théry et al., 2002; van Niel et al., 2018). Small RNA profiling revealed EVs contained small RNAs <200 nucleotides (Figure 5 in Appendix II), which was similar to other literature using uterine flush fluid samples from ewes (Burns et al., 2014). A follow up study by G. W. Burns, Brooks, and Spencer (2016) revealed that EVs originating from the uterus of ewes were not only able to be located in uterine flush fluid, but that they were also responsible for mediating conceptus-maternal interactions during pregnancy. Additionally, pregnancy-specific EV-derived miRNAs originating from the human placenta have been successfully detected in maternal circulation (Luo et al., 2009). Therefore, it is likely that pregnancy-related miRNAs in cattle will be located in uterine flush fluid and can migrate into circulation, which will ultimately allow for easier sampling that is required for the determination of pregnancy status.

Next generation sequencing confirmed the findings obtained by the Agilent 2100 Bioanalyzer. The majority of small ncRNAs corresponded to miRNAs (54%) and was followed by small nucleolar RNAs (snoRNAs) (20%) (Figure 6 in Appendix II). These results are similar to other literature reporting small ncRNAs distribution within serum from pregnant and non-pregnant beef cows at day 17 post-insemination (Pohler et al., 2017). G. Burns et al. (2014) did not report the exact distribution of small ncRNAs

located in uterine flush fluid from pregnant and non-pregnant ewes at day 14 post-insemination; however, the Agilent 2100 Bioanalyzer results from that study depicting small RNAs <200 nucleotides suggest the possibility of similar small ncRNAs present in ewe uterine flush fluid.

Differentially abundant miRNAs were observed on day 18 post-insemination between pregnant and non-pregnant heifers in all comparison groups except for the pregnant heifer serum vs. non-pregnant heifer serum group (Table 3 in Appendix II). The observation of no differentially abundant miRNAs for pregnant heifer serum vs. non-pregnant heifer serum is similar to a different study examining miRNAs from serum samples of beef cows at day 17 post-insemination, where the authors identified only one novel miRNA as being differentially expressed between non-pregnant and pregnant cows (Pohler et al., 2017). The single novel miRNA reported barely reached significance (FDR = 0.09) (Pohler et al., 2017), which was similar to our study where no miRNAs from the pregnant heifer serum vs. non-pregnant heifer serum group had an FDR of ≤ 0.1. Even though many different comparison groups were analyzed in this study, there is one group that holds the most promise for containing more ideal miRNA biomarkers than the miRNAs that were determined to be differentially expressed in the other groups. For instance, the ideal, pregnancy-associated miRNA candidate would be present in uterine flush fluid while still being conserved in circulation. One of our groups, different in pregnant heifers vs. non-pregnant heifers in both serum and uterine flush fluid, analyzed this specific subset of miRNAs which revealed two novel miRNAs at two

different loci (19:43923840-43923864 and 3:107486424-107486448) as being more abundant in pregnant heifers than non-pregnant heifers on day 18 post-insemination (Table 2 and 3 in Appendix II).

Interestingly, the two novel miRNAs (at loci 19:43923840-43923864 and 3:107486424-107486448) of interest had identical mature sequences (AAAUGGAUUUUUGGAGCAGGAAGUU) that most closely corresponded to the same known miRNA, which was bta-miR-1246 (mature sequence: AAAUGGAUUUUUGGAGCAGGAAG; loci: 1:116822078-116822100). Markkandan et al. (2018) reported bta-miR-1246 as being greater in abundance of pregnant Holstein cows at day 30 of gestation compared to their non-pregnant counterparts (*P*-value = 0.0013; FDR = 0.0287; FC = 0.960). The authors reported overall gene ontology and enriched pathways for the combined 29 miRNAs that were identified as differentiating pregnancy status at day 30 (Markkandan et al. 2018). Consequently, the functional role specific to bta-miR-1246 still remains elusive. MicroRNA sequences are highly conserved across species and often have similar functions (Ha, Pang, Agarwal, & Chen, 2008). Subsequently, studies associated with hsa-miR-1246 (mature sequence: AAUGGAUUUUUGGAGCAGG) and its predicted functions are relevant. In human pregnancy literature, decreased abundance of has-miR-1246 has been associated with severe preeclampsia (Muralimanoharan, Kwak, & Mendelson, 2018). Furthermore, when trophoblast cells of the placenta were cultured in hypoxic conditions, hsa-miR-1246 was also decreased in abundance (Muralimanoharan, Kwak, & Mendelson, 2018).

These negative pregnancy-related outcomes observed when miR-1246 is decreased further suggests the likelihood of favorable pregnancy outcomes when miR-1246 is increased in abundance. In addition to miR-1246 potentially playing a role in pregnancy, several human cancer patient and cell line studies have identified hsa-miR-1246 as functioning similar to an oncogene by targeting nuclear factor I/B (NFIB), thrombospondin-2 (THBS2), and cell adhesion molecule 1 (CADM1) for downregulation (Chen et al., 2014; Sun et al., 2014; Chai et al., 2016; Kim et al., 2016; Zhang et al., 2016; Cooks et al., 2018). It is intriguing to speculate that the two novel *Bos taurus* miRNAs closely related in structure to hsa-miR-1246 could aid in attachment of the elongating embryo to the uterine epithelium at day 18 post-insemination given the invasion and migration cancer cell properties associated with hsa-miR-1246 (Chen et al., 2014; Sun et al., 2014). Moreover, we cannot dismiss the possibility that the two novel *Bos taurus* miRNAs may be present and detectable earlier than day 18 post-insemination at which time they could act in embryonic development and growth as hsa-miR-1246 is related to increased cell proliferation (Chen et al., 2014). Future research should aim to elucidate whether or not the *Bos taurus* novel miRNAs identified in the present study are functioning in either capacity.

Conclusion

Results obtained from this study indicate EV-derived miRNAs are located in serum and uterine flush fluid from pregnant and non-pregnant beef heifers at day 18

post-insemination. Furthermore, some EV-derived miRNAs located in uterine flush fluid are also conserved and detectable in circulation, providing useful biomarkers. Additional research is warranted to further validate the two novel, EV-derived miRNAs identified in the current study that were able to differentiate pregnancy status in both serum and uterine flush fluid. Future research should aim to identify potential mRNA targets of the two *Bos taurus* novel miRNAs to gain an understanding of how these miRNAs may be functioning in relation to pregnancy. Furthermore, determining the potential ability of these two novel, EV-derived miRNAs to decipher embryonic viability as a way to detect compromised pregnancies that will fail as a result of embryonic mortality also would be valuable.

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Appendix II

Table 3. Number of significantly and differently expressed known and novel Bos taurus miRNAs according to group at day 18 post-insemination. NP = Non-Pregnant, P = Pregnant, S = Serum, and UF = Uterine Flush.

Group	Number of Known MiRNA	Number of Novel MiRNA
P-UF vs. NP-UF	13	44
P-S vs. NP-S	0	0
NP-S vs. NP-UF	155	89
P-S vs. P-UF	171	143
Different (P vs. NP; in S & UF)	0	2

Table 4. Differentially abundant miRNAs located in both uterine flush fluid and serum on day 18.

Day 18: Pregnant vs. Non-Pregnant					
Different (Pregnant vs. Non-Pregnant) in (Serum and Uterine Flush)					
Locus	**MiRNA**	**P-Value**	**FDR**	**Uterine Flush FC**	**Serum FC**
19:43923840-43923864	Novel	1.1587E-05	0.0014	55.36	1.58
3:107486424-107486448	Novel	7.5663E-06	0.0011	76.74	1.53

Table 5. Continuation from Table 4 of the differentially abundant miRNAs depicting counts per million for each sample according to their respective group.

Counts per Million (cpm)											
Non-Pregnant Uterine Flush			Pregnant Uterine Flush			Non-Pregnant Serum			Pregnant Serum		
5008_U	5019_U	5028_U	5017_U	5023_U	5025_U	5008_S	5019 S	5028_S	5017_S	5023_S	5025_S
36.4	69.6	67.2	6265.5	1009.8	2485.4	117.9	90.1	112.8	156.4	431.4	239.5
14.1	65	39.6	5889.6	982	2357.7	134.4	73.6	81.8	131.5	408.6	192.2

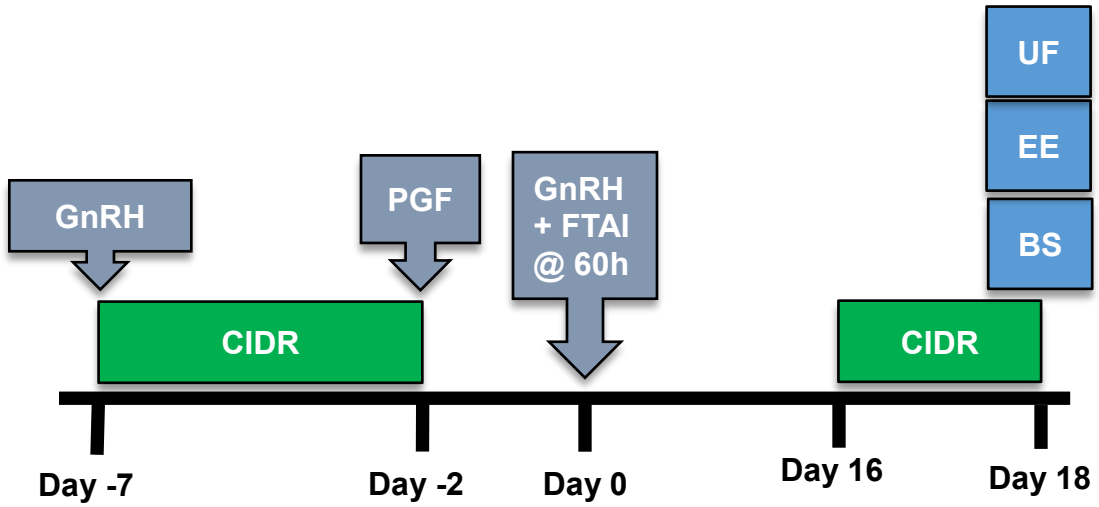
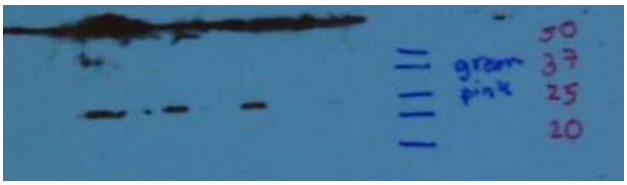


Figure 3. Illustration of the experimental design. Angus crossbred heifers (*n* = 20) were submitted to a modified 5-day CO-synch plus controlled intravaginal drug release (CIDR) protocol. On day -7, administration of CIDR and Gonadotropin-releasing hormone (GnRH); withdrawal of the CIDR and administration of prostaglandin F2α (PGF) on day -2; fixed-time artificial insemination (FTAI) and administration of GnRH on day 0. Administration of a second CIDR was applied on day 16 and removed on day 18, which represents the day of sacrifice, uterine flush fluid (UF) collection, endometrial explant (EE) collection, and blood sample (BS) collection. Pregnancy was confirmed by presence of conceptus and increased expression of at least 2 of 3 interferon-stimulated genes. Non-pregnant heifers were determined as having absence of a conceptus and no elevation in ISGs at day 18 post-insemination.

a



b

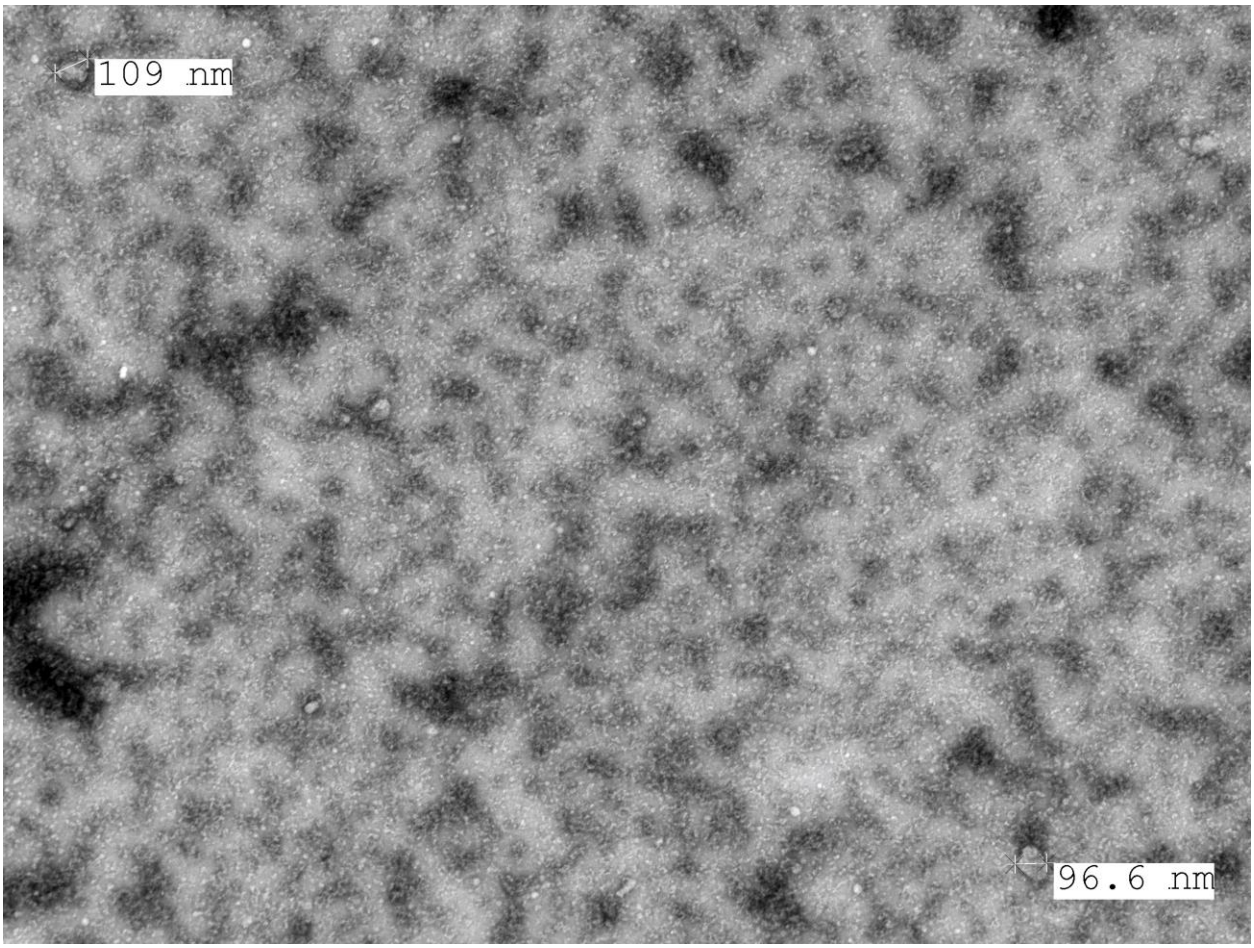
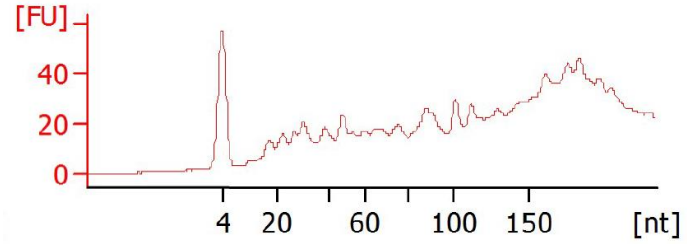
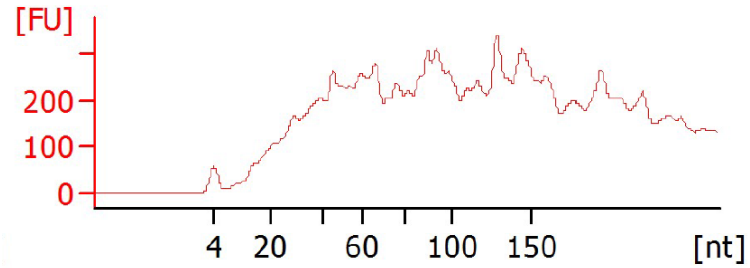


Figure 4. Identification of extracellular vesicles (EVs). (a) Western blot indicating the presence of CD81, a verified marker of EVs. (b) Representative transmission electron microscopy image of isolated EVs and their corresponding diameters (nm).

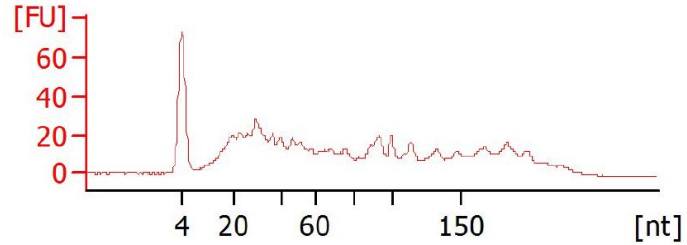
Serum Sample of Pregnant Heifer on Day 18



Uterine Flush Sample of Pregnant Heifer on Day 18



Serum Sample of Non-Pregnant Heifer on Day 18



Uterine Flush Sample of Non-Pregnant Heifer on Day 18

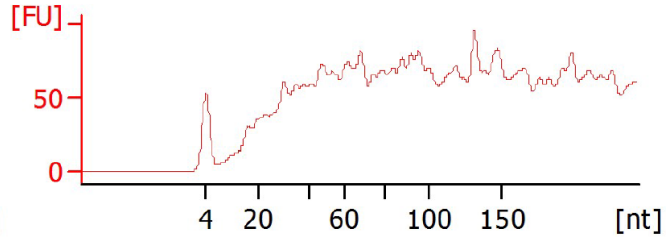


Figure 5. Small RNA profiles from all sample types at day 18 post-insemination determined by an Agilent 2100 Bioanalyzer. Peaks around 20 nucleotide correspond to miRNA length. FU = fluorescence units; nt = nucleotide.

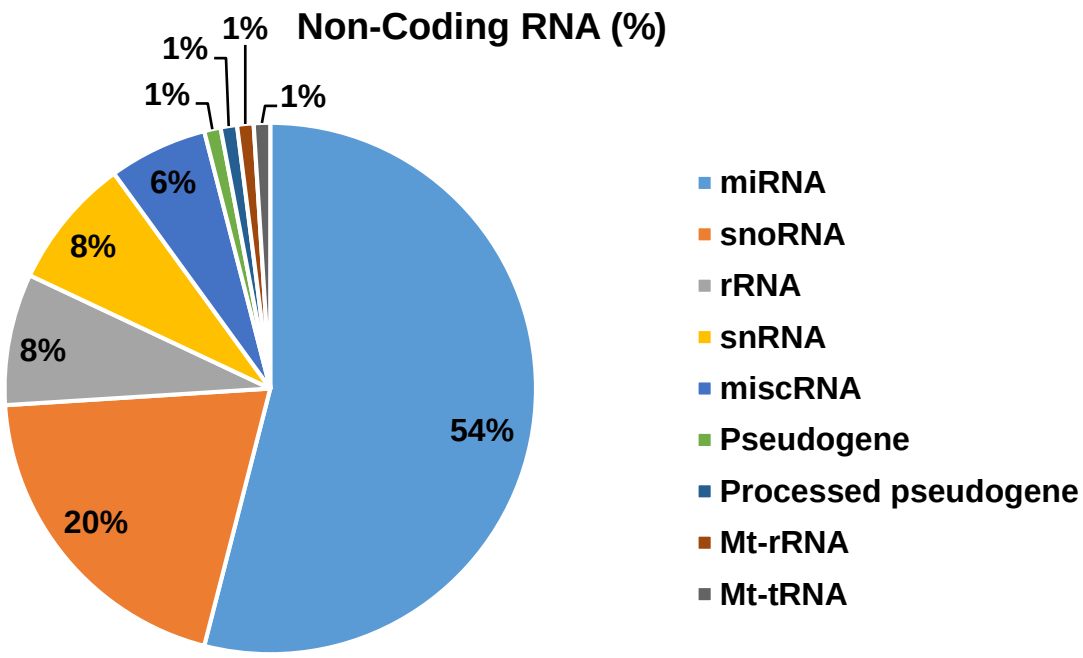


Figure 6. Distribution of extracellular vesicle derived small non-coding RNAs identified by deep sequencing. MiRNA = microRNA; snoRNA = small nucleolar RNA; rRNA = ribosomal RNA; snRNA = small nuclear RNA; miscRNA = miscellaneous RNA; Mt-rRNA = mitochondrial ribosomal RNA; Mt-tRNA = mitochondrial transfer RNA.

CHAPTER III: INFLUENCE OF ESTRUS ACTIVITY AND REPRODUCTIVE TRACT SIZE AND POSITION SCORES ON FERTILITY IN BOS INDICUS AND BOS TAURUS SUCKLED BEEF COWS

Abstract

The primary objective of this study was to determine if estrus activity and reproductive tract size and position score (SPS) are associated with fertility in *Bos indicus* (Nelore) and *Bos taurus* (Angus) beef cows. In study 1, multiparous Nelore cows (n = 1280) were artificially inseminated at a fixed time (FTAI, d 0) using an estradiol (E2) and progesterone (P4) FTAI protocol. In study 2, multiparous Angus cows (n = 764) were artificially inseminated at a fixed time (FTAI, d 0) using a gonadotropin-releasing hormone (GnRH) and P4 FTAI protocol. Estrus activity was qualified using Estrotect heat detector patches and scored on d 0 using the following scoring system: 1 (<25% activation), 2 (<50% activation), 3 (<75% activation), or 4 (>75% activation) where patch scores of 1 and 2 signified no or limited estrus activity, whereas scores of 3 and 4 had increased estrus activity. Reproductive tract SPS were assigned on d 0 (FTAI) as SPS1: small and compact resting within the pelvic cavity; SPS2: intermediate, resting partially outside the pelvic cavity; and SPS3: larger and resting outside the pelvic cavity. Pregnancy diagnosis was performed by ultrasound on day 30 and 100 after FTAI. Cows were determined as undergoing pregnancy loss if a viable embryo with heartbeat was detected at d 30 but was no longer present at d 100. Day 30 FTAI pregnancy rates were interactively influenced by estrus activity and reproductive tract SPS in both Nelore (P = 0.004) and Angus (P = 0.009) cows. Specifically, SPS1 cows with increased estrus activity had greater pregnancy rates when compared to SPS1 cows with little to no estrus activity regardless of breed. In Angus cows, pregnancy rate of SPS1 versus

SPS3 cows was greater in cows that exhibited estrus activity. In contrast, pregnancy loss between day 30 and 100 for both breeds was not interactively influenced by reproductive tract SPS and estrus activity (P = 0.194, Nelore; P = 0.832, Angus) or when individual main effects of estrus activity (P = 0.775, Nelore; P = 0.797, Angus) or reproductive tract SPS (P = 0.882, Nelore; P = 0.567, Angus) were analyzed. In summary, influence of reproductive tract SPS on day 30 FTAI pregnancy rates depends on estrus activity in beef cows. However, neither estrus activity nor reproductive tract SPS were associated with pregnancy loss in the present studies.

Introduction

A single service insemination in beef cattle is quite successful at fertilization (~95%) (Ayalon, 1978), yet fixed-time artificial insemination (FTAI) pregnancy rates resulting from estradiol (E2) or gonadotropin-releasing hormone (GnRH) based protocols average ~50% (Meneghetti et al., 2009; Whittier et al., 2013; Bó et al., 2016). Many factors including but not limited to the absence or presence of estrus at FTAI and embryonic mortality (early embryonic mortality, <28 d of gestation; late embryonic mortality, 28 to 42 d of gestation) contribute greatly to this decreased percentage (Santos et al., 2004; Perry et al., 2007; Sá Filho et al., 2011; Pohler et al., 2015). Management efforts, such as evaluating estrus expression and reproductive tracts, can be performed prior to breeding to potentially identify and select females with optimal fertility. Overall, these practices aim to increase the probability of pregnancy success.

Estrus expression at time of artificial insemination (AI) has been linked to increased pregnancy success across studies in both beef and dairy (Cerri et al., 2004; Galvão et al., 2004; Perry et al., 2005; Perry et al., 2007; Sá Filho et al., 2010; Sá Filho et al., 2011; Pitaluga et al., 2013; Pereira et al., 2014; Pereira et al., 2016; Pessoa et al., 2016; Pohler et al., 2016). This physiological association stems from a change in steroid hormones, particularly an increase in E2, which fosters conditions apt for follicular development and maturation (McNatty et al., 1979; Perry et al., 2007). Furthermore, E2 positively alters the uterine environment to influence uterine metabolic activities (Miller and Moore, 1976; Miller et al., 1977) and sperm transport (Hawk, 1983; Perry and Perry, 2008) in the female reproductive tract at insemination. Indeed, absence of estrus at FTAI has resulted in increased pregnancy loss in some studies (Galvão et al., 2004; Pereira et al., 2014; Pereira et al., 2016) but not others (Jinks et al., 2013; Franco et al., 2018).

Reproductive tract scoring systems have also been used in both the beef and dairy industries for assessing female reproductive potential and fertility (Andersen et al., 1991; Rosenkrans and Hardin, 2003; Stevenson et al., 2008; Holm et al., 2009; Gutierrez et al., 2014; Baez et al., 2016; Young et al., 2017). The most recent reproductive tract scoring system developed by Young et al. (2017) appears to be the most useful with a greater likelihood of implementation since it uses rectal palpation to determine reproductive tract size and position of mature, cycling females on a three scale basis (SPS1, SPS2, or SPS3). Reproductive tracts that were designated as SPS1

had small uterine horns resting within the pelvic cavity, SPS2 females had medium cervical and uterine horn diameter with larger uterine horns compared to SPS1 that rest partially outside of the pelvic cavity, and SPS3 females had larger uterine horns resting mostly outside of the pelvic cavity (Young et al., 2017). The authors reported in lactating dairy cows, as tract size increased there was a decrease in pregnancy per AI (P/AI). Baez et al. (2016) observed comparable findings where P/AI was greater for cows with a smaller uterine volume compared to a larger uterine volume, when using two-dimensional ultrasound. Similarly, humans with smaller uterine length, width, and volume measurements had increased clinical pregnancy rates compared to patients with larger measurements when undergoing assisted reproductive technologies (Hong Gao et al., 2019). Collectively, these data suggest that smaller reproductive tracts are associated with increased fertility and pregnancy success.

To our knowledge, estrus activity in relation to reproductive tract scoring has not yet been investigated in beef or dairy cows. Therefore, the objective of this study was to determine if estrus activity and reproductive tract SPS are associated with fertility in both Nelore and Angus beef cows. Two factors, pregnancy rate and pregnancy loss, were used as measures of fertility. We hypothesized, regardless of breed influence and corresponding synchronization protocol, pregnancy rates would be greater among all beef cows that were classified as exhibiting estrus activity as well as those with small and compact uterine horns resting within the pelvic cavity (SPS1) at FTAI.

Materials and Methods

Study 1: FTAI of Nelore Cows

This study was conducted on a commercial beef farm in Mato Grosso, Brazil following the recommendations of the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (FASS, 1999). All multiparous, Nelore cows (*n* = 1280) were on *Brachiaria brizantha* pastures and had ad libitum access to water and mineral salt. Cows were at least 37 d postpartum (ranged from 37 to 72 d; averaged 54 d) and averaged a body condition score (BCS) of 2.91 (ranged from 2 to 4.25) before subjected to an E2-based FTAI protocol as previously described by Meneghetti et al. (2009): an intravaginal progesterone (P4) insert (CIDR; Zoetis, São Paulo, Brazil) containing 1.9 g of P4, and 2.0 mg (i.m.) estradiol benzoate (2.0 mL of Gonadiol; Zoetis, São Paulo, Brazil) on d -11, CIDR withdrawal, 12.5 mg (i.m.) dinoprost tromethamine (PGF; 2.5 mL of Lutalyse; Zoetis, São Paulo, Brazil), 300 IU (i.m.) of equine chorionic gonadotropin (1.5 mL of Novormon; Zoetis, São Paulo, Brazil), and 0.6 mg (i.m.) of estradiol cypionate (0.3 mL of E.C.P.; Zoetis, São Paulo, Brazil) on d -2, and FTAI to 1 of 8 Nelore sires on d 0.

Study 2: FTAI of Angus Cows

All animal experimental procedures were approved by the University of Tennessee Institutional Animal and Care and Use Committee. All multiparous, Angus cows (*n* = 764) on mixed fescue pastures and had ad libitum access to water and

mineral salt. Cows were at least 21 d postpartum (ranged from 21 d to 94 d; averaged 54 d) and averaged a BCS of 5.76 (ranged from 3 to 8.5) before subjected to a 7-day CO-synch plus CIDR FTAI protocol: CIDR containing 1.38 g P4 (CIDR; Zoetis, New York, NY), and 100 μg (i.m.) of gonadotropin-releasing hormone (GnRH; 2 mL of Cystorelin; Merial, Duluth, GA) on d -9, 25 mg (i.m.) Prostaglandin F2α (PGF2α; 5 mL of Lutalyse; Zoetis, New York, NY), and CIDR withdrawal on d -2, a second injection of GnRH (2 mL of Cystorelin; Merial, Duluth, GA) 66 hr after PGF2α, and FTAI to 1 of 18 Angus sires on d 0.

Transrectal Ultrasonography

Uteri of all cows were examined by transrectal ultrasonography (Aloka 500V, Aloka, Wallingford, CT) using a 7.5-MHz transrectal linear probe at d 30 and 100 post-insemination for pregnancy detection. Presence or absence of a viable embryo with heartbeat was recorded in order to determine a positive or negative pregnancy status, respectively. Cows were determined as undergoing pregnancy loss if a viable embryo with heartbeat was detected at d 30 but was no longer present at d 100.

Estrotect Heat Detector Patch Scoring System

On d -2, Estrotect heat detector patches (Rockway Inc., Spring Valley, WI) were placed halfway between the hip and tail head on all cows. A patch scoring system, developed and validated by Pohler et al. (2016), was assessed and determined by AI

technicians at FTAI, d 0. Patch scores ranged from 0 to 4 on the basis of whether a patch was lost, most likely due to repeated mounting (score 0), or by the amount of patch surface physically activated by rubbing due to mounting (score 1, <25% activation; score 2, <50% activation; score 3, <75% activation; score 4, >75% activation). Cows that received patch scores of 1 and 2 were classified as having little to no estrus activity (labeled as no estrus), whereas cows with patch scores of 0, 3, or 4 were classified as having increased estrus activity (labeled as estrus).

Reproductive Tract Size and Position Scoring System

At FTAI (d 0) reproductive tracts were evaluated by skilled AI technicians to determine the reproductive tract size and position score (SPS) of each individual cow. Reproductive tract SPS were assigned based on a previously reported scoring system developed by Young et al. (2017): SPS1 cows had smaller and more compact reproductive tracts that rested within the pelvic cavity, SPS2 cows had intermediate sized tracts that rested partially outside of the pelvic cavity, and SPS3 cows had larger reproductive tracts that rested outside of the pelvic cavity.

Statistical Analyses

The FREQ procedure (SAS, 9.4, Institute Inc., Cary, NC) was used to determine separate frequencies for estrus activity and reproductive tract SPS in both Nelore and Angus cows. Separate generalized linear mixed models (GLIMMIX procedure, SAS 9.4,

Institute Inc., Cary, NC) for binary variables employing one-way ANOVA were used to determine differences in dependent variables of pregnancy rate and pregnancy loss for Nelore and Angus cows. For both Nelore and Angus analyses, fixed effects included estrus activity (estrus or no estrus), reproductive tract SPS (SPS1, SPS2, or SPS3), and respective interaction. A general Satterthwaite approximation was used to determine the denominator degrees of freedom for the tests of all fixed effects. Random effects for all analyses were sire and days postpartum. Cow represented the experimental unit and means were separated using LSMEANS and adjusted in accordance with the Tukey-Kramer test. Statistical significance was determined as $P \leq 0.05$ and tendencies were determined as $0.05 < P \leq 0.10$. Results are presented as mean ± SEM.

Results

Study 1: Nelore Cows

Overall FTAI day 30 pregnancy rate was 44.06% (564/1280) and overall pregnancy loss between day 30 and 100 was 6.92% (39/564). Out of 1280 cows, 11.48% (n = 147) exhibited estrus activity (Table 6 in Appendix III). The frequency of reproductive tract scores of SPS1, SPS2 and SPS3 for Nelore cows was 71.95%, 20.63%, and 7.42%, respectively (Table 7 in Appendix III).

Day 30 FTAI pregnancy rate was interactively influenced by reproductive tract SPS and estrus activity (P = 0.004; Figure 7 Appendix III). Increased estrus activity in SPS1 cows was associated with a greater pregnancy rate (69.71%). Pregnancy rate was similar for SPS2 and SPS3 cows regardless of estrus activity.

Pregnancy loss between days 30 and 100 of gestation did not differ between estrus activity (*P* = 0.775; 6.78% vs 7.61% for no or limited estrus activity vs. increased estrus activity, respectively) or reproductive tract SPS (*P* = 0.882; 6.81% vs. 6.62% vs. 8.7% for SPS1, SPS2, and SPS3, respectively). There was no significant interaction of estrus activity and reproductive tract SPS for pregnancy loss (*P* = 0.194).

Study 2: Angus Cows

Overall FTAI day 30 pregnancy rate was 56.8% (434/764) and overall pregnancy loss between day 30 and 100 was 5.71% (18/315). Out of 764 Angus cows, 37.57% (*n* = 287) exhibited increased estrus activity. (Table 6 in Appendix III). The frequency of reproductive tract scores of SPS1, SPS2, and SPS3 for Angus cows was 26.96%, 57.85%, and 15.18%, respectively (Table 7 in Appendix III).

Day 30 FTAI pregnancy rate was interactively influenced by reproductive tract SPS and estrus activity (*P* = 0.009; Figure 8 in Appendix III). Increased estrus activity in SPS1 cows was associated with a greater pregnancy rate (74.43%). Pregnancy rate of SPS1 versus SPS3 cows was greater in cows that exhibited estrus activity.

Pregnancy loss between days 30 and 100 of gestation did not differ between estrus activity (*P* = 0.797; 5.51% vs. 6.21% for no or limited estrus activity vs. increased estrus activity, respectively) or reproductive tract SPS (*P* = 0.567; 7.14% vs. 5.9% vs. 2.33% for SPS1, SPS2, and SPS3, respectively). There was no significant interaction of estrus activity and reproductive tract SPS for pregnancy loss (*P* = 0.832).

Discussion

To our knowledge, this is the first study where both estrus activity and reproductive tract SPS have been measured concurrently. Both estrus activity and reproductive tract SPS interactively influence day 30 FTAI pregnancy rates (Figure 7 and 10 in Appendix III). The positive association between estrus expression and pregnancy outcomes has been heavily investigated in both beef and dairy. A steroid hormone, E2, reaches peak concentration at estrus and has been identified to aid in pregnancy success through multiple roles including those related to sperm transport (Hawk, 1983), partial messenger RNA machinery inhibition for PGF2α synthesis (Davoodi et al., 2016), increased uterine receptivity (Spencer and Bazer, 1995; Spencer et al., 2008; Davoodi et al., 2016), increased preovulatory follicle diameter and subsequent corpus luteum diameter, and therefore, progesterone production (Bridges et al., 2012).

Previous literature using *Bos indicus* (Sá Filho et al., 2010; Sá Filho et al., 2011) and *Bos taurus* (Perry et al., 2005; Perry et al., 2007; Pessoa et al., 2016) cows, reported increased estrus expression corresponded to greater pregnancy rates. These data are similar to the present studies where we identified greater pregnancy rates in SPS1 cows with increased estrus activity compared to SPS1 cows with little to no estrus activity. The percentage of cows exhibiting increased estrus activity was 11.48% and 37.57% for Nelore and Angus cows, respectively (Table 2 in Appendix III). Previous Nelore cow estrus expression literature using cows that were subjected to an E2 (using

ECP at CIDR withdrawal) and P4 based FTAI protocol indicates that resulting estrus activity is ~65% (ranged from 52.8% to 78.7%) (Sá Filho et al., 2010; Sá Filho et al., 2011; Davoodi et al., 2016; Rodrigues et al., 2018; Cooke et al., 2019). This discrepancy is unusual as the demographic of cattle, geography, and farms as well as FTAI protocols were very similar between studies. In the present study, it is possible that cows reported as exhibiting little to no estrus activity may have come into estrus after day 0 (day of patch score evaluation) as patch scores were not evaluated from that point on. Estrus activity response for Angus cows in the present study is similar to previous Angus cow estrus expression literature using cows that were subjected to a GnRH and P4 based FTAI protocol where resulting estrus activity is ~44% (ranged from 23.1% to 57%) (Perry et al., 2005; Nash et al., 2012; Whittier et al., 2013; Thomas et al., 2014). Collectively, this could account for why we observed SPS1 cows with increased estrus activity having greater pregnancy rates than SPS3 cows with no or limited estrus activity in Angus cows but not Nelore cows (Figure 7 and 8 in Appendix III).

A couple lactating dairy cow studies investigated tract sizes on pregnancy rates without measuring estrus activity (Baez et al., 2016; Young et al., 2017). Young et al. (2017) identified a 15.6% difference in pregnancy rates between SPS1 cows (43.3%) and SPS3 cows (27.7%). Similarly, using two dimensional ultrasonography, Baez et al. (2016) observed a 12.9% increase in pregnancy rates in lactating dairy cows that had a smaller uterine volume (≤128 cm^3) compared to a larger uterine volume (>128 cm^3). The authors theorized that larger tracts could interfere with fertilization because sperm have

more surface area to travel in order to reach the oviduct. In some cases, previous literature identified deep cornual semen deposition was able to increase pregnancy rates (López-Gatius and Camón-Urgel, 1988; Meirelles et al., 2012), whereas other literature observed no differences according to deposition location (Williams et al., 1988; Sá Filho et al., 2012). In the present studies, day 30 FTAI pregnancy rates were greatest among females with smaller tract size and position (SPS1) when they exhibited increased estrus activity regardless of breed or FTAI protocol (Figure 1 and 2 in Appendix III).

In addition to pregnancy rate, pregnancy loss was also evaluated as a second measure of fertility. In both of the present experiments using beef cows, no relationship between pregnancy loss and the main effects of estrus activity or reproductive tract SPS was identified. Estrus activity did not influence pregnancy loss in both studies, consistent with other *Bos indicus* (Franco et al., 2018) and *Bos taurus* (Jinks et al., 2013) literature. These results regarding estrus activity and pregnancy loss in beef cattle differ to studies performed in lactating dairy cows where lack of estrus at AI corresponded with increased pregnancy loss (Galvão et al., 2004; Pereira et al., 2014; Pereira et al., 2016). This discrepancy could be due to lactating dairy cows having greater E2 metabolism as a result of increased feed consumption and liver blood flow in comparison to beef cattle (Sangsritavong et al., 2002; Vasconcelos et al., 2003; Wiltbank et al., 2006; Pereira et al., 2016). In turn, this could amount to decreased preovulatory E2 concentrations, which have been associated with premature luteloysis

(Mann and Lamming, 2000). However, more recent data by Franco et al. (2018) indicates that the sire used for insemination may have a greater influence on pregnancy loss after day 30 post-insemination, which suggests less importance of E2 in this role. Specifically, the authors were able to identify specific Angus sires (*n* = 6) that either contributed greatly (66.67% of total losses; *n* = 3) or slightly (33.33% of total losses; *n* = 3) to overall late embryonic loss (Franco et al., 2018). This large variation that exists between sires could be responsible for differences across studies regarding percentages reported for pregnancy loss.

In regard to reproductive tract SPS and pregnancy loss, these data are in agreement with a study using lactating dairy cows where authors found no correlation between uterine size or parity and pregnancy loss between day 32 to 67 (Baez et al., 2016). Interestingly, Angus cows had double the frequency of SPS3 cows compared to Nelore cows (15.18% vs. 7.42%) (Table 6 in Appendix III). It is unclear if the differences in FTAI protocols influenced this outcome since evaluation of reproductive tract SPS took place at FTAI.

Conclusion

In summary, results from both studies indicate that day 30 FTAI pregnancy rate is interactively influenced by reproductive tract SPS and estrus activity in both Nelore and Angus cows. Specifically, SPS1 cows with increased estrus activity had greater pregnancy rates compared to SPS1 cows with little to no estrus activity. Estrus expression and reproductive tract SPS and did not influence incidence of pregnancy

loss in either study. Therefore, from a management perspective, it may be beneficial to incorporate both a patch-based estrus detection system and assign reproductive tract SPS at breeding to make individual breeding decisions to increase pregnancy rates in beef females. Once estrus activity and reproductive tract SPS is determined at FTAI, producers could prioritize more valuable semen or embryos to be used in females with increased likelihood of pregnancy success (SPS1 females with increased estrus activity) whereas semen from a high fertility bull could potentially better serve those females that had both larger tracts and limited or no estrus activity at FTAI.

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Appendix III

Table 6. Frequency of estrus activity according to breed. No Estrus = no to limited estrus activity, Estrus = increased estrus activity.

Nelore *(Study 1)*		**Angus *(Study 2)***	
No Estrus	**Estrus**	**No Estrus**	**Estrus**
1113 (88.52%)	147 (11.48%)	477 (62.43%)	287 (37.57%)

Table 7. Frequency of reproductive tract SPS according to breed. SPS = size and position score.

Nelore *(Study 1)*			Angus *(Study 2)*		
SPS1	**SPS2**	**SPS3**	**SPS1**	**SPS2**	**SPS3**
921 (71.95%)	264 (20.63%)	95 (7.42%)	206 (26.96%)	442 (57.85%)	116 (15.18%)

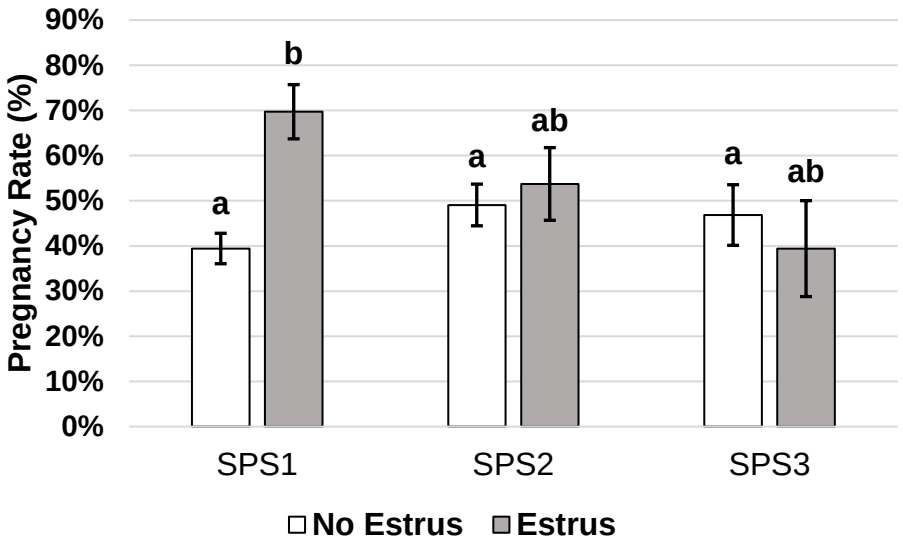

Figure 7. Influence of estrus activity and reproductive tract SPS on Nelore cow FTAI day 30 pregnancy rates (Study 1). Estrus activity was measured using a patch scoring system developed by Pohler et al. (2016). Reproductive tract size and position scores (SPS) were determined according to criteria developed by Young et al. (2017). SPS1 cows that exhibited increased estrus activity had greater pregnancy rates (69.71%) than all cows that did not exhibit or had limited estrus activity and that were classified as SPS1 ($P < 0.001$; 39.43%), SPS2 ($P = 0.035$; 49.07%), and SPS 3 ($P = 0.07$; 46.84%). Different letters (a, b) represents a tendency for difference ($P \leq 0.10$) across means. No Estrus = no or little estrus activity, Estrus = increased estrus activity.

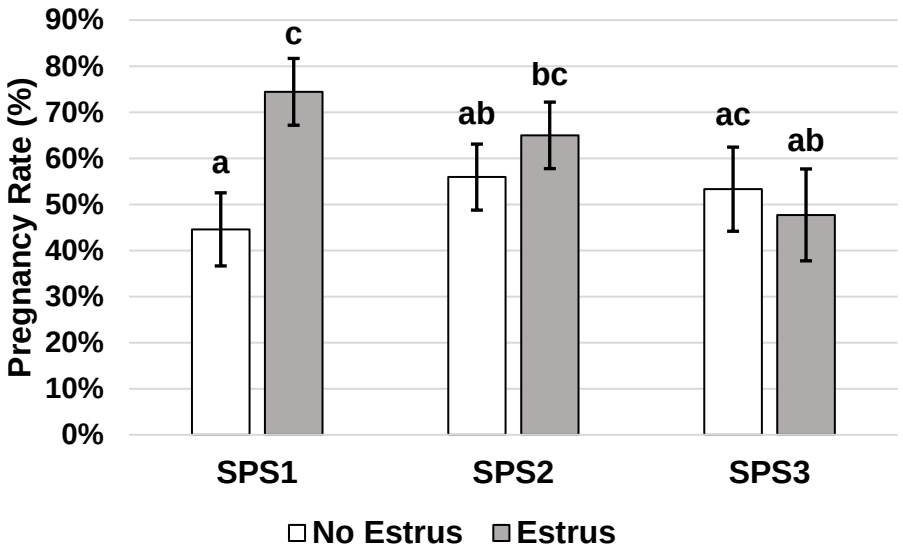

Figure 8. Influence of estrus activity and reproductive tract SPS on Angus cow FTAI day 30 pregnancy rates (Study 2). Estrus activity was measured using a patch scoring system developed by Pohler et al. (2016). Reproductive tract size and position scores (SPS) were determined according to criteria developed by Young et al. (2017). Cows designated as SPS1 and exhibiting increased estrus activity had greater pregnancy rates (74.43%) than SPS1 (P = 0.002; 44.59%) as well as a tendency for greater pregnancy rates compared to SPS2 (P = 0.069; 55.93%) cows that did not exhibit or had limited estrus activity. Cows designated as SPS1 with increased estrus activity tended to have greater pregnancy rates compared to SPS3 cows that had increased estrus activity (P = 0.056; 47.73% vs. 74.73%). Cows designated as SPS2 with increased estrus activity had increased pregnancy rates compared to SPS1 cows that had no or limited estrus activity (P = 0.018; 44.59% vs. 64.99%). Different letters (a, b, c) represents a tendency for difference ($P \leq 0.10$) across means. No Estrus = no or little estrus activity, Estrus = increased estrus activity.

CHAPTER IV: CONCLUSION

Embryonic mortality is a major contributing factor of pregnancy loss in cattle. The ability to detect that failure is critical to limit the number of days a non-pregnant cow continues to accrue labor and feed costs while expanding the calving interval and consequently decreasing pounds of calf weaned. Pregnancy-related microRNAs are closing the gap between the time a cow loses pregnancy and when that failure is first able to be detected. Furthermore, evaluating reproductive tract size and position scores and estrus expression at time of artificial insemination [AI] adds an extra measure that can be taken prior to breeding. Indication of presence or absence of estrus at AI along with using reproductive tract size and position scores can allow producers to make informed decisions regarding if expensive semen or semen from a high fertility sire is warranted to optimize the likelihood of pregnancy success.

VITA

Savannah L. Speckhart was born on April 28, 1995 to parents, Beth and Jeff Speckhart. She was raised in Princeville, Illinois, where she graduated from Princeville High School in 2013. Following high school, she attended Black Hawk College in Galva, Illinois, where she obtained an A.S. degree. She then transferred to Kansas State University in Manhattan, Kansas, where she completed a B.S. in Animal Sciences & Industry. In Fall 2017, she joined the lab of Dr. Ky Pohler at the University of Tennessee, Knoxville, to pursue a M.S. in Animal Science. Following completion of her M.S., Savannah will begin a doctoral degree at Virginia Tech in August 2019.