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Changes in Pregnancy - Associated Glycoproteins Associated with Fetal Age, Postpartum Interval and Evaluation of a Chute-side Lateral Flow Assay

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CHANGES IN PREGNANCY-ASSOCIATED GLYCOPROTEINS ASSOCIATED WITH FETAL AGE, POSTPARTUM INTERVAL AND EVALUATION OF A CHUTE-SIDE LATERAL FLOW ASSAY

BY

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THESIS ACCEPTANCE PAGE

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This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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"I can do all things through Christ who strengthens me"

Philippians 4:13

"Fear not, for I am with you; Be not dismayed, for I am your God. I will strengthen you, Yes, I will help you, I will uphold you with My righteous right hand".

Isaiah 41:10

CONTENTS

ABBREVIATIONS

AI	artificial insemination
AI2	second artificial insemination
AI3	third artificial insemination
BNGC	binucleate giant cell
boPAG-1	bovine pregnancy-associated glycoprotein-1
bPAG	bovine pregnancy-associated glycoprotein
bPSPB	bovine pregnancy-specific protein B
BW	birth weight
℃	degrees Celsius
CL	corpus luteum
d	day(s)
dpp	days postpartum
E_2	estradiol
FSH	follicle stimulating hormone
g	grams
GnRH	gonadotropin releasing hormone
h	hour(s)
ICM	inner cell mass
IFN-τ	interferon-tau
ISGs	interferon-tau stimulated genes
kg	kilogram
lateral flow	IDEXX Alertys OnFarm Pregnancy Test

LH	luteinizing hormone
min	minute(s)
mL	milliliter
MRP	maternal recognition of pregnancy
n	number
ng	nanogram
nm	nanometer
od	optical density
P_4	progesterone
PAG-1	pregnancy-associated glycoprotein subunit-1
PAGs	pregnancy-associated glycoproteins
PCR	polymerase chain reaction
PGF2α	prostaglandin $F_{2\alpha}$
PRP	prolactin-related proteins
PSP60	pregnancy-specific protein 60
PSPB	pregnancy-specific protein B
RNA	ribonucleic acid
RPT	IDEXX Alertys Ruminant Pregnancy test
RVPT	IDEXX Alertys Rapid Visual Pregnancy Test
SAS	statistical analysis system
TMB	tetramethylbenzidine
μL	microliter
ZP	zona pellucida

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ABSTRACT

CHANGES IN PREGNANCY-ASSOCIATED GLYCOPROTEINS ASSOCIATED WITH FETAL AGE, POSTPARTUM INTERVAL AND EVALUATION OF A CHUTE-SIDE LATERAL FLOW ASSAY

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Transrectal ultrasonography is known as the gold standard for pregnancy detection but requires costly equipment and technical skill. Access to a cheaper and more user-friendly method with similar accuracy to transrectal ultrasonography would be beneficial to individual cattle producers and to the cattle industry as a whole. Pregnancy diagnosis by detection of pregnancy-associated glycoproteins (PAGs) has the ability to accurately determine pregnancy in ruminants, however, usually requires specialized equipment for the assay. Pregnancy-associated glycoprotein pregnancy tests are gaining in popularity due to their ease of use and the unique feature of not requiring specialized training. Recently, a new lateral flow test (Alertys OnFarm Pregnancy Test) that does not require special equipment has been developed. A downside to blood pregnancy tests is they only provide an answer as to whether an animal is pregnant or nonpregnant. In addition, PAGs have a long half-life (80 to 100 d) in the maternal blood supply and can cause a false positive result. Thus the objectives of these studies were to determine whether a commercially available blood pregnancy test could be modified to detect differences in PAG concentrations to determine fetal age in cattle (study 1, chapter 2) and to determine factors that impact clearance of PAGs in postpartum beef females (study 2,

chapter 2) and also, to compare the accuracy of the IDEXX lateral flow, IDEXX Alertys Rapid Visual, and IDEXX Alertys Ruminant Pregnancy Test to transrectal ultrasonography (study 1, chapter 3) and to determine how many days postpartum (dpp) is necessary for the clearance of PAGs from the previous pregnancy to avoid false positives when utilizing the lateral flow test (study 2, chapter 3). In chapter 2, previously identified pregnant *Bos taurus* females from six different herds and postpartum females from one herd were utilized: (n = 1,753; study 1) between d 28 and 285 of gestation and (primiparous n = 418 and multiparous n = 657; study 2) once a week for up to 12 weeks after calving. In study 1, procedures were adapted to allow PAG concentrations to fall within the detectible range of the assay. Data were analyzed using the MIXED procedure of SAS with parity and gestational age (also divided into four gestational groups: 1) < 30 d; 2) 30-90 d; 3) 91-180 d; 4) > 180 d) in the model and then analyzed further using the REG procedure in SAS within gestational age group. In study 2, data were analyzed as repeated measure using the MIXED procedure of SAS with parity, days postpartum (dpp), and parity by dpp in the model, then data was analyzed further using the REG procedure in SAS. In study 1, there was a significant effect of gestational age and a parity by gestational age interaction ($P < 0.01$) as well as a tendency of parity ($P = 0.08$). Among nulliparous and multiparous females, serum PAG concentrations did not differ between gestational age groups 1 and 2 ($P > 0.84$); however, PAG concentrations differed between groups 2 and 3 ($P < 0.0001$) and 3 and 4 ($P < 0.0001$). There was a positive correlation between gestational age and PAG concentrations ($P < 0.01$; $r^2 = 0.2604$). In study 2, there was a significant effect of dpp ($P < 0.01$) on PAG concentrations; however, PAG concentrations were not influenced by parity ($P = 0.73$) or parity by dpp

($P = 0.55$). Concentrations of PAGs rapidly decreased from d 0 to 50 postpartum and then continued to gradually decrease ($P < 0.01$; $r^2 = 0.8083$). Prior to 42 dpp, PAG concentrations were sufficiently elevated which resulted in false positive readings. In chapter 3, blood samples were collected from six different *Bos taurus* herds between day 27 and 285 of gestation (nulliparous n = 1,205 and multiparous n = 1,539; study 1). Additional blood samples to determine PAG clearance interval were collected weekly postpartum for up to 12 weeks (primiparous n = 418 and multiparous n = 657; study 2). Serum was tested using the lateral flow test and were read by two technicians who were blind to animal pregnancy status. Level of agreement between the different methods of pregnancy detection was determined by Pearson's correlation and Kappa scores. The MIXED procedure of SAS was used to evaluate the effect of dpp and parity (primiparous or multiparous) on clearances. In study 1, there was a positive correlation between transrectal ultrasonography and the lateral flow test (r = 0.77; $P < 0.01$), and agreement between the two tests was good (Kappa = 0.84). Of the animals that were diagnosed nonpregnant by transrectal ultrasonography, 5.61% were called pregnant by the lateral flow test. Of the animals diagnosed pregnant by transrectal ultrasonography, 2.00% were called not pregnant by the lateral flow test. Thus, a 92.38% agreement occurred between the two methods. In study 2, for postpartum samples, there was no effect ($P = 0.21$) of parity, but there was an effect of dpp ($P < 0.01$) and a tendency for a dpp by parity interaction ($P = 0.06$). All animals were still considered pregnant from the previous pregnancy through 35 dpp (100 ± 2.58%). The percentage of females receiving a false positive test result further decreased with time postpartum, by 77 dpp there were 13.72 ± 3.16% of the females positive for pregnancy and at 84 dpp there were 4.11 ± 4.39%

positive for pregnancy detection. In conclusion, there is very good agreement between transrectal ultrasonography and the lateral flow PAG test, but if the test is used at less than 40 dpp the likelihood of false positive result is extremely likely. Thus, the test should be utilized at least 42 dpp to prevent gaining false positive results.

CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

In order to find success in the beef cow/calf industry, reproductive management practices must be implemented and held to a high standard in an operation. There are multiple reproductive management strategies which operations should try to implement early on in the breeding season: insemination of all cows towards the beginning of breeding season, detection of all non-pregnant cows as soon as possible, and any of the non-pregnant cows should be rebred as early as possible or sent to be culled or to a feedlot (Bó et al., 2016). Reproductive efficiency plays a critical role in influencing the economic standing within a cow-calf operation (Smith et al., 2005). The economic impact of reproduction is at least five times as more important than calf growth in commercial operations (Trenkle and Willham, 1977).

Most of the income for an operation comes from calves that are born alive, thus there is a direct dependency on reproductive efficiency (Santos et al., 2009). Increasing the calf crop only one percent (86 to 87 percent) by applying reproductive technologies there is a gain of either an additional 200,000 calves, or 340,000 fewer cows are required to produce the same number of calves (Trenkle and Willham, 1977). To have maximum efficiency within a herd a cow must produce a calf every year. The gestational length in *Bos taurus* cows ranges from 280 to 285 days in length (Paschal et al., 1991), leaving only 80 to 85 days for uterine involution, normal ovarian cyclicity, and conception to occur, which typically a cow requires about 40 days for the uterus to recover from

parturition (Caldow et al., 2005). So, this puts greater importance on getting cows bred and making sure they are pregnant earlier in the breeding season to allow further management decisions to not only enhance the herd but also add more profitability to the operation.

Following insemination in beef cows, fertilization rates are 90 percent, by time of parturition the total amount of embryo deaths account for more than 30 percent of the reproductive failure, of which the largest proportion of embryo deaths occur between days 8 to 16 (Diskin and Sreenan, 1980). The quantity of pregnancy losses continues to decrease each day as the pregnancy advances and becomes much lower after day 60 of gestation in cattle (Diskin and Morris, 2008; Wiltbank et al., 2016). According to Bellows et al. (2002), the reproductive condition in beef cow-calf herds becoming the costliest is infertility. The reason infertility is the greatest financial setback is because producers tend to cull any infertile females on their operation, which means the acquired cost producers have gained from either raising or purchasing a female are currently not generating any revenue (Bellows et al., 2002). There is also decreased salvage value, due to infertility compared to selling a pregnant female (Bellows et al., 2002). Thus, making reproductive technologies important to implement into an operation.

The purpose of this review is to establish the physiological events that occur during embryo and fetal development. The concepts considered within this review will add to the discussion of maximizing reproductive efficiency, management, and economic decisions by implementing early pregnancy detection and giving producers a better understanding of pregnancy detection methods available to implement the one that best fits their operation.

EMBRYO AND FETAL DEVELOPMENT

A female who is unable to produce a calf early post-breeding may lack proper ovulation, fertilization, or normal growth of the existing conceptus, which can drastically decline productivity by increasing the number of days required for a female to produce a calf on the ground at the end of the gestational period (Warnick and Hansen, 2010). The two main origins for reproductive failure in inseminated cattle are due to failure to fertilize or absence of embryo survival (Santos et al., 2004). According to Diskin and Sreenan (1980) fertilization rates in cows are about 90 percent, but calving rates are only about 55 percent. Early embryonic death occurring from day 8 to 16 following insemination accounts for about 40 percent of all pregnancy loss in cattle (Ayalon, 1978; Diskin and Sreenan, 1980; Farin et al., 2001; Diskin et al., 2011).

A cow's estrous cycle length is of normal length (~21 days) when embryonic mortality occurs before maternal recognition of pregnancy (day 16), but if it occurs later than day 16, the estrous cycle will be lengthened (Northey and French, 1980; Farin et al., 2001). Although late embryo mortality does not have as many losses as early embryo mortality, it does have a greater negative effect on a herd, especially in herds with a distinct breeding season (Diskin et al., 2011). Late embryonic mortality causes severe economic loss within an operation because by the time it is recognized it is too late to rebreed those females within the herd, so ultimately these open females get culled from the herd (Diskin et al., 2011). During early embryonic development, luteolytic mechanisms need to be stopped in order to allow for continual growth and development of the embryo, so steroidal hormones need to be secreted in order to stop luteolysis

(Mann and Lamming, 2001). When an embryo survives beyond the point of maternal recognition of pregnancy luteal regression becomes delayed, which allows for return to estrus to be suspended and the embryo can grow and develop further (Diskin et al., 2011).

The health status of an individual cow or herd affects the efficiency of the operation and also reproductive performance either in a direct or indirect manner, so there needs to be awareness of the female's health so the embryo can remain healthy and viable (Diskin et al., 2011). The majority of pregnancy loss in cattle occurs during the preimplantation stage of embryo development, which is why it is commonly considered as the critical period (Valadão et al., 2018). Due to this high amount of implantation failure typically two ovulation cycles are necessary in order to achieve pregnancy (Valadão et al., 2018). Thus, to have the most success in cattle operations, reproductive management advancements need to be made in the future by concentrating on boosting fertilization rates and reducing embryonic loss in efforts of improving conception rates (Santos et al., 2004). The subsequent sections will discuss the role they play in early embryo and fetal development.

Fertilization

Most cows remain in estrus for 18 hours (Trimberger and Davis, 1943). Once a cow has exhibited estrus, ovulation typically occurs between 10 to 18 hours later (Nalbandov and Casida, 1942; Brackett et al., 1980). Once a cow begins to show signs of standing estrus, she becomes receptive to be bred either by natural service or artificial insemination and semen can be deposited into the vagina (natural service) or uterine body (artificial insemination; López-Gatius, 2000). The development of the Graafian follicle

on the threshold of ovulation dictates the harmonization of two gametes, sperm and oocyte (Hunter and Wilmut, 1984). Adequate amounts of spermatozoa navigate during early estrus into the anterior portion of the oviducts around 8 to 12 hours or more after mating (Hunter and Wilmut, 1983, 1984; Wilmut and Hunter, 1984). In order to achieve successful fertilization *in vivo*, mammalian spermatozoa must experience capacitation first and then undergo the acrosome reaction (Fraser, 1998). During capacitation, there are three significant changes that could occur on the exterior of the sperm, which include: molecules being lost, rearranged, or unmasked (Oliphant et al., 1985; Fraser, 1998). Capacitation occurs at a relatively slow rate (hours), while the acrosome reaction is comparatively rapid, minutes, to complete (Fraser, 1998).

When mammalian spermatozoa leave the male reproductive tract, they are extremely motile, but at this time they are not able to fertilize an oocyte yet (Austin, 1951; Chang, 1951; Fraser, 1998). Once capacitation and the acrosome reaction have occurred in sperm cells it is critical that sperm bind with the oocyte (Betteridge and Fléchon, 1988; Fraser, 1998). After ejaculation via natural service the number of spermatozoa that reach the site of fertilization, become limited to only a small proportion of the ejaculated sperm (Fraser, 1998). The myometrium contractions help move sperm when high estradiol concentrations are present at estrus, but it is unlikely that many reach the fertilization site; although, motile sperm, by swimming, reach the site of fertilization (Hunter and Wilmut, 1984; Wilmut and Hunter, 1984; Senger, 2003).

Once sperm cells reach the oocyte, sperm cells must penetrate the zona pellucida, a protective ring that surrounds the oocyte. The zona pellucida (ZP) is made up of three glycoproteins, known as ZP1, ZP2, and ZP3 (Wassarman, 1988). Two glycoproteins, ZP1

and ZP2, give the zona pellucida its structure, while ZP3 provides a binding site for the head of the sperm (Wassarman, 1988). The acrosome reaction is initiated once the sperm becomes bound to the zona pellucida binding region and acrosome promoting ligand (Wassarman, 1988). The acrosome reaction is an exocytotic event which allows a sperm cell to penetrate the zona pellucida where it can now fuse with the oocyte plasma membrane (Yanagimachi, 1988; Fraser, 1998).

Early Embryo Development

When the nucleus of the sperm enters the cytoplasm it commences a process called syngamy, fusion of male and female pronuclei, which is the last step in fertilization (Hansen, 2002). After syngamy, the male and female pronuclei fuse within a singular diploid nucleus, and the fertilized oocyte is known as a zygote (Betteridge and Fléchon, 1988; Hansen, 2002). The zygote undergoes mitotic divisions, or cleavage divisions. The first cleavage division takes place around 48 hours post-estrus and creates a two-cell embryo (Shea, 1981), and further cleavage divisions occur later, creating 4, 8, and 16 daughter cells (Senger, 2003). Each cell at the 2, 4, and 8 cell stages are totipotent, meaning they have potential to mature into a fully formed calf. Mitotic divisions continue and each cell decreases in size (Chavatte-Palmer and Guillomot, 2007). Morula stage embryos are a tight mass of blastomeres formed in a rounded mass around d 4 to 5 (Betteridge and Fléchon, 1988).

There is a continuation of cellular divisions within the cells of the morula until a blastocyst is formed. As more fluid begins to accumulate in the embryo a fluid filled cavity is formed known as a blastocoele, and both the inner cell mass (ICM) and

trophoblast cells can be identified (Negrón-Pérez, 2017). A blastocyst is composed of an inner cell mass and a single layer of outer cells, the trophoblast (Forde and Lonergan, 2012). Within the inner cell mass the gap junctions allow for intercellular communication, but among trophoblast cells the tight junctions allow for the accumulation of fluid on the inside of the embryo (Shimada et al., 2001; Houghton, 2005; Senger, 2003). The blastocoele can be seen around d 7 of gestation (Shea, 1981; Senger, 2003). The formation of the first hypoblast cells, also known as primitive endoderm, occur in the inner cell mass by d 8 (Maddox-Hyttel et al., 2003). By d 10 the hypoblast cells have a fused hypoblast lining in the inside of the trophoblast (Maddox-Hyttel et al., 2003).

By d 8 to 11 the cells of the blastocyst hatch from the ZP due to the ZP being weakened from the increased pressure inside causing it to rupture (Fléchon and Renard, 1978; Shea, 1981; Garrett et al., 1988; Forde and Lonergan, 2012). Once hatched, the conceptus becomes completely reliant on the uterine environment to assist and support in survival and attachment of the conceptus (Garrett et al., 1988; Geisert et al., 1991).

By d 12 the inner cell mass is transformed and is referred to now as the epiblast, a thin overlying trophoblast lining known as the Rauber's layer, which establishes the embryonic disk (Fléchon and Renard, 1978; Maddox-Hyttel et al., 2003). The combination of the epiblast and hypoblast are known as the embryonic disk (Vejlsted et al., 2006). Simultaneous to elongation occurring, the ICM is developing and differentiating from a spherical disk into an oval embryonic disk (Greenstein and Foley, 1958; Blomberg et al., 2008). Once the disk becomes converted into an oval shape, the mesodermal cells can be distinguished from the ICM and begin to migrate out between

the trophectoderm and endoderm by d 14 to 16 (Winters et al., 1942; Greenstein and Foley, 1958; Betteridge and Fléchon, 1988). The conceptus then gets transformed into an elongated conceptus which has a direct correlation between length and time, as the duration of time increase so does length of the conceptus (Forde and Lonergan, 2012). During elongation the conceptus increases in size by greater than one-thousand-fold (Maddox-Hyttel et al., 2003). The blastocyst is a free-floating embryo until around d 20 to 23 and is found in the lumen of the uterus (King et al., 1981; Negrón-Pérez, 2017). The conceptus becomes elongated and occupies space in both of the uterine horns by d 20 to 25 of gestation (Assis Neto et al., 2009). It is imperative for the trophectoderm to elongate so it can make as much contact with the endometrium as possible (Goff, 2002).

Extraembryonic Development

Later in embryo development the ICM evolves into the body of the embryo and a portion of its extraembryonic tissues, while the trophoblast will evolve into the extraembryonic ectoderm once attachment has occurred and it forms the chorion of the placenta (Senger, 2003; Hue et al., 2015; Negrón-Pérez, 2017). Further transformation of the ICM occurs to form the epiblast, developing later into fetal tissues, and the hypoblast, developing later into extraembryonic tissues (Kuijk et al., 2012). The conceptus develops its extraembryonic membranes before attachment can even occur (Degrelle et al., 2005; Hue et al., 2015). There are four membranes which are formed during this extraembryonic period that consist of the trophoblast, endoderm, mesoderm, and the embryo (Greenstein and Foley, 1958). When the conceptus elongates it allows extraembryonic differentiation to occur: yolk sac and allantois development, necessary

for embryonic survival, and establishment of the placenta (Guillomot, 1995; Hue et al., 2012; Spencer and Hansen, 2015).

The mesodermal cells become separated into two layers: outer cell layer, which lines the trophectoderm, and inner cell layer, which acts as the wall around the endoderm (Betteridge and Fléchon, 1988). The outer layer forms the chorion, while the inner layer creates the outer wall of the yolk sac (Betteridge and Fléchon, 1988). The embryonic endoderm and mesoderm develop both the chorion and amnion. The embryonic endoderm matures into the yolk sac when both ends of the hypoblast become joined together (Negrón-Pérez, 2017). The amnion is a fluid filled cavity that aids in protection and allows for growth and development of the fetus; while the chorion is what will later attach to the uterus.

The yolk sac begins to form when the embryo begins its own blood circulation (Assis Neto et al., 2010). Around d 18 the amniotic sac is formed (Greenstein and Foley, 1958). The layer between the two mesoderm layers is known as the coelom, which is where the allantois buds into this space; around d 20 (Fléchon and Renard, 1978; Betteridge and Fléchon, 1988). The second and most apparent blood vascular system is produced by the allantois, which is observable as early as d 20 to 21 as a bud (Fléchon and Renard, 1978; Maddox-Hyttel et al., 2003; Assis Neto et al., 2010). Day 20 to 25 the amnion is not completely fused with the allantois, so at this stage they remain distinguishable (Assis Neto et al., 2009). There are three distinct layers of membranes in contiguity, the chorion, allantois, and amnion (Assis Neto et al., 2009). The chorion is the external layer, allantois is the intermediate transparent layer, and the amnion is a thin membrane that wraps around the embryo (Assis Neto et al., 2009). Around d 32 to 39,

sexual development of the fetal gonads can be distinguishable (Erickson, 1966). By d 40 to 50, the allantois and chorion become fused with each other to form the chorioallantois (Assis Neto et al., 2009). The formation of these extraembryonic structures is necessary for strong embryo attachment to the uterus of the dam.

Maternal Recognition of Pregnancy

Maternal recognition of pregnancy (MRP) is referred to when the uterus, either recognizes that the conceptus is present and continuing with the pregnancy; no embryo is present and resetting the estrous cycle; or it thinking the conceptus is a foreign object and eliminating both the conceptus and the source of progesterone by regressing the corpus luteum (CL; Wiltbank et al., 2016). The term MRP was created by Robert Short in 1969 defined as the physiological process where the conceptus signals to the maternal system that it is present and needs the maternal system to lengthen the existence of the CL in order to maintain elevated concentrations of progesterone (Spencer and Hansen, 2015). The enlargement of the trophoblast increases placental surface area to facilitate communication between dam and conceptus, it also aids in nutrient exchange essential for the survival of the conceptus (Stroband and Van der Lende, 1990; Blomberg et al., 2008). According to Wiltbank et al. (2016), the mortality rate during this period, MRP, is about 30 percent. The time period around MRP is known as the critical period during gestation, because the uterus needs to identify the existence of the conceptus so it is maintained (Wiltbank et al., 2016). While elongation is occurring, there are multiple molecules expressed such as, interferon-tau (IFN-τ); placental lactogen; and pregnancy-associated glycoproteins (PAGs; Degrelle et al., 2005; Hashizume, 2007; Blomberg et al.,

2008). The conceptus becomes entirely reliant on maternal secretions in order to ensure its survival during this critical time period (Forde and Lonergan, 2012). At d 16 of gestation the embryo needs to influence the maternal system in order to prevent regression of the CL with adequate amounts of IFN- τ and to allow for MRP to be completed (Northey and French, 1980; Forde and Lonergan, 2012).

In ruminants, the signaling molecule for MRP is interferon-tau, produced specifically by the trophoblast during extraembryonic development, acting within the uterus to prevent luteolysis and extend secretions of progesterone (Stewart et al., 1992; Guillomot, 1995; Matsuyama et al., 2012). Interferon-tau plays a large role in the establishment of pregnancy (Matsuyama et al., 2012). Expression of IFN-τ rises during the elongation stage of gestation, peaking just before the conceptus attaches to the uterine epithelium, and then dissipates once implantation has occurred (Hansen et al., 1988; Guillomot, 1995; Matsuyama et al., 2012).

In order to have successful recognition of pregnancy, adequate levels of embryonic development and production of IFN-τ is needed, which is reliant upon progesterone secretion after ovulation (Mann and Lamming, 2001). Progesterone concentrations are elevated until about d 18 post-breeding, which corresponds with the period of MRP (Kerbler et al., 1997). Progesterone also plays an important role in evolving the histotrophic environment for the nourishment of the conceptus (Rani et al., 2018). The conceptus is critical for the prevention of luteolysis and maintaining progesterone production by the CL (Goff, 2002). If a conceptus is not present, then both progesterone and estradiol become involved in regulating the uterus to prepare it to regress the CL by secreting prostaglandin $F_{2\alpha}$ (PGF2α; Robinson et al., 2001; Rani et al.,

2018). A decrease in progesterone and an increase in estrogen causes a sequence of sporadic periods of oxytocin secretion (McCracken et al., 1999). This increase in estrogen upregulates the endometrial oxytocin receptors, causing the combination of neurohypophysial oxytocin and the oxytocin receptors found in the endometrium to secrete luteolytic pulses of uterine PGF2α (McCracken et al., 1999). Interferon-tau blocks the increase of oxytocin receptors in the endometrium, which causes a decrease in the amount PGF2α secreted (Spencer and Bazer, 1996; Robinson et al., 1999; Mann and Lamming, 2001; Goff, 2002).

There is a correlation between elongation of the embryo (specifically the trophectoderm) and increased concentrations of IFN-τ, which decreases PGF2α secretion (Hansen et al., 1988; Goff, 2002). In order to gain successful establishment of pregnancy within a cow, the embryo must develop adequately so it can produce sufficient quantities of IFN-τ by d 16 to avoid luteolysis (Mann et al., 2006). Once IFN-τ becomes absent from the maternal circulation, new molecules, such as PAGs, placental lactogens and prolactin-related proteins (PRPs), need to be produced to secure maintenance of pregnancy (Degrelle et al., 2005; Hashizume, 2007; Blomberg et al., 2008; Valadão et al., 2018). Pregnancy-associated glycoproteins, placental lactogens, and prolactin-related proteins are found in binucleate giant cells, which appear in the trophoblast of the chorion on d 16 to 18 (Greenstein and Foley, 1958). A known function of PAGs is they can direct the CL to survive, expand and continue to produce progesterone to allow suppression of cyclicity and create a proper environment for embryo development (Valadão et al., 2018). Binucleate giant cell migration occurs as early as d 20 and continues throughout gestation (Wooding and Wathes, 1980). Without MRP and hormone signaling, then the dam would

not recognize the pregnancy, and the conceptus would be lost, hence it being called the "critical period".

Placentome Development and Attachment

The uterine wall can be divided into two sections: myometrium and endometrium (Spencer and Hansen, 2015). The endometrium has two distinct areas: the aglandular caruncular and glandular intercaruncular regions (Atkinson et al., 1984; Spencer and Hansen, 2015). The aglandular caruncularis region is the site of superficial implantation and placentation, while the glandular intercaruncular regions supplies early nourishment to the embryo through secretions of large molecules (Atkinson et al., 1984; Spencer and Hansen, 2015). Remodeling of the endometrium is essential for successful attachment and placentation (Hashizume, 2007).

Normal bovine placental attachment to the endometrium progresses throughout almost all of the first trimester (Hill et al., 2000). The blastocyst anchors the embryo to the wall of the uterus (King et al., 1980); this occurs when elongating embryos attach to the uterus around d 20 to 22 of gestation (Blomberg et al., 2008). This initial attachment is replaced by attachment through the chorion which occurs between d 28 and 35 of gestation (King et al., 1980). Attachment takes place from about 20 to 30 d post-insemination up to 70 d, and placentome enlargement occurs through growth, vascularization, and number of cotyledons (fetal) opposite to endometrial caruncles (maternal; Assis Neto et al., 2010). During early attachment, the epithelium of the carunclar and intercarunclar regions near the embryo expand to each end of the blastocyst and attach to the chorion by microvillous interdigitation for placental formation (King et

al., 1980; Wooding and Wathes, 1980; Atkinson et al., 1984). Placentomes develop in the caruncle region of the endometrium and not in the intercaruncular region of the endometrium (Atkinson et al., 1984; Hashizume, 2007). Placentomes contain both fetal and maternal information because they are formed with the fetal trophoblasts and the maternal endometrium (Hashizume, 2007). A histological examination revealed there are placentomes, microvilli, and a fragile attachment of maternal and fetal epithelia by d 30 of gestation (King et al., 1979; Hill et al., 2000).

The initial placental connection with maternal caruncle tissue stimulates hypertrophy and hyperplasia to form cotyledons, observed between d 30 to 40 of gestation, that are developed by increasing the number, size and thickness of the placentomes so they become larger and more complexed (Hill et al., 2000; Assis Neto et al., 2009). Cotyledonary formation extends gradually from the chorionic surface of the embryonic zone to the chorionic tips, although, there is not a tight chorio-endometrial connection until at least d 50 (Assis Neto et al., 2010). Assis Neto et al. (2010), found that around d 37 to 40 there was a maximum of 20 cotyledons present on the chorionic surface of the embryo but by d 70 that number increased averaging about 80 cotyledons. Large cotyledons are first observed around d 60 to 70 of gestation (Assis Neto et al., 2009).

Each caruncle has crypts (maternal component) that increases the surface area contact with chorionic villi (fetal component; Atkinson et al., 1984; Aires et al., 2014). The crypts of the caruncle and chorionic villi interlock to maintain both structure and position (Atkinson et al., 1984). There were multiple caruncles throughout the endometrium some protruded into the lumen, when combined with cotyledons they

develop into placentomes (Leiser et al., 1998; Aires et al., 2014). Placentomes act as a communication source between the dam and the fetus (Hashizume, 2007). The role of placentomes is to allow transfer of maternal/fetal gaseous, nutrient and metabolic waste (Aires et al., 2014). Placentomes are also the primary site for small molecular transfer such as oxygen, carbon dioxide, amino acids, and glucose, whereas macromolecules are carried through interplacentomal areas adjacent to the uterine gland openings (Hill et al., 2000). This fetal-maternal transfer of nutrients and gases is vital to maintain the pregnancy (Stice et al., 1996). There is about a 12 percent loss in pregnancies during this phase namely due to delays or defects in chorioallantoic placentomes or the fetus, which eventually results in CL regression or fetal death during this period (Wiltbank et al., 2016).

Fetal Period

Around d 50 to 60 of gestation marks the beginning of the fetal phase and it ends at parturition (Assis Neto et al., 2009; Valadão et al., 2018). Beginning this period is rapid body growth and maturation of organs and systems (Valadão et al., 2018). The transition from an embryo to a fetus occurs when an elongated neck and the umbilical cord are distinctly visible (Assis Neto et al., 2010). By the third trimester of gestation fetal length increases slowly, while fetal weight and fetal energy requirements continue to increase (Valadão et al., 2018). Fetal nutrient source also switches from uterine histotroph through the yolk-sac, placenta, or choriovitelline nutrition to gather nutrients from the chorioallantoic placental nourishment for the remainder of gestation (Wiltbank et al., 2016).

PREGNANCY DETECTION

Early pregnancy detection is essential to properly manage a cattle herd, but it is only effective if it is accurate in determining which females are pregnant and which ones are not pregnant. The greatest single loss in potential calf crop is the failure of cows to become pregnant during the breeding season (Wiltbank et al., 1961). For every missed estrus there is a loss of about three weeks of production waiting for that particular animal to cycle again (Zemjanis, 1962). This is critically important to the profitability of any beef operation as age at weaning is the single greatest factor that impacts weaning weight. An analysis of 3,700 calves at the USDA-Meat Animal Research Center indicated for each day of age after the beginning of the breeding season a calf is born there is 1.09 kg of weaning weight that is lost (personnel communication R. Cushman).

The possibility of distinguishing between pregnant and non-pregnant animals also allows for further management decisions to be made. By identifying which females are non-pregnant or are pregnant they can be sorted into separate groups and managed for their specific needs, which allows for better utilization of labor and time. Typically, when early pregnancy detection occurs calves have not been weaned from the dam yet, which must be taken into consideration with management decisions when a female is non-pregnant until she has weaned her calf. Non-pregnant females may either be rebred, sent to a feedlot, or culled. Without pregnancy detection, a non-pregnant heifer or cow who has weaned a calf would be treated like a pregnant female, which would result in a larger monetary loss. There are multiple methods of pregnancy detection for cattle, including: calving, visual signs of return to estrus, transrectal palpation, blood collection for

interferon-stimulate genes, blood and milk collection for analysis of progesterone, blood and milk collection for analysis of pregnancy-associated glycoproteins, and transrectal ultrasonography. Each of these pregnancy detection methods have their own benefits and downfalls. The ideal pregnancy detection method should accurately identify both pregnant and non-pregnant females in the herd (Romano and Larson, 2010). Each producer must determine what pregnancy detection method best suits their herd/operation. Whatever the method, pregnancy detection is a useful tool to make informed management decisions and gain improved reproductive and economic success among your cow-calf herd.

Calving

Calving is the simplest and least time-consuming method of pregnancy detection there is. The gestational period for a cow on average is 283 days or about nine months long (Paschal et al., 1991; Senger, 2003). A good indication of a pregnant female close to calving is when her vulva is swollen and bouncy, an udder has been developed, and there is mucosal discharge. Consequently, if a producer waits for the female to calve at the end of 283 days to determine whether she is pregnant or not there is a potential to miss out on a multitude of other economic and management decisions. In the United States, 21.9 percent of larger operations (200 or more cows) do not use any pregnancy detection methods and in smaller operations (less than 50 cows) there is a greater percentage (69.6 percent) of operations that do not use any pregnancy detection method (USDA, 2017). Especially amongst smaller herds there is a larger number of producers who wait for their females to calve to determine whether they are pregnant or not. Of those producers who

do not use pregnancy detection the most common reason for not using one was due to the amount of time and labor required (USDA, 2009). Although calving is ultimately the cheapest (by direct costs) and least time-consuming method and used frequently in many herds, it also represents the method that has the greatest indirect cost. These indirect costs include: $380 to $900 per head for an additional year kept in the herd, also the loss of productivity by the land for maintaining a cow that is not producing a calf is 1.8 acres per head (USDA, 2009; Gray et al., 2012). When the indirect costs are evaluated, there are better and more cost-efficient methods for pregnancy detection.

Return to Estrus

While waiting to see if a cow calves has the lowest direct cost and has the least amount of labor involved, monitoring cows for return to estrus is the most labor intensive. The estrous cycle is a recurring pattern of ovarian activity in a female that begins and ends with periods of sexual receptivity. Estrus behavior (sexual receptivity) in cattle occurs about every 21 d, and is necessary to allow for mating and potential pregnancy establishment (Forde et al., 2011). Beef cows nursing calves will initiate estrous cycles between 30 to 130 d after calving (Short et al., 1990; Forde et al., 2011). Each estrous cycle consists of two phases: the luteal phase, 14 to 18 d in length, and the follicular phase, 4 to 6 d (Forde et al., 2011). The luteal phase occurs after ovulation and the follicular phase occurs prior to ovulation. There are six hormones that have a role in the estrous cycle: follicle stimulating hormone (FSH), luteinizing hormone (LH), progesterone (P_4), estradiol (E_2), gonadotropin releasing hormone (GnRH), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Follicle stimulating hormone is responsible for the

recruitment of follicles from the pool of growing follicles on the ovaries (Adams, 1999). When previously dominant follicles ovulate or lose dominance, the inhibitory factors that allowed it to be dominant are lost resulting in an increase in FSH allowing for a new wave to begin to grow (Fortune, 1994; Adams, 1999). Following ovulation of a dominant follicle a CL is formed. The CL secretes progesterone which inhibits females from exhibiting estrus (Milvae et al., 1996). Elevated concentrations of progesterone are inhibitory to LH secretion, resulting in the inhibition of a surge of estradiol and a preovulatory LH surge and ovulation (Adams, 1999). If maternal recognition of pregnancy is not established around d 16, there are no signals to save the CL (Forde et al., 2011), and prostaglandin $F_{2\alpha}$ is released from the uterus in a pulsatory manner, which results in regression of the CL, known as luteolysis (Shirasuna et al., 2004). Once luteolysis is initiated, P_4 concentrations decrease (Fortune, 1994; Ginther et al., 2011), and the inhibitory effect of P_4 is removed. This allows the next dominant follicle to produce increased concentrations of E_2, and behavioral estrus to occur when E_2 concentrations reach their peak (Staigmiller et al., 1982; Frandson et al., 2003). Without $PGF_{2\alpha}$, a female would not return to standing estrus during the breeding season (Burfening et al., 1978; Diskin et al., 2002).

During the late follicular phase, estrus is expressed (expression ranges from 2 to 24 h; Forde et al., 2011). Cows from d 5 to 16 of their cycle or pregnant are less likely to mount a cow in estrus (Diskin and Sreenan, 2000). Thus, normal estrous cyclicity and estrus are disrupted when animals are pregnant or when they have other underlying physiological conditions. Because of this, return to estrus or the non-return to estrus can be used as a pregnancy detection method. It is important for observers to know what

signs to look for when observing for standing estrus so they can accurately determine which females are not pregnant. When females are coming into estrus, they become restless, bellow more, and mount other females who are in standing estrus (Thomas and Dobson, 1989). Those in estrus walk 2 to 4 times more than a non-estrous animal (Diskin and Sreenan, 2000). Some visual signs that females have returned to estrus include standing to be mounted by other animals, clear mucus discharge from the vagina, ruffed up tail head, and a swollen vulva (Williamson et al., 1972). Observation of a female standing while being mounted by another female is an accurate visual sign to observe that indicates she is in estrus (Williamson et al., 1972).

Estrus mount detectors are a great tool to use to help aid in the detection of females in estrus (Williamson et al., 1972). Most females exhibit estrus either early in the morning or later in the evening, when temperatures are cooler (Diskin and Sreenan, 2000). According to Diskin and Sreenan (2000) when females are observed every 4 to 5 h for expression of estrus there is a 90 percent detection rate, however, with the knowledge of when females are most likely to display estrus, producers can focus their labor by detecting estrus in the morning and evenings (only 2x per day) and still achieve a 70 percent detection rate (Diskin and Sreenan, 2000). However, this means 30 percent of non-pregnant animals will be considered pregnant or not in estrus. Thus, the more time allowed for observing for estrus the greater the accuracy of this method will be. Failure to detect estrus can be attributed to 10 percent cow problems and the other 90 percent attributed to management problems such as, human error or inadequate time allotted for detection of estrus (Diskin and Sreenan, 2000). False positives can also be attributed to pregnant females showing estrus in a herd, which accounts for about 5 to 7 percent in a

herd (Thomas and Dobson, 1989). The behavioral signs that pregnant females display is indistinguishable from non-pregnant females, aside from the shorter duration of standing behavior, the intensity of standing behavior in a pregnant female is equivalent to that of a true estrus in a non-pregnant female (Thomas and Dobson, 1989). If pregnancy detection is inaccurate, animals showing signs of estrus may be considered repeat breeders or infertile and then sold; if insemination occurs in an already pregnant cow it is very likely embryo or fetal loss will occur, which would result in a longer calving timeframe (Esslemont et al., 1985; Thomas and Dobson, 1989).

Detecting return of estrus is cost effective and requires little to no equipment, but those who are observing for estrus need to know what to look for and be willing to put the time in to be successful at detecting estrus. Tools such as estrus mount detectors, tail chalk, and radio telemetric devices may aid in the accuracy of heat detection (Diskin and Sreenan, 2000). Return to estrus is a good method of identifying whether a female is pregnant or non-pregnant but is costly in terms of time and can have very high false positive rates due to human errors. Also, it will most likely need to work in concert with another pregnancy detection method to ensure pregnancy status later in gestation.

Palpation

Waiting for a cow to calve or observing for the return to estrus are indirect methods to determine pregnancy; rectal palpation is the oldest form of direct pregnancy detection (Cowie, 1948; Purohit, 2010). It is conducted by manually palpating through the rectal and uterine walls for various structures such as fetal membranes, amniotic vesicle, fetus, or cotyledons within the uterus (Sasser et al., 1986). Wisnicky and Casida

(1948) were the first to report pregnancy detection in cattle by rectal palpation at d 35 or later in gestation (Sasser et al., 1986). Typically, rectal palpation examinations are performed between d 40 and 60 (Jaśkowski et al., 2019). The downfall to manual rectal palpation, is accurate pregnancy diagnosis is dependent upon the technician's familiarity of the bovine reproductive tract, stage of gestation, and ability of the technician to detect differences between pregnant and non-pregnant animals (Zemjanis, 1962). The most common diagnosis errors are mistaking certain structures of the reproductive tract, such as, the uterus or parts of the fetus for an organ or bone structure (Zemjanis,1962). If palpation is performed too early during pregnancy the embryo and/or signs of pregnancy could be easily missed (Zemjanis, 1962; Gunn and Hall, 2018) or the embryo could be damaged or even aborted (Zemjanis, 1962; Balhara et al., 2013). In addition, it is not possible to determine fetal sex or viability by palpation. Structures such as: fetal membranes, amnionic vesicle, cotyledons, and/or a fetus would be a positive sign of pregnancy within the female (Zemjanis, 1962). Those who do not have a strong technical sense for palpation should identify multiple structures indicative of pregnancy when making their determinations (Zemjanis, 1962).

Rectal palpation is one of the most cost-effective methods, because it requires minimal equipment (a palpation glove and lubricant) and gives you a real time answer. Rectal palpation is solely based on feel and having the knowledge to know what is being felt is truly an accurate representation. Implementation of manual palpation within smaller herds (less than 50 head of beef cows) was only 14.2 percent of the herds surveyed, while in larger herds (greater than 200 head) it was utilized in about 53.6 percent of the herds surveyed (USDA, 2017). Although there are other pregnancy

detection methods available, this method is the most frequently practiced in detecting pregnancy in beef cattle due to it being cost-effective, easy to perform (if knowledgeable), and quick.

Interferon-tau stimulated gene (ISGs)

Interferon-tau is the pregnancy recognition signal in ruminant animals (Hansen et al., 1988), and is produced between d 13 and 16 post-insemination by embryonic trophoblast cells (Thatcher et al., 1995; Green, 2010). It is present in the uterus from d 13 to 21 after ovulation (Senger, 2003), and preserves the CL by decreasing the luteolytic pulses of uterine $PGF_{2\alpha}$ (Meyer et al., 1995; Spencer and Bazer, 2004; Shirasuna et al., 2015). Leukocytes in circulation respond to IFN-τ by expressing interferon stimulated genes (ISGs). These genes can be used as a pregnancy detection method by taking a blood sample from females. Expression of ISGs in the buffy coat (white blood cells; (Gifford et al., 2007; Shirasuna et al., 2015) can be measured by extracting ribonucleic acid (RNA) and performing a polymerase chain reaction (PCR) to determine whether the female is pregnant or non-pregnant (Gifford et al., 2007). Increased concentrations of blood ISGs around d 18 to 22 following insemination are indicative of a conceptus being present (Wijma et al., 2016). If blood ISG concentrations are low around d 18 to 22 there is a good chance the female is not pregnant, but all ISGs are completely gone by d 30 of pregnancy (Lucy et al., 2011).

Determining pregnancy status early is extremely beneficial, and the use of ISGs is the earliest method currently available for determining if a cow is pregnant. Since this pregnancy detection method can be determined as early as 18 to 22 d after insemination

that means rebreeding non-pregnant females could happen sooner. Detecting pregnancy in females early is imperative to shorten the calving interval by enabling producers to identify non-pregnant animals and either treat, rebreed or cull them at the earliest opportunity possible (Balhara et al., 2013). There is however, a 20 to 40 percent chance that the embryo present on d 17 to 20 will fail to survive until term, which is a downfall to determining pregnancy this early (Sreenan and Diskin, 1986; Senger, 2003). The majority of embryonic loss occurs within 60 d after insemination before the placenta has a chance to be fully formed (Santos et al., 2004; Lucy et al., 2011). Another downfall is that this pregnancy detection method would never be done by a producer on the farm or ranch. This method requires time, skill, and a lab that would allow for RNA extractions and PCR. Early pregnancy detection means a producer has a greater economic advantage and management decisions can be made earlier, however, if ISGs are used exclusively then pregnancy losses and changes in pregnancy status among females may go undetected.

Blood Progesterone Analysis

Progesterone is important for pregnancy maintenance, inhibiting the expression of estrus, normal cyclic function, and hypophyseal-gonad interrelationships (Gomes and Erb, 1965). Progestins are also important for regulating the uterus necessary for implantation, blastocyst development, and maintenance of the fetus during pregnancy (Gomes and Erb, 1965). Lucy et al. (2011), reported the first true example of chemical pregnancy testing identifying non-pregnant cows by measuring progesterone in blood or milk. Laing and Heap (1971) were the first to suggest the use of milk progesterone for the

determination of pregnancy in cows. Milk progesterone testing using a direct rapid radioimmunoassay for pregnancy diagnosis has been available to dairy farmers in the United Kingdom since 1975 (Booth et al., 1979). Before radioimmunoassay's, which use large amounts of blood to determine the progesterone levels in cows, reproductive tissues collected at time of surgery or slaughter were used to determine progesterone levels (Short, 1958; Gomes and Erb, 1965).

If a cow is non-pregnant, then her progesterone concentrations will be decreased at approximately 21 d after insemination, but if a cow is pregnant her progesterone concentrations will remain elevated (Lucy et al., 2011). This method is more accurate if performed between 21 to 24 d post-artificial insemination to determine if a cow is truly non-pregnant (Mortimer and Hansen, 2006). Progesterone testing has a high negative predictive value (concentrations below 1 ng/mL), which is why it is accurate in determining if a cow is not pregnant (Booth et al., 1979; Lucy et al., 2011), but it has a low positive predictive value, so if progesterone values are high, she may or may not be pregnant (Lucy et al., 2011). The reason for a false positive predictive value for a progesterone chemical test could be due to an embryonic loss between d 18 and 24 or the female was never synchronized for artificial insemination (AI) properly (Lucy et al., 2011).

Measuring progesterone concentrations can be performed by utilizing an ELISA or RIA assay, although an ELISA assay does not require expensive equipment. It also does not require the knowledge that is required for transrectal ultrasonography or palpation and requires less time than detecting estrus (Nebel, 1988). In 1965 when Gomes and Erb (1965) worked on this method it was hindered by the lack of chemical

techniques adequately sensitive and specific enough to aid in the quantitation of progesterone in small plasma samples. Throughout the decades, scientists have created methods with a greater sensitivity without losing reliability of progesterone (Gomes and Erb, 1965). Today, progesterone chemical tests use methods to measure small quantities of progesterone and are still able to attain high sensitivity and specificity (Spieler et al., 1972). Progesterone rapid visual tests are a good indication of non-pregnant cows during early gestation, but past d 24 there are more accurate methods available.

Pregnancy-associated glycoproteins (PAGs)

Pregnancy-associated glycoproteins are a part of the large family of aspartic peptidases (Piechotta et al., 2011). Some members of the aspartic peptidase family include lysosomal enzymes, cathepsin D and rennin and also digestive enzymes such as pepsin and gastricsin, which all help to maintain salt homeostasis and blood pressure within animals (Davies, 1990; Telugu et al., 2009). According to Butler et al. (1982), the first pregnancy protein discovered in cattle was pregnancy-specific protein-B (PSPB) in 1982. The work performed by Sasser et al. (1986) was the first to reveal a simple, yet precise and accurate serological test to determine pregnancy in cattle. Later PSPB and PAG were classified under a new name together known as bovine pregnancy-associated glycoprotein-1 (boPAG-1; Green et al., 2005; Silva et al., 2007). Although, PAGs have been studied extensively in cattle, there are still many things we do not know about them aside from their use for pregnancy detection in blood or milk. Among bovine, there are 22 transcribed genes within the PAG gene family along with other variants (Telugu et al., 2009). There are two main groupings of PAGs, ancient and modern PAGs (Telugu et al.,

2009). Ancient PAGs are those that are transcribed in all cotyledonary trophoblast cell types, predicted to be active enzymes, and possess the activity of typical aspartic proteinases (Telugu et al., 2009). Modern PAGs, are members that are constructed only by a particular type of trophoblast cell called binucleate giant cells (BNGC; Green et al., 2000; Telugu et al., 2009) in the ruminant placenta (Eckblad, 1985; Piechotta et al., 2011). Once the giant trophoblastic cells migrate from the trophectoderm to fuse with the maternal epithelial cells, their granular contents are released into the maternal circulation (Wooding and Wathes, 1980; Piechotta et al., 2011; Figure 1.1). At this time, determination of pregnancy status of the female can be verified by PAG concentrations. Pregnancy-associated glycoproteins enter into maternal circulation around d 22 to 24, but the earliest and most reliable pregnancy diagnosis can be made on d 28, and the production of PAGs continue throughout gestation (Pohler et al., 2016).

The first radioimmunoassay to provide a serological method of pregnancy detection in bovine was created in 1986 measuring PSPB (Sasser et al., 1986). Since the development of the original PAG test, other commercial pregnancy tests have been created. There are four different tests currently on the market which include, 1) BioPRYN (BioTracking LLC.), 2) DG29 Pregnancy Test (Genex Cooperative Inc.), 3) IDEXX Alertys Ruminant Pregnancy Test, and 4) IDEXX Rapid Visual Pregnancy Test (IDEXX Laboratories Inc.). These commercial PAG tests are 95 to 99 percent accurate for true positive readings with only a 1 to 5 percent false positive rate compared to transrectal ultrasonography (Pohler et al., 2016). A PAG blood test costs about 3 to 5 dollars per female and takes up to 4 d (depending on location and if nearby vet clinic services PAG testing) to get results back from the lab (excluding rapid visual, which only

takes 30 minutes) once they are sent off (Pohler et al., 2016). Currently these tests only determine whether a female is pregnant or not pregnant. An advantage of this method is it does not have the physical demands on the body that transrectal palpation or transrectal ultrasonography does.

Caution does have to be used when using a PAG blood test due to the long half-life of PAGs in the blood postpartum, which ranges from 80 to 100 d (Green et al., 2005; Northrop et al., 2019). If the pregnancy blood test is performed within 80 to 100 d after calving, the producer should be aware that the results may give a false positive, so if possible, testing outside of that 100-d window is preferred.

Transrectal Ultrasonography

Transrectal ultrasonography is considered the gold standard for pregnancy detection. Unlike palpation, transrectal ultrasonography allows the user to see what they would be feeling inside the animal instead of just making a judgement call based off of feel. According to Ginther (1986), transrectal ultrasonography has been a revolutionary research tool in the field of reproductive biology. Ultrasonography allows for real-time visualization of internal structures that are otherwise difficult to evaluate. High-frequency soundwaves, produced by vibrations of piezoelectric crystals in the ultrasound transducer, are utilized in order to produce images of tissues and internal organs (Griffin and Ginther, 1992). Fluids do not reflect sound waves, so images containing follicles, embryonic vesicles, and the bladder are seen as black on the screen. Dense tissues, such as bone appear to be a white or light gray color on the screen, because they are the most

echogenic. Soft tissues and parts of the reproductive tract appear on the screen as shades of gray. It takes a skilled eye to determine what structures are being visualized.

The first reports of transrectal ultrasonography used in bovine appeared in 1984, and between years 1985 to 1990 the first reproductive biology manuscripts using transrectal ultrasonography appeared in the *Journal of Animal Science* (Griffin and Ginther, 1992). This technology has extensively increased our knowledge in both the research and production world. Transrectal ultrasonography aids in the selection of animals to be kept in the breeding herd, increasing the likelihood of reproductive success, by identifying cyclicity status of animals through observation of dominant follicles and/or corpora lutea (Perry and Cushman, 2016). Not only does this technology determine whether or not a female is pregnant, but it can also determine fetal age, sex, number of fetuses, and if they are still alive, via visualization of a fetal heartbeat, throughout pregnancy (Griffin and Ginther, 1992; Perry and Cushman, 2016). According to Perry and Cushman (2016), one of the biggest benefits to ultrasonography over other techniques is the capability to determine embryo viability and the status of the developing fetus. Accurate determination of pregnancy status can be made as early as d 26 post-insemination; however, there is a high incidence of pregnancy loss among early pregnancies (Kastelic et al., 1991; Perry and Cushman, 2016), thus it is important to confirm pregnancy is maintained later in gestation.

The downfall to transrectal ultrasonography is the wear and tear on the technician's body and it requires technical skills to make an accurate conclusion. Ultrasound equipment also costs within the range of $7,000 to $14,000 (Gunn and Hall, 2018). The reliability of using a transrectal ultrasound depends on the ability of the

operator to be able to recognize structures and organs shown on the screen (Zemjanis, 1962). It also requires expensive equipment, acute observational skills, and proper utilization of the ultrasound. Transrectal ultrasonography can be more time consuming to ultrasound than a blood sample, but results are in real time. This technology allows producers to increase their scope of knowledge and make better management decisions. Transrectal ultrasonography will not be going anywhere in the foreseeable future, as it will continue to have a large role in the success of reproductive management within cattle herds (Perry and Cushman, 2016).

CONCLUSIONS

Early pregnancy detection methods (palpation, ISGs, progesterone, PAGs, and transrectal ultrasonography) provide accurate methods for the detection of pregnancy and aid the study of late embryonic/early fetal mortality in cattle (Santos et al., 2009). The majority of embryo mortality occurs before d 16 of gestation (Diskin and Morris, 2008), but late embryonic losses in cattle, although not as numerous, have a greater economic impact on producers, because it often is too late to rebreed those females (Diskin and Morris, 2008). A pregnancy detection method should be used that can detect pregnancy status as early as possible and also, be used later in gestation to ensure the pregnancy is still being maintained. Wiltbank et al. (2016), suggested that there are two primary goals during early pregnancy: first, to initiate, establish and preserve a healthy pregnancy; and second, when the first does not work, reinitiate the procedure to allow for another chance for pregnancy at the earliest time possible. The subsequent chapters will contribute to

existing research and advance the possibilities of recently developed commercial pregnancy detection methods via pregnancy-associated glycoproteins.

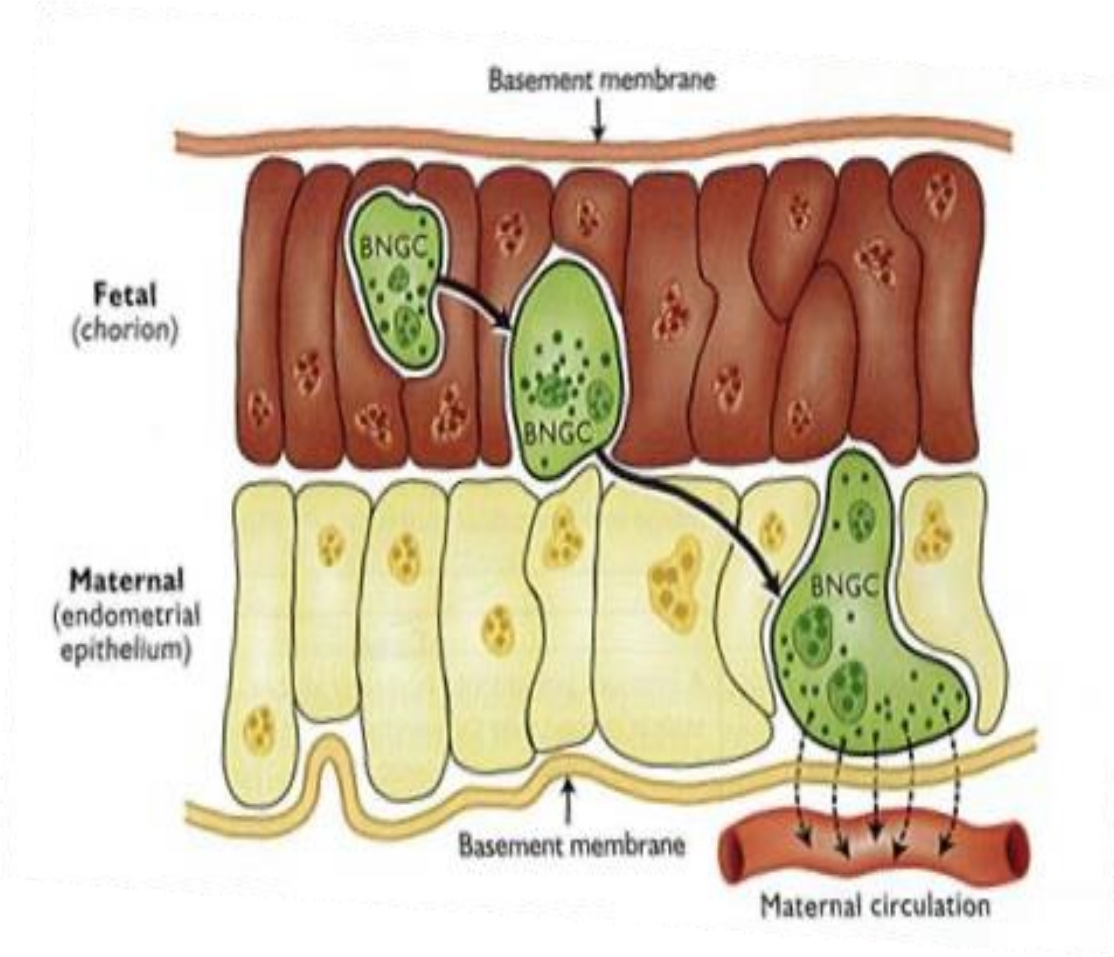

Figure 1.1. Binucleate giant cell (BNGC) migration. Reprinted from Pathways to Pregnancy and Parturition, 3rd Ed. from Current Conceptions, Inc. Senger (2003).

CHAPTER 2

USE OF PREGNANCY-ASSOCIATED GLYCOPROTEINS TO DETERMINE FETAL AGE THROUGHOUT GESTATION AND CLEARANCE RATE IN POSTPARTUM BEEF CATTLE

ABSTRACT

Blood pregnancy-associated glycoproteins (PAGs) pregnancy tests are gaining in popularity due to their ease of use and the unique feature of not requiring equipment or special training. A downside to blood pregnancy tests is they only provide an answer as to whether an animal is pregnant or nonpregnant. In addition, PAGs have a long half-life (80 to 100 d) in the maternal blood supply. Therefore, the objectives of these studies were to determine whether a commercially available blood pregnancy test could be modified to detect differences in PAG concentrations to determine stage of pregnancy in cattle and to determine factors that impact clearance of PAGs in postpartum beef females. Previously identified pregnant females from six different herds and postpartum females from one herd were utilized. Blood samples were collected (n = 1,753; study 1) between d 28 and 285 of gestation and (primiparous n = 418 and multiparous n = 657; study 2) once a week for up to 12 weeks after calving. Serum was tested in duplicate using a commercially available blood pregnancy test, IDEXX Alertys Pregnancy Test. In study 1, procedures were adapted to allow PAG concentrations to fall within the detectible range of the assay. Data were analyzed using the MIXED procedure of SAS with parity and gestational age (also divided into four gestational age groups: 1) < 30 d; 2) 30-90 d; 3) 91-180 d; 4) > 180 d) in the model and then analyzed further using the REG procedure in SAS within gestational age group. In study 2, data were analyzed as repeated measure using the

MIXED procedure of SAS with parity, days postpartum (dpp), and parity by dpp in the model, then data was analyzed further using the REG procedure in SAS. In study 1, there was a significant effect of gestational age and a parity by gestational age interaction ($P < 0.01$) as well as a tendency of parity ($P = 0.08$). Among nulliparous and multiparous females, serum PAG concentrations did not differ between gestational age groups 1 and 2 ($P > 0.84$), however, PAG concentrations differed between groups 2 and 3 ($P < 0.0001$) and 3 and 4 ($P < 0.0001$). There was a positive correlation between gestational age and PAG concentrations ($P < 0.01$; $r^2 = 0.2604$). In study 2, there was a significant effect of dpp ($P < 0.01$) on PAG concentrations; however, PAG concentrations were not influenced by parity ($P = 0.73$) or parity by dpp ($P = 0.55$). Concentrations of PAGs rapidly decreased from d 0 to 50 postpartum and then continued to gradually decrease ($P < 0.01$; $r^2 = 0.8083$). Prior to 42 dpp, PAG concentrations were sufficiently elevated which resulted in false positive readings. In conclusion, PAG concentrations using this modified pregnancy test would not make a reliable marker for gestational age due to high variability. Additionally, residual PAGs decreased to undetectable concentrations after 42 dpp in most females; thus, minimizing the likelihood of false positives.

INTRODUCTION

In order to be more profitable and have a complete and successful reproductive management program, pregnancy diagnosis within an operation is not only important, but necessary (Oltenacu et al., 1990). Fertilization occurs greater than 90% of the time following insemination of beef cows that have been detected in estrus, but calving rates to a single insemination are usually only about 55% (Diskin and Sreenan, 1980), so

without an accurate pregnancy detection method an operation will miss out on both monetary and management decisions. Transrectal ultrasonography is considered the gold-standard for pregnancy detection, but it is costly and requires a skilled technician (Perry and Cushman, 2016). An alternative method to determine pregnancy is with blood tests utilizing pregnancy-associated glycoproteins (PAGs; Sasser et al., 1986; Humblot et al., 1988; Zoli et al., 1992; Mialon et al., 1993). These glycoproteins can be detected in the maternal bloodstream as early day 22 to 24 of gestation (Pohler et al., 2013), and are extremely accurate with a 95 to 99% true positive rate and a 1 to 5% false positive rate compared to transrectal ultrasonography (Pohler et al., 2016). Blood pregnancy tests are also increasing in popularity due to ease of use and the unique feature of not requiring costly equipment or special training.

Pregnancy-associated glycoproteins are synthesized by binucleate giant cells (BNGCs) of the trophectoderm in the ruminant placenta (Wooding, 1992). Binucleate giant cells then migrate to fuse with maternal uterine epithelial cells where the granular content within the BNGC is released into the maternal circulation. Once the granular content is released in the maternal circulation, PAGs can be measured in either milk or blood samples to determine pregnancy status (Sousa et al., 2006; Ricci et al., 2015).

A downside to blood pregnancy tests is they do not provide any additional results such as fetal age. Pregnancy-associated glycoproteins do steadily increase throughout gestation, are elevated at time of parturition, and then decrease after parturition (Sasser et al., 1986; Zoli et al., 1992; Green et al., 2005). They also have a long-half life in the blood of postpartum females which ranges from 80 to 100 d before they become undetectable in the maternal blood supply (Zoli et al., 1992; Kiracofe et al., 1993).

Because PAGs peak at parturition and have a long half-life, residual concentrations can still exist in both primiparous and multiparous animals at the start of the subsequent breeding season. Thus, when trying to use PAG concentrations as a marker for early pregnancy diagnosis these residual concentrations can impact the results. Therefore, the objectives of these studies were to 1) determine if a commercially available blood pregnancy test could be modified to detect differences in PAG concentrations to indicate stage of pregnancy or fetal age and 2) determine factors that impact clearance of PAGs in postpartum beef females.

MATERIALS AND METHODS

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

Experimental Design

In study 1, blood samples (n = 1,753) were collected from *Bos taurus*, Angus and Angus-cross, beef females previously identified as pregnant via transrectal ultrasonography from four herds in South Dakota.

In study 2, Angus and Angus-cross postpartum females (n = 114; primiparous n = 48 and multiparous n = 66) from one of the previously mentioned herds were utilized. Animals were managed similarly in two groups; however, females were in separate pastures based on sex of calf. Blood samples were collected once a week for up to 12

weeks post calving (range of first and last sample was 1-7 to 84-91 days postpartum; dpp).

Blood Sampling

In study 1, blood samples were only collected in females identified as pregnant by transrectal ultrasonography. In study 2, blood samples were only collected in females who had calved. Blood samples were collected by venipuncture of either the tail or jugular vein into 10-mL Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and stored at room temperature (20 ℃) for approximately two hours until centrifuged. Samples were centrifuged at 2,000 x g for 30 min, serum was collected and stored at -20 ℃ until PAG assays were conducted.

Bovine Pregnancy Tests

Each serum sample was examined in duplicate using a commercially available blood pregnancy test, IDEXX Alertys Ruminant Pregnancy Test (IDEXX, Westbrook, ME).

In study 1, since all females were pregnant, PAG concentrations surpassed the maximum linear threshold of the IDEXX Alertys Ruminant Pregnancy Test assay. Thus, procedures were adapted to allow PAG concentrations to fall within the linear detectable range of the assay. All reagents were brought to room temperature. Sample diluent (25 mL) was dispensed into every well that a sample would be added to. Samples (8 μL) were then pipetted in duplicate into the appropriate wells. Plates were gently tapped on the counter to mix and then tightly covered and incubated for 60 min at 37 ℃. Post

incubation, solution was removed, and each well was washed 3 times with 300 µL of Wash Solution using a BioTek ELx50 plate washer. Plates were not allowed to dry out between washing or prior to the addition of the next reagent. Absorbent material was used after the final wash to remove any residual wash fluid. Detector Solution (100 µL) was added to each well, plates were covered, and incubated for 30 min at 37 ℃. Plates were washed as previously described, and then 100 µL of Conjugate Solution was added to each well. Plates were again covered and incubated for 30 mins at 37 ℃. Plates were washed as previously described, 100 µL of tetramethylbenzidine (TMB) substrate was added to each well, and plates were covered and incubated for 15 min at 37 ℃. After incubation Stop Solution (100 µL) was pipetted into each well. Optical density was measured at Absorbance of 450 nm and 650 nm using a Molecular Devices SpectraMax 190 microtiter plate reader (San Jose, California) and recorded. In study 2, all samples were analyzed according to the manufacturer's instructions with 100 µL of sample used.

Statistical Analysis

In study 1, blood samples were grouped by parity (nulliparous and multiparous) and were also grouped into 4 gestational age groups (group 1: < 30 d, group 2: 30- 90 d, group 3: 91- 180 d, and group 4: >180 d) to further statistically analyze the data. Groups were established where a natural break in gestational age occurred. Serum PAG concentrations were analyzed using the MIXED procedure of SAS with parity and gestational age in the model. Correlations between PAG concentrations and gestational age were analyzed using the REG procedure in SAS.

In study 2, samples were grouped by week of collection and PAG concentrations were analyzed as a repeated measure using the MIXED procedure of SAS (9.4) with parity, days postpartum (dpp), and parity by dpp in the model. Since there was no effect ($P > 0.10$) of parity, effect of dpp on PAG concentrations were analyzed using the REG procedure in SAS. Statistical significance was determined at a $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.10$ for analysis.

RESULTS

Study 1

There was a significant impact of gestational age on PAG concentrations among pregnant females. Pregnancy-associated glycoproteins had a linear increase in both nulliparous and multiparous animals as gestational age increased ($P < 0.01$); however, there was a weak correlation ($r^2 = 0.3009$; Figure 2.1). There was an interaction between parity and gestational day ($P < 0.05$), so blood samples were divided into gestational age groups by trimester to further analyze the data. Concentrations of PAGs had a linear increase in both nulliparous and multiparous animals as gestational age group increased ($P < 0.01$); however, there was a weak correlation ($r^2 = 0.2604$) to gestational age (Figure 2.2). Among animals, there was no difference in PAG concentrations between groups 1 to 2 ($P = 0.84$), but there was an increase between groups 2 to 3 ($P < 0.0001$) and then a further increase between groups 3 to 4 ($P < 0.0001$; Figure 2.3.A). A comparison of age by group was made between nulliparous and multiparous females (Figure 2.3.B). Among nulliparous females there was a linear increase from group 1 to 2, group 2 to 3, and then further from group 3 to 4 ($P < 0.0001$; Figure 2.3.B). Among multiparous females there

was no difference between group 1 and 3, but there was a decrease, between group 1 to 2, then an increase from group 2 to 3, and a further increase from 3 to 4 ($P < 0.0001$; Figure 2.3.B). Between nulliparous and multiparous females there was a tendency between group 1 ($P = 0.06$) and a difference between groups 2, 3, and 4 ($P < 0.0001$; Figure 2.3.B).

Study 2

There were no differences detected in PAG concentrations between primiparous and multiparous females (parity; $P = 0.73$) and parity by dpp ($P = 0.55$). There was, however, a significant effect of dpp on PAG concentrations in postpartum females ($P < 0.01$). There was a linear decrease in PAG concentration from day 0 (calving) to approximately day 40 postpartum; as dpp increased, PAG concentrations decreased before plateauing from approximately 40 to 100 dpp (Figure 2.4; $P < 0.01$). Days postpartum accounted for 67.08% of the variation in PAG concentrations. Females were further separated into 7-d interval groups based on dpp for further analysis; dpp were grouped in blocks of 7 days from 0 to 84 dpp (Figure 2.5). There was a linear decrease from 0 to 50 dpp and then PAG concentrations plateaued from 50 to 84 dpp (Figure 2.5). There was again a strong correlation between dpp group and PAG concentrations, accounting for 67.48% of the variance (Figure 2.5). Since PAGs plateaued from 50 dpp onward further statistical analysis was performed to determine the clearance from 0 (calving) to 50 dpp (Figure 2.6). Analysis of females when separated into 7-d interval groups was also made from 0 to 50 dpp (Figure 2.7). Elimination of the samples when they begin to plateau allowed for a stronger correlation between PAG concentrations and

dpp, accounting for 81.73% and 80.83% of the variance (Figure 2.6 and 2.7, respectively; $P < 0.01$). When determining average PAG concentrations of samples broken down into 7 d intervals through day 84, clearance occurred by 42 dpp (od = 0.26 ± 0.036; Figure 2.8.A). Animals are considered pregnant when optical density (od) were ≥ 0.3. There was no significant difference between multiparous and primiparous females determining the clearance of PAGs with the IDEXX Alertys Ruminant Pregnancy Test ($P = 0.55$).

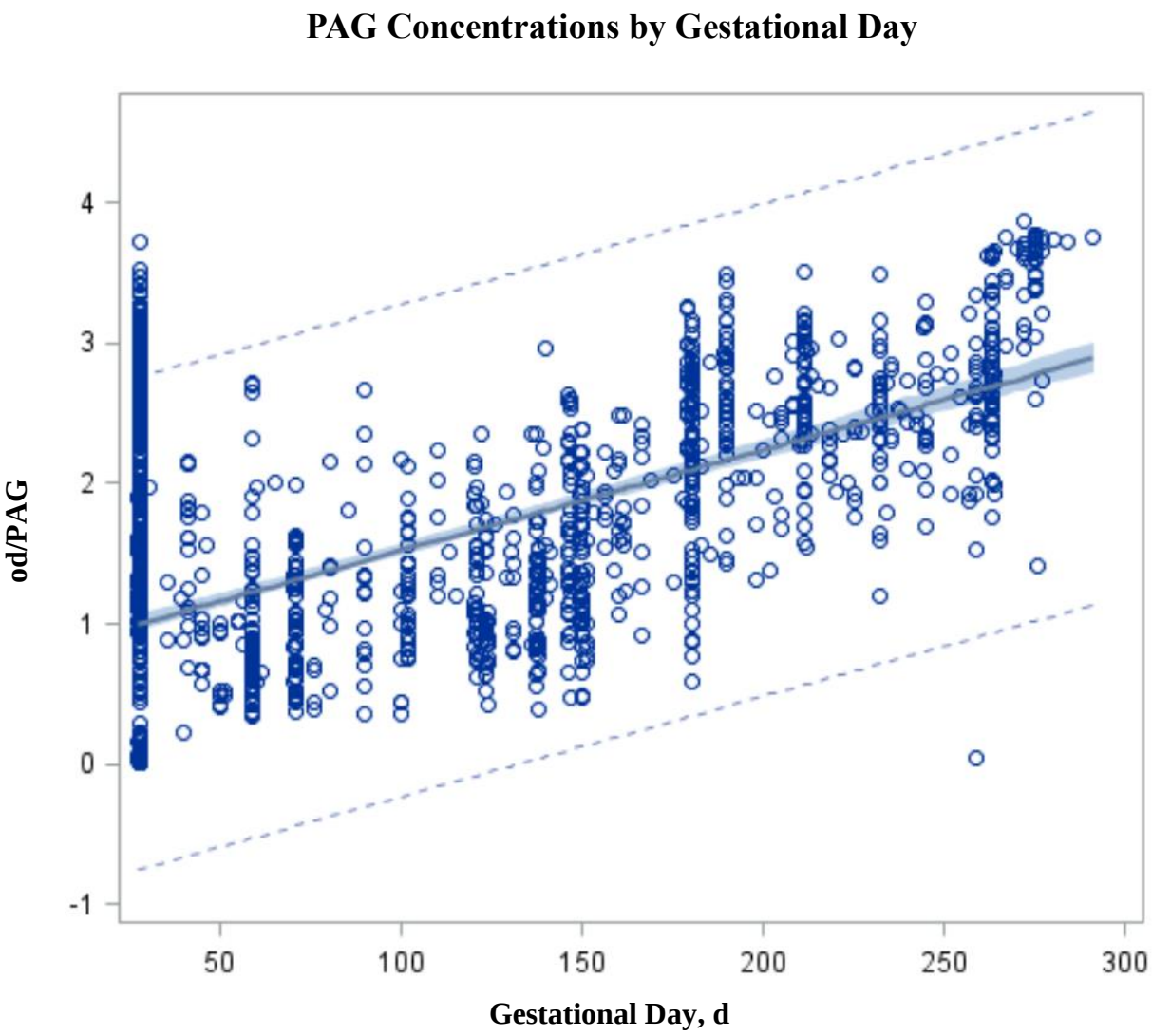

Figure 2.1. Regression analysis of gestational age on circulating concentrations of pregnancy-associated glycoproteins (PAG) in nulliparous and multiparous animals. Each circle indicates an individual sample. The solid line is the calculated regression line, the blue shaded area is the 95% confidence interval, and the dashed line is the 95% prediction limit. Gestational age 30 and greater ($P < 0.01$; $r^2 = 0.3009$).

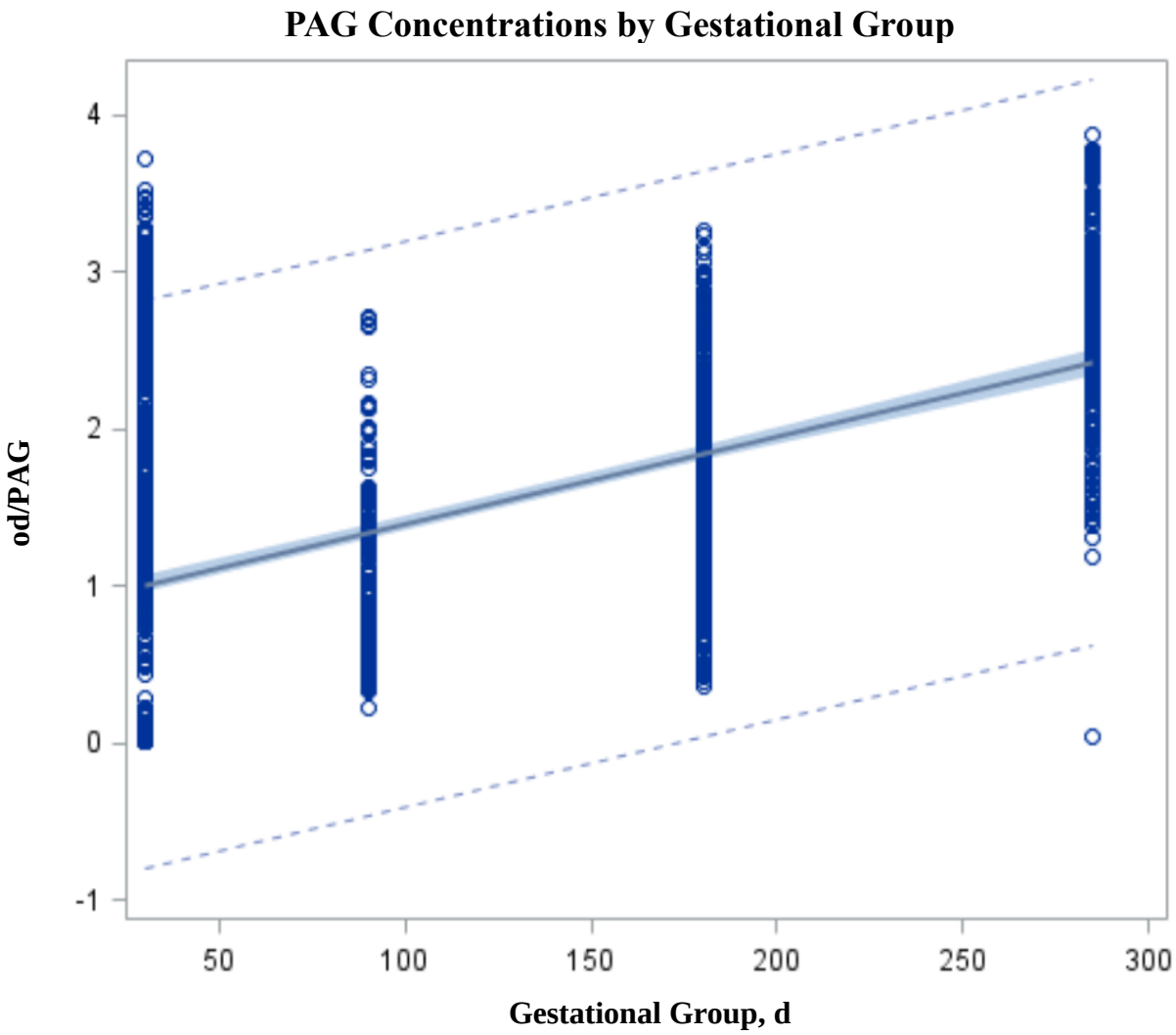

Figure 2.2. Increase of pregnancy-associated glycoproteins (PAG) concentrations among four different gestational groups (1) < 30 d; 2) 30-90 d; 3) 91-180 d; 4) > 180 d) in nulliparous and multiparous animals. Regression analysis of gestational age group on circulating concentrations of PAG. Each circle indicates an individual sample. The solid line is the calculated regression line, the blue shaded area is the 95% confidence interval, and the dashed line is the 95% prediction limit ($P < 0.01$; $r^2 = 0.2604$).

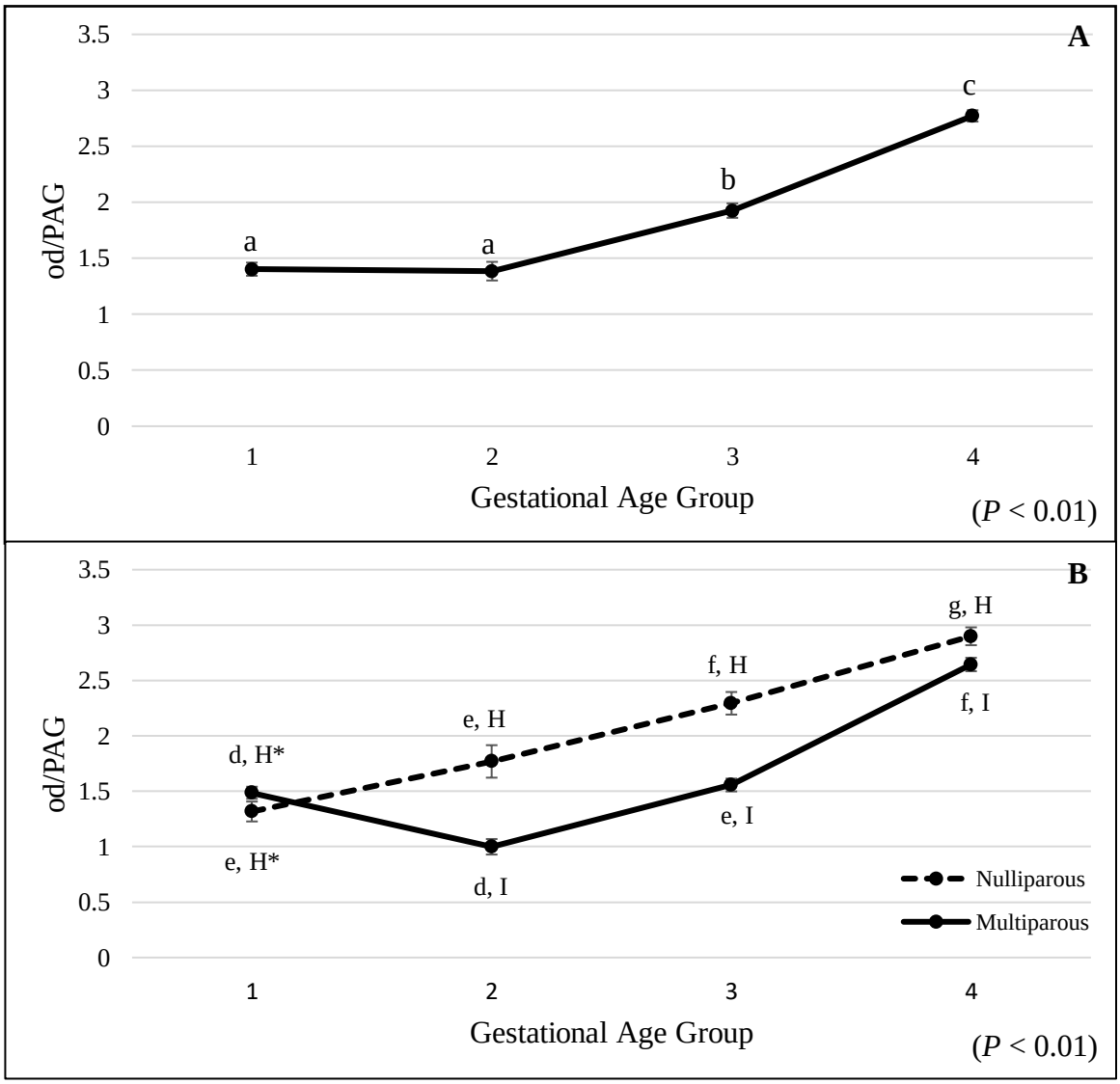

Figure 2.3. Effect of Group (A) and Parity by Group (B) Interaction on PAG Concentrations. Mean (± SEM) serum pregnancy-associated glycoprotein (PAG) among four different gestational groups (1) < 30 d; 2) 30-90 d; 3) 91-180 d; 4) >180 d) of nulliparous and multiparous females in figure A. Figure B, age represents nulliparous (dashed line) and multiparous (solid line). Different superscripts a-c not sharing the same superscripts differ ($P < 0.01$). Different superscripts d-g differ between groups within age not sharing the same superscript ($P \leq 0.01$). Different superscripts H, I represent values between age within group not sharing the same superscript differ ($P \leq 0.01$). Superscript * represents values tended to differ between gestational group ($P = 0.06$).

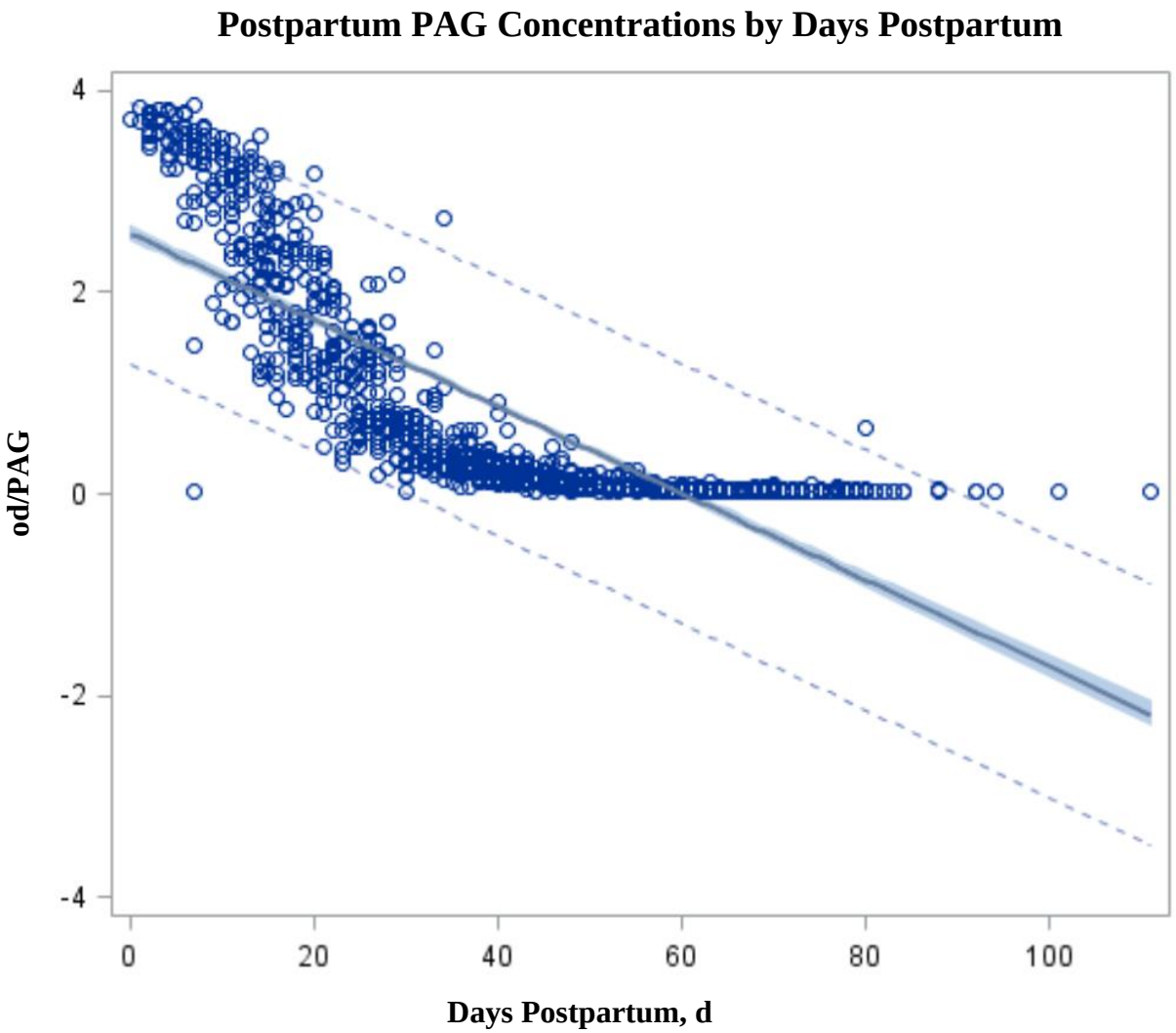

Figure 2.4. Regression analysis of days postpartum (dpp) on circulating concentrations of pregnancy-associated glycoproteins (PAG) in postpartum primiparous and multiparous beef cattle. Each circle indicates an individual sample. The solid line is the calculated regression line, the blue shaded area is the 95% confidence interval, and the dashed line is the 95% prediction limit ($P < 0.01$; $r^2 = 0.6708$).

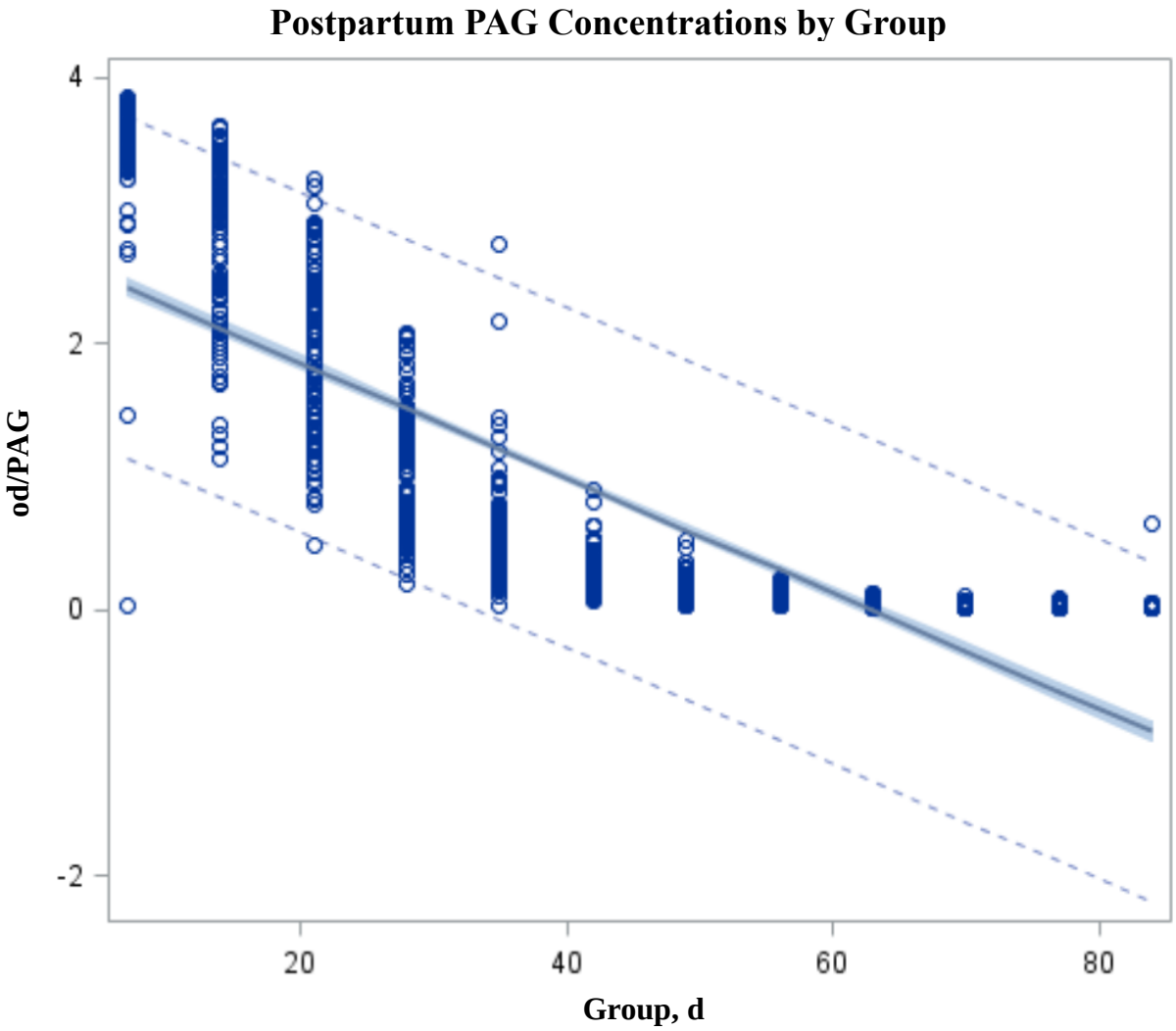

Figure 2.5. Regression analysis of group (7-day intervals) on circulating concentrations of pregnancy-associated glycoproteins (PAG) in postpartum primiparous and multiparous beef cattle. Each circle indicates an individual sample. The solid line is the calculated regression line, the blue shaded area is the 95% confidence interval, and the dashed line is the 95% prediction limit ($P < 0.01$; $r^2 = 0.6748$).

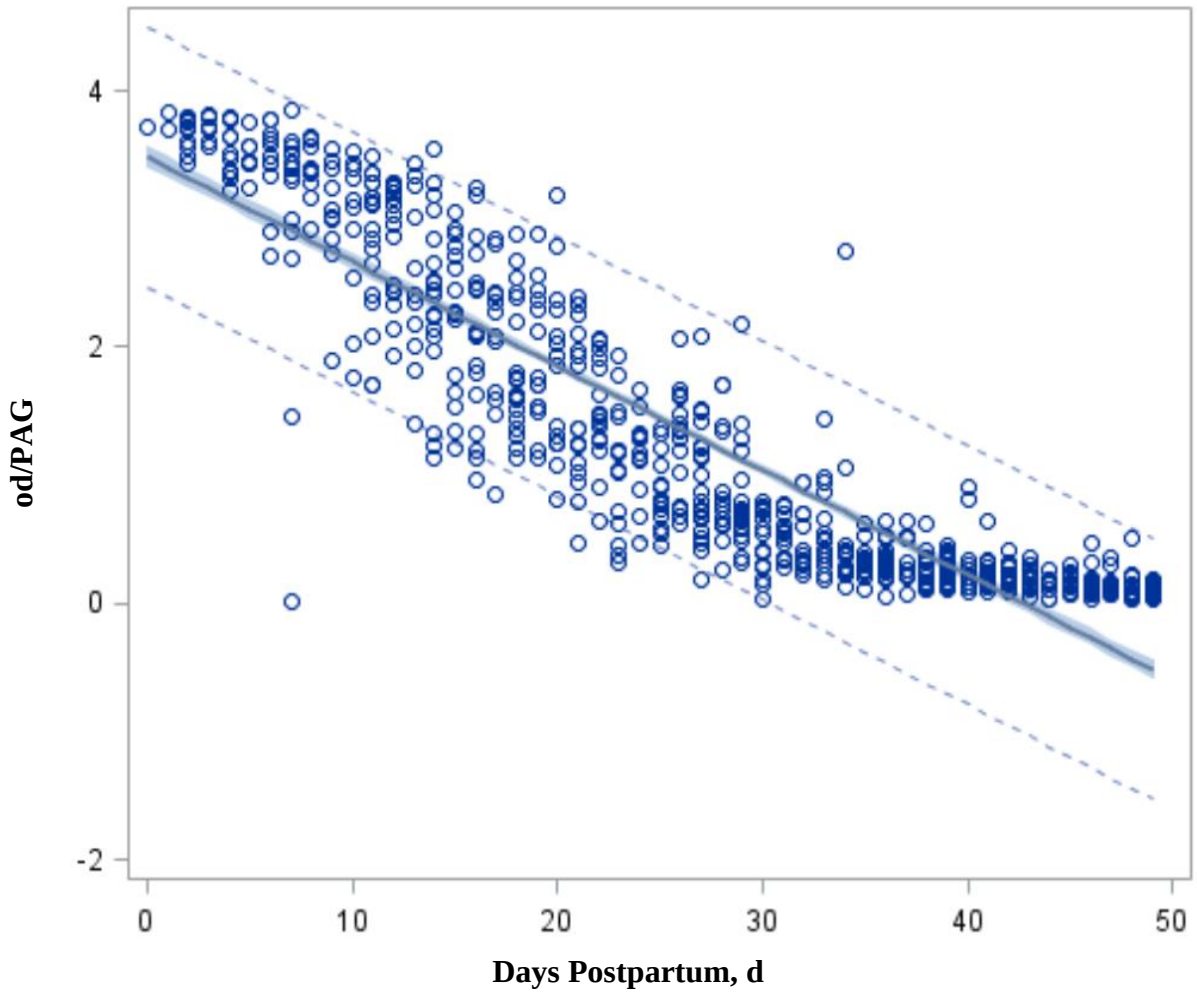

Figure 2.6. Regression analysis of days postpartum (dpp) on circulating concentrations of pregnancy-associated glycoproteins (PAG) in postpartum primiparous and multiparous beef cattle through d 50. Each circle indicates an individual sample. The solid line is the calculated regression line, the blue shaded area is the 95% confidence interval, and the dashed line is the 95% prediction limit ($P < 0.01$; $r^2 = 0.8173$).

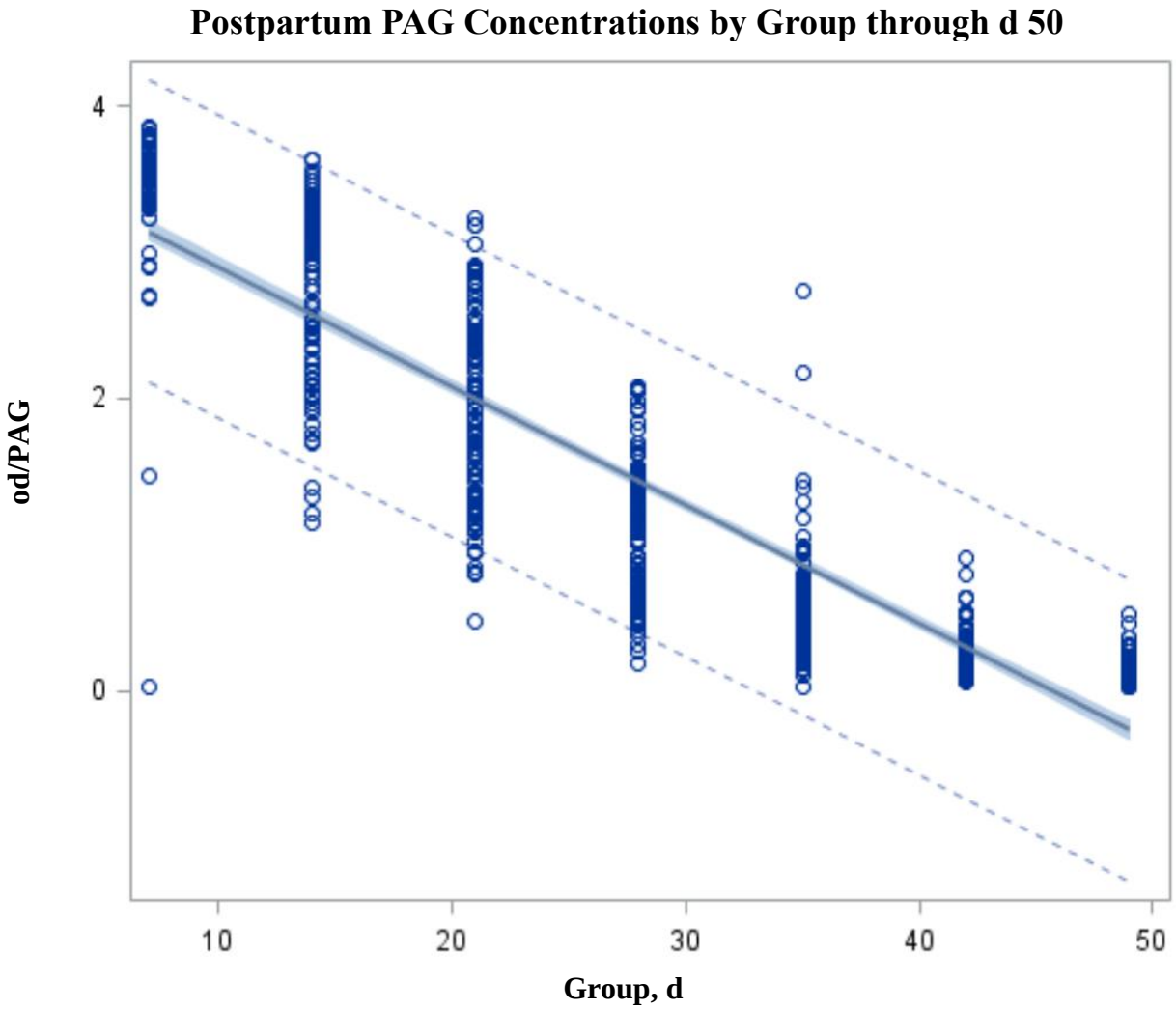

Figure 2.7. Regression analysis of group (7-day intervals) on circulating concentrations of pregnancy-associated glycoproteins (PAG) in postpartum primiparous and multiparous beef cattle through d 50. Each circle indicates an individual sample. The solid line is the calculated regression line, the blue shaded area is the 95% confidence interval, and the dashed line is the 95% prediction limit ($P < 0.01$; $r^2 = 0.8083$).

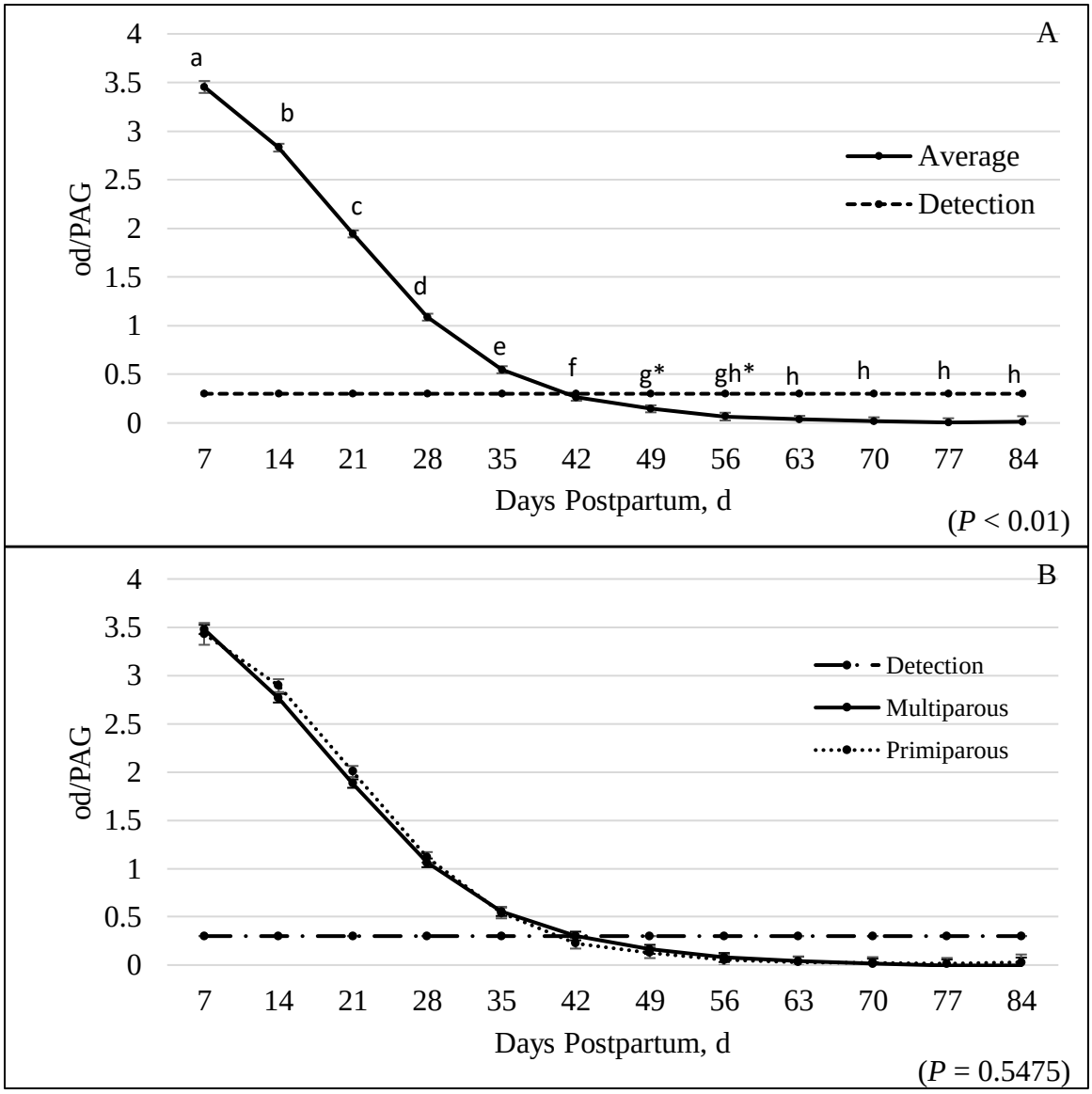

Figure 2.8. Overall clearance of PAG concentrations in postpartum beef cattle (A) and overall comparison between parity (B). Mean (± SEM) serum pregnancy-associated glycoprotein (PAG) concentration levels among postpartum beef females (A). PAG concentration levels fall below the undetectable range by 42 dpp (optical density (od) = 0.2636; A). Different superscripts a-h not sharing the same superscripts differ ($P < 0.01$; A). Superscript * represents values tended to differ ($P = 0.08$; A). In multiparous and primiparous beef females there is no significant difference found postpartum ($P = 0.55$; B).

DISCUSSION

To be enticing for producers to implement blood pregnancy testing into their program, the method should accurately identify both pregnant and non-pregnant females (Romano and Larson, 2010). Pregnancy-associated glycoprotein blood pregnancy tests provide an alternative to transrectal ultrasonography for determining whether females in the herd are pregnant or non-pregnant. Determining other possible capabilities of this test would make it more competitive compared to a transrectal ultrasonography.

Study 1

The present study agreed with findings from Zoli et al. (1992) who reported an increase of PAG concentrations with increasing gestational age, but concentrations also tended ($P = 0.08$) to be different due to parity. This makes defining a cutoff for different gestational ages difficult. When data was analyzed age by group nulliparous PAG concentrations were greater than multiparous females. Also, nulliparous females had a positive linear increase, while multiparous females decreased from group 1 to 2 and increased from group 2 to 3 and then further increased from group 3 to 4. We hypothesized nulliparous females have greater PAG concentrations than multiparous females due to decreased body weight, meaning PAGs are not diluted as much in nulliparous females. Also, nulliparous females are not lactating, while multiparous females are lactating and peak lactation occurs during gestational group 2, which could also cause a greater dilution of PAGs in multiparous females. Lobago et al. (2009) found nulliparous females had greater PAG concentrations than multiparous females (19.9 ± 1.5 ng/mL and 14.0 ± 1.3 ng/mL, respectively) and females having smaller average body

weights (< 275 kg) had greater PAG concentrations than females with larger average body weights (> 315 kg; 19.2 ± 1.3 and 11.2 ± 1.5, respectively). Nulliparous females have a tendency to have smaller body weights than multiparous females since they have not reached their mature body weight yet (Short et al., 1994), thus the study by Lobago et al. (2009) supports the hypothesis that nulliparous females have greater PAG concentrations than multiparous females due to body weight.

Sasser et al. (1986) discovered if pregnancy specific protein B (specific type of PAG) values are below 5 ng/mL in the serum then it would suggest that the fetal age would be less than 80 days, but if it is above 10 ng/mL the fetus would be greater than 80 days. Pregnant females were grouped into gestational groups by trimester in order to determine PAG concentration levels for specific gestational age groups (30, 90, 180, 285 dpp). Unlike Sasser et al. (1986), this study could not determine a specific value to distinguish a fetal age. There was a weak correlation found between day and group (r^2 = 0.3009 and 0.2604, respectively), indicating that factors other than gestational age had a bigger effect on concentrations, thus making this modified test difficult to use PAG concentrations to determine gestational age. Thus, the use of the IDEXX Alertys Ruminant Pregnancy Test, RPT, is not effective at determining fetal age. Transrectal ultrasonography remains the most optimal tool to determine age of the fetus, but with further modifications and research commercial blood pregnancy tests may be a comparable alternative.

Study 2

Commercial PAG tests have a 1 to 5% false positive rate compared to transrectal ultrasonography (Pohler et al., 2016). These false positives could be due to residual PAG concentrations from the previous pregnancy. Pregnancy-associated glycoproteins increased in female beef cattle throughout gestation (study 1), peak around time of parturition, and then decreased until they become undetectable. The cause for the peak of PAG concentrations around time of parturition is unknown (Sasser et al., 1986), but these elevated concentrations and the fact that PAGs have a long half-life in circulation (Zoli et al., 1992; Kiracofe et al., 1993) could lead to these false positive results when using PAGs as a pregnancy test.

Due to these residual concentrations of PAGs after parturition blood pregnancy tests need to have enough time elapsed between parturition and pregnancy test to accurately determine pregnancy status. Previous research suggests that PAG pregnancy blood tests should not be utilized until 91 to 120 dpp (Zoli et al., 1992; Mialon et al., 1993). Specifically Zoli et al. (1992) used boPAG67kDa, which is a PAG part of the boPAG-1 group that is detected in the maternal bloodstream on 28 through 100 dpp (Sousa et al., 2006). Others found that PAG pregnancy tests should not be performed until 70 to 90 dpp or later (Kiracofe et al., 1993; Szenci et al., 1998; Sousa et al., 2003). However, after 80 dpp, Kiracofe et al. (1993), found PAG concentrations were < 1 ng/mL, which indicates PAGs from the previous pregnancy would not produce a false positive result. The differences in these studies could be due to the different PAGs that were detected in each study. This study using a commercially available assay found that tests could be performed as early as 42 dpp without resulting in false positives.

Differences in the clearance of PAGs could be related to different breeds of cattle and/or the specific PAGs that were being detected. Pregnancy-associated glycoproteins can be expressed at different times throughout gestation, some are expressed earlier, while others are expressed when the pregnancy advances (Sousa et al., 2006). For example, Ruder and Sasser (1986), found that bovine pregnancy-specific protein B (bPSPB) remained in the circulation of cows for approximately 56 to 63 dpp. A study by Szenci et al. (1998), found that bPSPB and bPAG tests had 56.7% and 44.9%, respectively, false positives due to them being utilized too early (within 70 dpp). According to Ruder and Sasser (1986), bPSPB has a very slow disappearance rate postpartum; leading Kiracofe et al. (1993) to believe bPSPB in the maternal epithelium may be caused by a prolonged disappearance rate of migratory cells. The test used in this study, IDEXX Alertys Ruminant Pregnancy Test, uses polyclonal antibodies, which measures multiple PAGs throughout gestation. The antibody implemented in this assay is directed towards a collection of PAGs (e.g. PAGs 4, 6, 9, 16, 18, 19; described in US Patent no. 7,604,950B2; Ketchum, 2020). Specifically, PAGs 4, 9, and 6 are secreted from d 25 (PAG 4 and 9) and d 45 (PAG 6) through to d 250 of gestation (Green et al., 2000).

Different breed types may also have a role in the clearance of PAG. Mialon et al. (1993) found pregnancy serum protein of M_r60kDa (PSP60) in the maternal blood postpartum had different residual concentrations (105, 87, and 85 dpp) in Charolais, Holstein, and Normande breeds respectively. It was concluded by Mialon et al. (1993) Charolais tend to have elevated residual concentrations due to Charolais having calves with heavier birth weights (BW) than Normande and Holstein breeds, therefore causing

an extended clearance compared to the two other breeds. It has been reported that PAG concentrations were decreased at time of parturition in Azawak Zebu (1,095.6 ng/mL) compared to *Bos taurus* cattle (1,352.8 to 1,462.4 ng/mL), but Zebu have a longer clearance rate (14 weeks) than Taurine cattle (12 weeks; Kiracofe et al., 1993; Patel et al., 1997; Sousa et al., 2003). Regardless of varying clearance of specific PAGs developed from previous research, PAGs still follow the same pattern from this study of decreasing rapidly and then gradually decreasing until becoming undetectable. In a study performed on Azawak Zebu, *Bos indicus* cattle, it was found that two weeks post calving PAG concentration had decreased significantly, but then decreased slowly until week 10 where they reached their lowest concentrations, which is similar to what has been reported in *Bos taurus* females (Sousa et al., 2003). This study, using *Bos taurus* primiparous and multiparous females, had similar results with a rapid decrease from day 0 to 40 and then a slow decrease from day 40 to 100. Concentrations reached a level in which an animal would not be called pregnant around 42 dpp. It was hypothesized that primiparous animals would take longer to reach baseline concentrations since they began at greater concentrations, however, parity not influencing clearance rate agrees with Kiracofe et al. (1993).

Mialon et al. (1993) concluded that PAG tests should be utilized around day 27 post-artificial insemination (AI) and not before 91 to 105 dpp. Haugejorden et al. (2006) discovered that a PAG early pregnancy test could be performed by d 28 post-AI if animals were > 60 days post calving. The reason for waiting to breed a female post calving is because the following must occur first in order to gain greater conception rates: uterine involution, endometrium regeneration, regular ovarian cyclic activity and removal

of bacterial contamination (Short et al., 1990; Sheldon, 2004). Kiracofe (1980) suggested within 21 dpp a female is not fertile, by d 35 to 42 post-partum there is a chance of fertility. This is confirmed with animals bred between 0 and 30 dpp having conception rates of only 33%, but when animals were bred between 31 and 60 dpp there was an increase in conception rates to 58% (Casida et al., 1968; Wiltbank, 1970). Thus, it is unlikely to breed cattle earlier than 30 dpp, and it is recommended to wait until at least 30 dpp to allow for uterine involution to occur. If this recommendation is followed and pregnancy determination occurs at day 28 post-AI that would mean detection occurs at 58 dpp and any residual PAGs from the previous pregnancy would be below the pregnancy detection level.

In conclusion, PAG concentrations did increase with gestational age. However, with the variability in concentrations it is difficult to use, PAG as a marker for fetal age. Concentrations of PAGs decreased rapidly for the first 3 weeks after parturition and after 42 dpp concentrations fell below the concentrations for pregnancy detection. Thus, more confidence can be gained from the results received from a PAG blood pregnancy test when it is performed at least 42 days after parturition.

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CHAPTER 3

COMPARISON OF LATERAL FLOW TO OTHER PREGNANCY DETERMINATION METHODS IN ORDER TO DETERMINE ACCURACY OF PREGNANCY STATUS IN BEEF CATTLE PRE AND POSTPARTUM

ABSTRACT

Transrectal ultrasonography is known as the gold standard for pregnancy detection but requires costly equipment and technical skill. Pregnancy detection by use of pregnancy-associated glycoproteins (PAGs) has the ability to accurately determine pregnancy in ruminants, and recently a new lateral flow test (Alertys OnFarm Pregnancy Test) that does not require special equipment has become commercially available. The objective of this study was to compare the accuracy of multiple PAG tests to transrectal ultrasonography, and to determine how many days postpartum (dpp) is necessary for the clearance of PAGs from the previous pregnancy to avoid false positives when utilizing the lateral flow test. Blood samples were collected from six different *Bos taurus* herds between day 27 and 285 of gestation (nulliparous n = 1,205 and multiparous n = 1,539). Blood samples to determine PAG clearance interval were collected weekly postpartum for up to 12 weeks (primiparous n = 418 and multiparous n = 657). Serum was tested using the lateral flow test and were read by two technicians who were blind to pregnancy status. Level of agreement between the tests were determined by Pearson's correlation coefficients and Kappa scores. The MIXED procedure of SAS was used to evaluate the effect of dpp and parity (primiparous or multiparous) on postpartum test results. There was a positive correlation between transrectal ultrasonography and the lateral flow test ($r^2 = 0.71$; $P < 0.01$), and agreement between the two tests was good (Kappa = 0.84). Of

the animals that were diagnosed nonpregnant by transrectal ultrasonography, 5.61% were called pregnant by the lateral flow test. Of the animals diagnosed pregnant by transrectal ultrasonography, 2.00% were called not pregnant by the lateral flow test. Thus, a 92.38% agreement occurred between the two methods. For postpartum samples, there was no effect ($P = 0.21$) of parity, but there was an effect of dpp ($P < 0.01$) and a tendency for a dpp by parity interaction ($P = 0.06$). All animals were still considered pregnant from the previous pregnancy through 35 dpp (100 ± 2.58%). The percentage of females receiving a false positive test result further decreased with time postpartum, by 77 dpp there were 13.72 ± 3.16% of the females positive for pregnancy and at 84 dpp there were 4.11 ± 4.39% positive for pregnancy detection. In conclusion, there is very good agreement between transrectal ultrasonography and the lateral flow PAG test, but if the test is used at less than 40 dpp the likelihood of false positive result is extremely likely. Thus, the test should be utilized at least 42 dpp to prevent gaining false positive results.

INTRODUCTION

Within a beef operation, there are reproductive management strategies which can be implemented to increase the success of the breeding season: insemination of all cows towards the beginning of breeding season, detection of all nonpregnant cows as early as possible, and rebreed those nonpregnant females as soon as possible (Bó et al., 2016). In the United States, within large (≥ 200 cows) and small operations (≤ 50 cows) there are 21.9% and 69.6%, respectively, who do not use any type of reproductive technology, which include artificial insemination, breeding soundness exam, pregnancy detection, body condition scoring to name a few (USDA, 2017). Time and labor were the most cited reasons for not implementing reproductive technologies within an operation (USDA,

2017). In order to increase the percentage of operations that utilize a pregnancy detection method and improve in their reproductive management strategies, the method needs to be accurate and easy to use.

Annual cost (feed, fuel, and labor) to maintain a female ranges from $380 to $900 per head (USDA, 2009; Gray et al., 2012), so without early pregnancy detection monetary and management decision could be lost if particular females are not pregnant. Transrectal ultrasonography has become the gold standard for pregnancy detection, as it can accurately determine pregnancy status as early as d 26 post-insemination (Curran et al., 1986). There are however, high incidences of pregnancy losses that occur after d 25 of pregnancy (Kastelic et al., 1991; Perry and Cushman, 2016), so determination later in gestation is beneficial to confirm pregnancy is maintained. Even though transrectal ultrasonography can determine pregnancy early in gestation it requires specialized training, is physically demanding, and is costly, which can deter the use of this method (Kastelic et al., 1991; Perry and Cushman, 2016).

Determining presence of pregnancy-associated glycoproteins is another pregnancy detection method that has increased in popularity because it is easy to perform and is highly accurate (95 to 99%) with only a 1 to 5% false positive rate (Pohler et al., 2016) compared to transrectal ultrasonography. Pregnancy-associated glycoproteins increase in concentration throughout gestation, peak at parturition, and then decrease postpartum (Sasser et al., 1986; Zoli et al., 1992; Green et al., 2005). Previous studies (Zoli et al., 1992; Kiracofe et al., 1993) have reported that PAGs have a long half-life in circulation; thus, determining the clearance of PAGs is essential to increasing the accuracy of the test by decreasing false positive rate due to PAG concentrations from the

previous pregnancy. Previously, a downside to PAG tests have included the time and/or equipment needed to conduct the assay, which can impact the use or acceptance of this method. A new method of pregnancy determination using PAGs has become commercially available (IDEXX Alertys OnFarm Pregnancy Test), which is a chute side test with results within 20 minutes. Therefore, the objective of this study was to determine if the IDEXX Alertys OnFarm Pregnancy Test is comparable to other pregnancy detection methods in accuracy, and to determining when concentrations have cleared post calving to not result in false positives.

MATERIALS AND METHODS

All procedures were approved by the South Dakota State University Institutional Animal Care and Use Committee.

Experimental Design

Blood samples from six different *Bos taurus* herds in the state of South Dakota were utilized in this study. Pregnancy detection was performed by transrectal ultrasonography between 30- and 75-days post-insemination in all animals.

Blood Sampling

Blood samples [(nulliparous n = 1,205 and multiparous n = 1,539) and postpartum n = 1,066] were collected over a three-year period (2018, 2019, and 2020). Postpartum samples were collected from one group of animals (primiparous n = 48 and multiparous n

= 66) and were collected once a week for up to 12 weeks post calving (range of first and last sample was 1-7 to 84-91 days postpartum; dpp). All blood samples were collected from either the coccygeal or jugular vein into 10-mL Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and stored at room temperature (20 ℃) for approximately two hours until centrifuged. Samples were centrifuged at 2,000 x g for 30 min for serum collection and stored at -20 ℃ until testing.

Ultrasonography

All animals were evaluated for pregnancy by transrectal ultrasonography by a trained technician with an Ibex EVO ultrasound and linear probe on d 28 following their first insemination. Pregnancy diagnosis was based on the visualization of a fetus or absence of one. A final pregnancy diagnosis occurred between 31 and 80 d following the end of the breeding season to determine if embryonic loss occurred.

IDEXX Alertys OnFarm Pregnancy Test (lateral flow)

Samples were tested using commercially available blood pregnancy tests, IDEXX Alertys OnFarm Pregnancy Test (IDEXX, Westbrook, ME) according to the manufacturer's directions. Briefly, 100 μL of serum or plasma was pipetted into the well of the lateral flow test followed with 25 μL of chase buffer. After waiting 20 min the tests were read by the same two technicians that were blind to ultrasonography pregnancy status. Interpretation of IDEXX Alertys OnFarm Pregnancy Test with one line visible

indicates the female was not pregnant, while two visible lines means the female was pregnant at time of blood sample.

IDEXX Alertys Rapid Visual Pregnancy Test (RVPT)

Whole blood samples were collected on d 28 and tested using the commercially available blood pregnancy tests, IDEXX Alertys Rapid Visual Pregnancy Test (IDEXX, Westbrook, ME) according to the manufacturer's directions. Briefly, positive/negative controls, and whole blood samples were pipetted into coated plates, and plates were washed and treated with reagents according to the manufacturer's instructions. Visual evaluation of the plates based on a numerical scale, established by color intensity were made upon completion of the procedure by one technician. Color intensity evaluation and description was according by Northrop et al. (2019). The scoring system included a yes/no assignment and numerical value from 0 - 3 based on color in comparison to the controls.

IDEXX Alertys Ruminant Pregnancy test (RPT)

Samples were tested using the commercially available blood pregnancy tests, IDEXX Alertys Ruminant Pregnancy Test (IDEXX, Westbrook, ME) according to the manufacturer's directions. Briefly, controls and blood samples were pipetted in duplicate into wells of the coated plates, and plates were washed and treated with reagents according to the manufacturer's instructions. The results from the RPT were analyzed on a Molecular Devices SpectraMax 190 microtiter plate reader (San Jose, California).

Statistical Analysis

Samples were analyzed as a correlation coefficient using the CORR procedure of SAS (9.4) to assess the agreement between tests: ultrasound, RVPT, RPT and lateral flow in the model. Since all variables had a significant correlation between tests further analysis was made using the FREQ procedure in SAS evaluating the frequency between each one comparatively to each other. The Kappa scoring scale is as follows: 0.80 - 1.00 = Very good, 0.60 - 0.80 = Good, 0.40 - 0.60 = Moderate, 0.20 - 0.40 = Fair, and < 0.20 = Poor. Statistical significance was considered at $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.10$ for analysis.

RESULTS

Agreement based on Kappa scores was very good amongst all tests in the study (Table 3.1). Additionally, there was a positive correlation among all tests (Table 3.2).

Of the 1,096 animals that were diagnosed nonpregnant by transrectal ultrasonography, 5.61% were diagnosed pregnant by the lateral flow test. Of the 1,648 animals diagnosed pregnant by transrectal ultrasonography, 2.00% were diagnosed nonpregnant by the lateral flow test. Thus, an 92.38% agreement occurred between the two methods (Table 3.3). In comparison the RPVT had a 90.73% agreement and the RPT had a 92.61% agreement with transrectal ultrasonography (Table 3.3).

Comparisons were also made between the lateral flow test to the two other IDEXX blood tests to determine accuracies amongst the tests. Of the 816 animals that were diagnosed nonpregnant by the RVPT, 3.56% were called pregnant by the lateral flow test, and the 644 animals diagnosed as pregnant by RPVT, 1.37% were called nonpregnant by the lateral flow test. Thus, a 95.07% agreement occurred between RVPT

and lateral flow (Table 3.3). Lastly, the 724 animals that were diagnosed as nonpregnant by RPT, 1.31% were called pregnant by lateral flow test. Of the 1,636 animals diagnosed as pregnant by RPT, 2.46% were called nonpregnant by the lateral flow test. Thus, 96.22% agreement occurred between RPT and lateral flow test (Table 3.3). Both qualitative and quantitative measures have been made to compare the differences made between each of the four pregnancy detection methods discussed in this chapter (Table 3.4).

For postpartum samples utilizing the lateral flow test, there was not a difference ($P = 0.21$) in parity, but there was an effect of dpp ($P < 0.01$) and a tendency for a dpp by parity interaction ($P = 0.06$). All animals regardless of parity were still considered pregnant from the previous pregnancy through 35 dpp (100 ± 2.58%), whereas by 42 dpp, 98.16 ± 2.55% were considered pregnant (Figure 3.1.A). The percentage of females receiving a false positive test result further decreased with time postpartum. By 77 dpp, there were 13.72 ± 3.16% of the females positive for pregnancy, and at 84 dpp, there were 4.11 ± 4.39% positive for pregnancy (Figure 3.1.A). The detection of false positives rapidly decreased from 42 to 70 dpp then slowly decreased from 70 to 84 dpp (Figure 3.1.A). There was a tendency for a parity by dpp interaction ($P = 0.06$; Figure 3.1.B). Between 63 to 77 dpp there was a greater decrease in false positives among primiparous compared to multiparous animals (at 77 dpp 5.12 ± 4.26% and 22.31 ± 4.67%; respectively). At 84 dpp 4.66 ± 6.03% of multiparous and 3.56 ± 6.38% of primiparous females were still considered pregnant (Figure 3.2).

Test	Ultrasound	RVPT	RPT	Lateral Flow
Ultrasound		0.8108	0.8344	0.8388
RVPT	very good		0.9472	0.9005
RPT	very good	very good		0.9122
Lateral Flow	very good	very good	very good	

Table 3.1. Agreement between Transrectal Ultrasonography (Ultrasound), IDEXX Alertys Rapid Visual (RVPT), IDEXX Alertys Ruminant Pregnancy test (RPT), and IDEXX Alertys OnFarm Pregnancy Test (Lateral Flow) to determine accuracy of pregnancy detection. Values depicted above the diagonal line are the Kappa scores, while values below the diagonal are the overall agreement of the tests based on the Kappa score. The Kappa scoring scale is: 0.80 - 1.00 = Very good, 0.60 - 0.80 = Good, 0.40 - 0.60 = Moderate, 0.20 - 0.40 = Fair, and < 0.20 = Poor.

Test	Ultrasound	RVPT	RPT	Lateral Flow
Ultrasound		0.81311	0.83856	0.84126
RVPT	< 0.0001		0.94739	0.90138
RPT	< 0.0001	< 0.0001		0.91257
Lateral Flow	< 0.0001	< 0.0001	< 0.0001	

Table 3.2. Correlation between Transrectal Ultrasonography (Ultrasound), IDEXX Alertys Rapid Visual (RVPT), IDEXX Alertys Ruminant Pregnancy test (RPT), and IDEXX Alertys OnFarm Pregnancy Test (Lateral Flow) to determine accuracy of pregnancy detection. Values depicted above the diagonal line is the correlation coefficient of the tests, while values below the diagonal are the *P*-values of all the tests. A positive correlation and significant difference were found amongst the tests in comparison to each other.

Test[1]	Agreement, %	False Positive[2], %	False Negative[3], %	Samples, n
Ultrasound[4]:Lateral Flow[5]	92.38	5.61	2.00	2,744
Ultrasound[4]:RVPT[6]	90.73	6.46	2.80	1,533
Ultraound[4]:RPT[7]	92.61	5.91	1.48	2,436
RVPT[6]:Lateral Flow[5]	95.07	3.56	1.37	1,460
RPT[7]:Lateral Flow[5]	96.22	1.31	2.46	2,360
RPVT[6]:RPT[7]	97.36	1.80	0.83	1,443

Table 3.3. Agreement between pregnancy detection tests to determine accuracy amongst tests.
[1]Comparison between tests first:second
[2] False Positive = a result that shows a female is pregnant when she is actually non-pregnant
[3]False Negative = a result that shows a female is non-pregnant when she is actually pregnant
[4]Ultrasound = transrectal ultrasonography
[5]Lateral Flow = IDEXX Alertys OnFarm Pregnancy Test
[6]RVPT = IDEXX Alertys Rapid Visual Test
[7]RPT = IDEXX Alertys Ruminant Pregnancy Test

Test	First Pregnancy Detection	Experienced Technician Needed?	Cost/Cow	When Results Known
Ultrasound	30 d	Yes	~$140 hr	Immediately
RVPT	28 to 30 d	Yes/No*	$4.00	30 min
RPT	28 to 30 d	No	$3.50	1 to 4 d
Lateral Flow	28 to 30 d	No	$7.00	1 to 20 min

Table 3.4. Contrasts between the tests Transrectal Ultrasonography (Ultrasound), IDEXX Alertys Rapid Visual (RVPT), IDEXX Alertys Ruminant Pregnancy test (RPT), and IDEXX Alertys OnFarm Pregnancy Test (lateral flow).
*Dependent on equipment available.

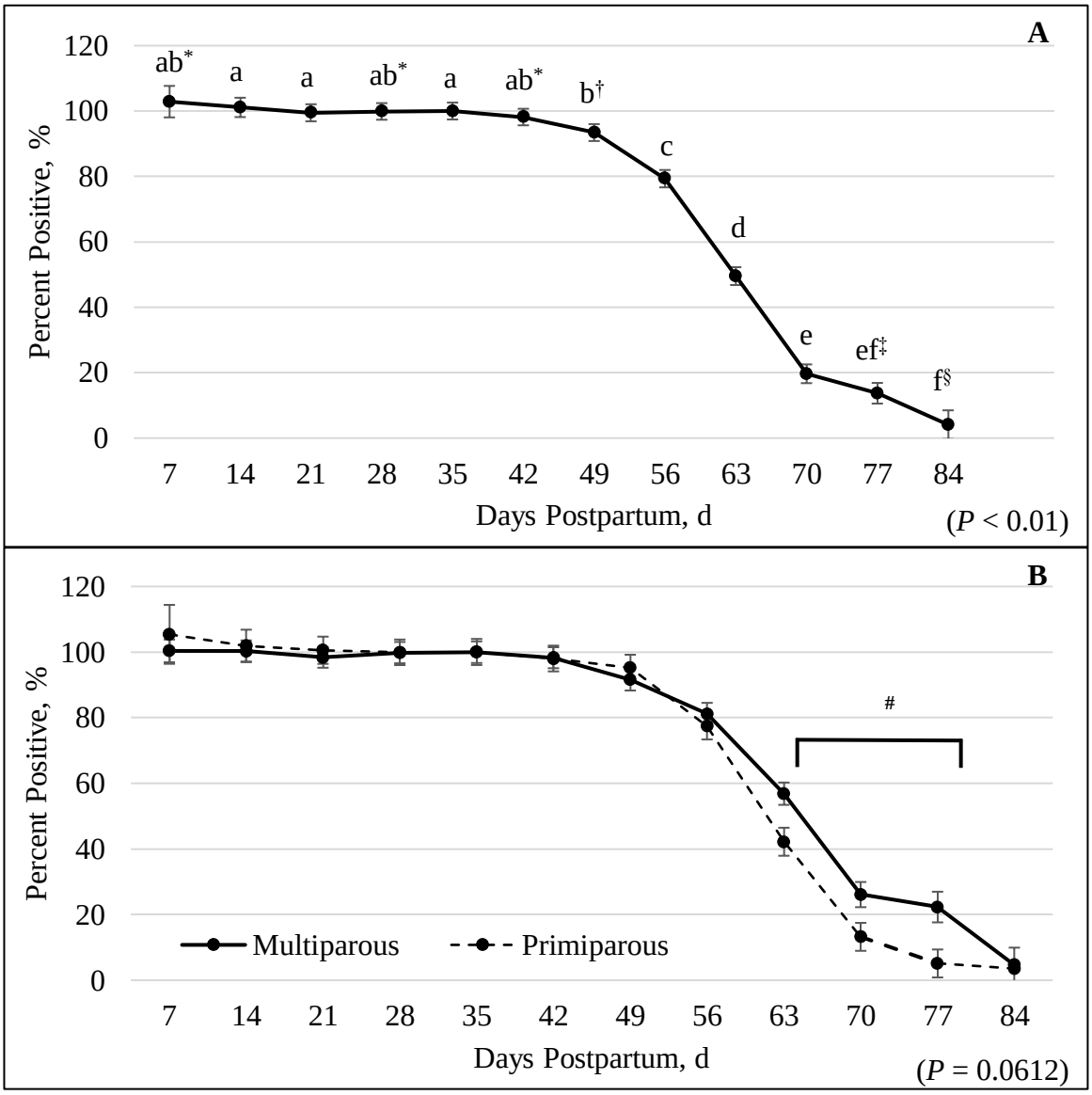

Figure 3.1. False positive percentage of days postpartum (A) and parity (B) on clearance utilizing the lateral flow test. Postpartum samples were analyzed by the IDEXX Alertys OnFarm Pregnancy Test, lateral flow, to determine an accurate timeframe to test pregnant females without getting a false positive test from the residual pregnancy-associated glycoproteins (PAGs) from the previous pregnancy. There was a significant effect of dpp ($P < 0.01$; A). All animals were still considered pregnant from the previous pregnancy on 35 dpp (100%; A). Days postpartum by parity tended to be different (P = 0.06; B).
a-f values not sharing the same superscripts differed $P < 0.05$
*† values not sharing the same superscripts differed $P \leq 0.08$
‡§ values not sharing the same superscripts differed $P \leq 0.07$
# values between multiparous and primiparous between 63 to 77 dpp differed $P < 0.05$

DISCUSSION

Currently, there is no method of pregnancy detection that is 100% accurate for pregnancy determination without being invasive, which makes it difficult to evaluate the accuracy of a new pregnancy detection method (Romano and Larson, 2010). The most accurate, non-invasive method currently known as the gold standard, is transrectal ultrasonography. It has been an ongoing task of trying to find a method that accurately determines pregnancy status and is also easy and quick to use. IDEXX laboratories have created a series of blood pregnancy tests, (Alertys Rapid Visual; Alertys Ruminant Pregnancy Test; and Alertys OnFarm Pregnancy Test), to help producers determine the pregnancy status of females within their herd. The RVPT and RPT are polyclonal tests, meaning they detect various PAG members that are secreted, some at different times throughout gestation, and found in the maternal bloodstream to determine pregnancy status. The lateral flow test is a monoclonal test, meaning that they use one antibody that binds to a specific PAG member that is secreted in order to determine the pregnancy status of the female.

In order for a pregnancy detection test to be beneficial, it must be sensitive, meaning it is accurate in identifying pregnant females, and specific, meaning it also accurately identifies females who are nonpregnant. Because transrectal ultrasonography is known as the gold standard, it was used as a comparative measure to determine the accuracy of all the IDEXX PAG pregnancy tests.

Epperson et al. (2020) conducted a comparison between RVPT, ultrasonography, RPT and resynchronization pregnancy diagnosis. Blood samples were taken on d 28 and the final pregnancy diagnosis was made 31 to 80 d post-second artificial insemination

(AI2). The kappa statistic scores amongst the comparisons between rapid visual pregnancy test, ultrasonography, and Alertys ruminant pregnancy test against the final resynchronization pregnancy diagnosis were very good; 0.90, 0.82, and 0.90, respectively. In a study completed by Silva et al. (2007), they compared the accuracy of PAG ELISA to transrectal ultrasonography on d 27 post-AI, d 39 post-AI2, and d 39 post-AI3 (post-third artificial insemination) utilizing dairy cows. This comparison found the PAG blood pregnancy test to have a kappa statistic score of 0.87 to 0.90, which is very good, when compared to ultrasonography. Similarly, a study by Romano and Larson (2010) compared PSPB (pregnancy specific protein B; PAG-1 subgroup) ELISA to transrectal ultrasonography on d 28, 30, and 35 post-AI. Romano and Larson (2010) found between d 28 to d 35 PSPB compared to transrectal ultrasonography had a very good kappa statistic score of 0.93 for accurately detecting pregnancy status. Piechotta et al. (2011) utilizing dairy cows, compared two ELISA blood pregnancy tests for PSPB (PAG-1 subgroup) against transrectal ultrasonography between d 26 to 58 post-AI. Between the two tests there was no significant difference found in comparison to transrectal ultrasonography. Northrop et al. (2019) also found similar results when comparing the ruminant pregnancy and rapid visual pregnancy test to transrectal ultrasonography in beef cows between d 28 to 40 post-AI and found them to have very good agreement, 0.86 and 0.85 respectively. This study found similar results when comparing RPVT and RPT to ultrasonography with very good kappa statistic scores (0.81 and 0.83, respectively) and agreement (81.3% and 83.9%, respectively). In validating the new IDEXX Alertys OnFarm Pregnancy Test compared to ultrasonography, RVPT, and RPT our lab found it had very good kappa statistic scores (0.84, 0.90, 0.91 respectively)

and agreement (84.1%, 90.1%, and 91.3%, respectively), which falls in range with the previous mentioned results from the other studies. The results from this study along with the previous research validates, blood pregnancy tests RPT and RVPT are highly accurate to transrectal ultrasonography; since the lateral flow test has a very good kappa score and agreement amongst all three tests used in this present study, it would make it a great pregnancy detection alternative.

Although, caution should be used when implementing this pregnancy diagnosis test to minimize false positives from performing the test too early in gestation (< 70 dpp). The RPT allows for a diminished rate of false positives at 42 dpp, thus allowing testing to be made earlier in gestation compared to the lateral flow test evaluation in our laboratory (Chapter 2). Waiting 30 dpp or more before breeding increases conception rates in beef females (Casida et al., 1968; Wiltbank, 1970). The utilization of the IDEXX Alertys Ruminant Pregnancy Test is recommended to be implemented as early as d 28 of gestation, so the RPT could be conducted at 58 dpp or 28 d post-AI to allow for uterine involution to occur (Short et al., 1990) and improve accuracy of results. Clearance of residual PAGs from the previous pregnancy determined by the lateral flow test took longer than the clearance within the RPT. Also, the clearance of residual PAGs in the lateral flow test surpassed the length of time needed for uterine involution to occur and the manufacturers recommended utilization day, d 28 post-AI. Thus, in order to gain the most accurate results an allotment of 70 dpp or 40 d post-AI should be made before performing the lateral flow test. This means between the RPT and lateral flow there is at least a 12 dpp difference if the RPT is utilized on d 28 and the lateral flow is utilized on d 40 of gestation. The RPT uses a polyclonal antibody against several PAGs (e.g. PAGs 4,

6, 9, 16, 18, 19; described in US Patent no. 7,604,950B2; Ketchum, 2020). Specifically, PAGs 4, 9, and 6 are secreted from d 25 (PAG 4 and 9) and d 45 (PAG 6) through to d 250 of gestation (Green et al., 2000). The lateral flow test is a monoclonal test where the exact PAG identified are unknown. Thus, the difference in detection of PAGs between the two tests may be due to the influence in clearance of different PAGs from circulation. Performing this test later in gestation when there would be fewer false positives would detect more embryo loss that occurs during late embryo mortality through d 45 (Diskin and Morris, 2008), and may improve more management decisions and opportunities. Implementation of the lateral flow test on postpartum females, may potentially increase the calving interval compared to the RPT test since pregnancy determination would occur later in gestation, causing the possibility of rebreeding, if utilizing AI, to be later as well. In nulliparous females, implementation of this test can occur on d 28 of gestation as the manufacturer's instructions state since there are no residual PAGs from a previous pregnancy.

For practical and research use in the cattle industry, any female that is detected by a pregnancy detection method as nonpregnant, the female may receive an injection of prostaglandin-$F_{2\alpha}$, which would cause the CL to regress decreasing progesterone concentrations and allow for the dominant follicle to increase estrogen concentrations and estrus to be initiated (Hafs et al., 1974). Females falsely classified as nonpregnant by a pregnancy detection method may potentially receive a shot of prostaglandin-$F_{2\alpha}$, which could cause the female to abort. Thus, producers would have an economic loss from the loss of a live calf, resynchronization drugs (if chosen to rebred the female), time, and labor which would range from $550 to $800 lost (De Vries, 2006; Romano and Larson,

2010). Silva et al. (2007) found the PAG test to have a great negative predicted value from the three different AI days, d 27 post-AI, d 39 post-AI2, and d 39 post-AI3 (97.1%, 96.9%, 97.7% respectively). This study also found a great negative predictive value between ultrasound:lateral flow, ultrasound:RVPT, and ultrasound:RPT (98.0%, 97.2%, and 98.5%, respectively). Having a greater negative predictive value decreases the likelihood of giving prostaglandin-$F_{2\alpha}$ to a female who is truly pregnant. On the other hand, early and accurate pregnancy detection allows for more management decisions to be made by identifying females who are nonpregnant post breeding. Animals that are accurately detected as nonpregnant can be rebred or culled earlier to reduce the number of days a nonpregnant female is cared for which ultimately would result in a reduction of an operation's financial losses (Whitlock and Maxwell, 2008).

In conclusion, the utilization of the IDEXX Alertys OnFarm Pregnancy Test, lateral flow, is a competitive alternative in pregnancy detection compared to the gold standard transrectal ultrasonography with an 92.38% agreement comparison in postpartum females. Of the three IDEXX Laboratories tests available, the lateral flow test is the most user-friendly method. Additionally, it provides the tools necessary for performing the test included in the kit once the blood has been collected. With the exception of transrectal ultrasonography where the pregnancy detection results are immediate, the time required for results from the lateral flow compared to the other two PAG pregnancy tests is shorter and does not require any specialized training or equipment. Caution should be used with the utilization of the lateral flow test postpartum in either primiparous or multiparous females until at least 70 dpp due to the remaining residual PAGs. Thus, allowing 30 dpp for uterine involution to occur before breeding a

female then testing females for pregnancy status should be performed at least an additional 40 dpp (or d 40 of gestation) to decrease the amount of false positive results.

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LITERATURE CITED

Adams, G. 1999. Comparative patterns of follicle development and selection in ruminants. Journal of Reproduction and Fertility Supplement 54:17-32.

Aires, M., K. Dekagi, V. Dantzer, and A. Yamada. 2014. Bovine placentome development during early pregnancy. Microscope 1:390-396.

Assis Neto, A., F. Pereira, T. Santos, C. Ambrosio, R. Leiser, and M. Miglino. 2010. Morpho-physical recording of bovine conceptus (Bos indicus) and placenta from days 20 to 70 of pregnancy. Reproduction in Domestic Animals 45(5):760-772.

Assis Neto, A., E. Santos, F. Pereira, and M. Miglino. 2009. Initial development of bovine placentation (Bos indicus) from the point of view of the allantois and amnion. Anatomia, Histologia, Embryologia 38(5):341-347.

Atkinson, B., G. King, and E. Amoroso. 1984. Development of the caruncular and intercaruncular regions in the bovine endometrium. Biology of Reproduction 30(3):763-774.

Austin, C. 1951. Observations on the penetration of the sperm into the mammalian egg. Australian Journal of Biological Sciences 4(4):581-596.

Ayalon, N. 1978. A review of embryonic mortality in cattle. Reproduction 54(2):483-493.

Balhara, A. K., M. Gupta, S. Singh, A. K. Mohanty, and I. Singh. 2013. Early pregnancy diagnosis in bovines: current status and future directions. The Scientific World Journal 2013:1-10.

Bellows, D., S. Ott, and R. Bellows. 2002. Cost of reproductive diseases and conditions in cattle. The Professional Animal Scientist 18(1):26-32.

Betteridge, K., and J. E. Fléchon. 1988. The anatomy and physiology of pre-attachment bovine embryos. Theriogenology 29(1):155-187.

Blomberg, L., K. Hashizume, and C. Viebahn. 2008. Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation. Reproduction 135(2):181-195.

Bó, G. A., J. J. de la Mata, P. S. Baruselli, and A. Menchaca. 2016. Alternative programs for synchronizing and resynchronizing ovulation in beef cattle. Theriogenology 86(1):388-396.

Booth, J., J. Davies, and R. Holdsworth. 1979. Use of the milk progesterone test for pregnancy determination. British Veterinary Journal 135(5):478-488.

Brackett, B. G., Y. K. Oh, J. F. Evans, and W. J. Donawick. 1980. Fertilization and early development of cow ova. Biology of Reproduction 23(1):189-205.

Burfening, P., D. Anderson, R. Kinkie, J. Williams, and R. Friedrich. 1978. Synchronization of estrus with PGF2α in beef cattle. Journal of Animal Science 47(5):999-1003.

Butler, J., W. Hamilton, R. Sasser, C. Ruder, G. Hass, and R. Williams. 1982. Detection and partial characterization of two bovine pregnancy-specific proteins. Biology of Reproduction 26(5):925-933.

Caldow, G., B. Lowman, and I. Riddell. 2005. Veterinary intervention in the reproductive management of beef cow herds. In Practice 27(8):406-411.

Casida, L., W. Graves, E. Hauser, J. Lauderdale, J. Riesen, S. Saiduddin, and W. Tyler. 1968. Studies on the postpartum cow. Research Bull 270:48-54.

Chang, M. C. 1951. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. Nature 168(4277):697-698.

Chavatte-Palmer, P., and M. Guillomot. 2007. Comparative implantation and placentation. Gynecologic and Obstetric Investigation 64(3):166-174.

Cowie, A. T. 1948. Pregnancy diagnosis tests: a review. Commonwealth [Bureau] of Animal Breeding and Genetics 13:1-283.

Curran, S., R. Pierson, and O. Ginther. 1986. Ultrasonographic appearance of the bovine conceptus from days 20 through 60. Journal of American Veterinary Medical Association 189(10):1295-1302.

Davies, D. R. 1990. The structure and function of the aspartic proteinases. Annual Review of Biophysics and Biophysical Chemistry 19(1):189-215.

De Vries, A. 2006. Economic value of pregnancy in dairy cattle. Journal of Dairy Science 89(10):3876-3885.

Degrelle, S. A., E. Campion, C. Cabau, F. Piumi, P. Reinaud, C. Richard, J. P. Renard, and I. Hue. 2005. Molecular evidence for a critical period in mural trophoblast development in bovine blastocysts. Developmental Biology 288(2):448-460.

Diskin, M., E. Austin, and J. Roche. 2002. Exogenous hormonal manipulation of ovarian activity in cattle. Domestic Animal Endocrinology 23(1-2):211-228.

Diskin, M., and D. Morris. 2008. Embryonic and early foetal losses in cattle and other ruminants. Reproduction in Domestic Animals 43:260-267.

Diskin, M., M. Parr, and D. Morris. 2011. Embryo death in cattle: an update. Reproduction, Fertility and Development 24(1):244-251.

Diskin, M., and J. Sreenan. 1980. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. Reproduction 59(2):463-468.

Diskin, M. G., and J. M. Sreenan. 2000. Expression and detection of oestrus in cattle. Reproduction Nutrition Development 40(5):481-491.

Eckblad, W. 1985. Localization of pregnancy-specific protein B (PSPB) in bovine placental cells using a glucose oxidase-anti-glucose oxidase immunohistochemical stain. Proceedings American Society of Animal Science West Section 36:396-397.

Epperson, K. M., J.J.J. Rich, S. Menegatti Zoca, E.J., Northrop, S.D. Perkins, J.A. Walker, J.R. Rhoades, and G.A. Perry. 2020. Effect of progesterone supplementation in a resynchronization protocol on follicular dynamics and pregnancy success. Theriogenology 157:121-129.

Erickson, B. 1966. Development and senescence of the postnatal bovine ovary. Journal of Animal Science 25(3):800-805.

Esslemont, R. J., J. H. Bailie, and M. J. Cooper. 1985. Fertility management in dairy cattle. London:Collins:1-143.

Farin, P., A. Crosier, and C. Farin. 2001. Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 55(1):151-170.

Fléchon, J. E., and J. P. Renard. 1978. A scanning electron microscope study of the hatching of bovine blastocysts in vitro. Reproduction 53(1):9-12.

Forde, N., M. E. Beltman, P. Lonergan, M. Diskin, J. F. Roche, and M. A. Crowe. 2011. Oestrous cycles in Bos taurus cattle. Animal Reproduction Science 124(3):163-169.

Forde, N., and P. Lonergan. 2012. Transcriptomic analysis of the bovine endometrium: What is required to establish uterine receptivity to implantation in cattle? Journal of Reproduction and Development 58(2):189-195.

Fortune, J. 1994. Ovarian follicular growth and development in mammals. Biology of reproduction 50(2):225-232.

Frandson, R. D., W. Lee Wilke, and A. Dee Fails. 2003. A. The Ovary and Estrous Cycles. Anatomy and Physiology of Farm Animals. Philadelphia:Lippincott Williams & Wilkins 6: 1-481.

Fraser, L. R. 1998. Sperm capacitation and the acrosome reaction. Human Reproduction 13(suppl_1):9-19.

Garrett, J., R. Geisert, M. Zavy, and G. Morgan. 1988. Evidence for maternal regulation of early conceptus growth and development in beef cattle. Reproduction 84(2):437-446.

Geisert, R., G. Morgan, M. Zavy, R. Blair, L. Gries, A. Cox, and T. Yellin. 1991. Effect of asynchronous transfer and oestrogen administration on survival and development of porcine embryos. Reproduction 93(2):475-481.

Gifford, C., K. Racicot, D. Clark, K. Austin, T. Hansen, M. Lucy, C. Davies, and T. Ott. 2007. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. Journal of Dairy Science 90(1):274-280.

Ginther, O., M. Fuenzalida, H. Shrestha, and M. Beg. 2011. The transition between preluteolysis and luteolysis in cattle. Theriogenology 75(1):164-171.

Ginther, O. J. 1986. Ultrasonic imaging and reproductive events in the mare. Ultrasonic imaging and reproductive events in the mare. Equiservices:1-378.

Goff, A. K. 2002. Embryonic signals and survival. Reproduction in Domestic Animals 37(3):133-139.

Gomes, W., and R. Erb. 1965. Progesterone in bovine reproduction: A review. Journal of Dairy Science 48(3):314-330.

Gray, W. C., K. Painter, and N. Rimbey. 2012. Livestock costs and returns estimates, University of Idaho, University of Idaho Agricultural Economics and Rural Sociology.

Green, J., C. Okamura, S. Poock, and M. Lucy. 2010. Measurement of interferon-tau (IFN-τ) stimulated gene expression in blood leukocytes for pregnancy diagnosis within 18–20 d after insemination in dairy cattle. Animal Reproduction Science 121(1-2):24-33.

Green, J. A., T. E. Parks, M. P. Avalle, B. P. Telugu, A. L. McLain, A. J. Peterson, W. McMillan, N. Mathialagan, R. R. Hook, and S. Xie. 2005. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. Theriogenology 63(5):1481-1503.

Green, J. A., S. Xie, X. Quan, B. Bao, X. Gan, N. Mathialagan, J.-F. Beckers, and R. M. Roberts. 2000. Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. Biology of Reproduction 62(6):1624-1631.

Greenstein, J., and R. Foley. 1958. Early embryology of the cow. I. Gastrula and primitive streak stages. Journal of Dairy Science 41(3):409-421.

Griffin, P., and O. Ginther. 1992. Research applications of ultrasonic imaging in reproductive biology. Journal of Animal Science 70(3):953-972.

Guillomot, M. 1995. Cellular interactions during implantation in domestic ruminants. Journal of Reproduction and Fertility-Supplements only (49):39-52.

Gunn, D., and J. B. Hall. 2018. Pregnancy Testing in Beef Cattle, The University of Idaho.

Hafs, H., T. Louis, P. Noden, and W. Oxender. 1974. Control of the estrous cycle with prostaglandin F2α in cattle and horses. Journal of Animal Science 38(suppl_1):10-21.

Hansen, P. 2002. Embryonic mortality in cattle from the embryo's perspective. Journal of Animal Science 80(E-suppl_2):E33-E44.

Hansen, T. R., K. Imakawa, H. Polites, K. Marotti, R. Anthony, and R. Roberts. 1988. Interferon RNA of embryonic origin is expressed transiently during early pregnancy in the ewe. Journal of Biological Chemistry 263(26):12801-12804.

Hashizume, K. 2007. Analysis of uteroplacental-specific molecules and their functions during implantation and placentation in the bovine. Journal of Reproduction and Development 53(1):1-11.

Haugejorden, G., S. Waage, E. Dahl, K. Karlberg, J.-F. Beckers, and E. Ropstad. 2006. Pregnancy associated glycoproteins (PAG) in postpartum cows, ewes, goats and their offspring. Theriogenology 66(8):1976-1984.

Hill, J. R., R. C. Burghardt, K. Jones, C. R. Long, C. R. Looney, T. Shin, T. E. Spencer, J. A. Thompson, Q. A. Winger, and M. E. Westhusin. 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biology of Reproduction 63(6):1787-1794.

Houghton, F. D. 2005. Role of gap junctions during early embryo development. Reproduction 129(2):129-135.

Hue, I., S. A. Degrelle, and N. Turenne. 2012. Conceptus elongation in cattle: genes, models and questions. Animal Reproduction Science 134(1-2):19-28.

Hue, I., D. Evain-Brion, T. Fournier, and S. A. Degrelle. 2015. Primary bovine extra-embryonic cultured cells: a new resource for the study of in vivo peri-implanting phenotypes and mesoderm formation. PloS one 10(6):1-21.

Humblot, P., S. Camous, J. Martal, J. Charlery, N. Jeanguyot, M. Thibier, and G. Sasser. 1988. Diagnosis of pregnancy by radioimmunoassay of a pregnancy-specific protein in the plasma of dairy cows. Theriogenology 30(2):257-267.

Hunter, R., and I. Wilmut. 1983. The rate of functional sperm transport into the oviducts of mated cows. Animal Reproduction Science 5(3):167-173.

Hunter, R., and I. Wilmut. 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reproduction Nutrition Developpement 24(5A):597-608.

Jaśkowski, J. M., M. Kaczmarowski, J. Kulus, B. M. Jaśkowski, M. Herudzińska, and M. Gehrke. 2019. Rectal palpation for pregnancy in cows: A relic or an alternative to modern diagnostic methods. Medycyna Weterynaryjna 75(05):259-264.

Kastelic, J., D. Bergfelt, and O. Ginther. 1991. Ultrasonic detection of the conceptus and characterization of intrauterine fluid on days 10 to 22 in heifers. Theriogenology 35(3):569-581.

Kerbler, T., M. Buhr, L. Jordan, K. Leslie, and J. Walton. 1997. Relationship between maternal plasma progesterone concentration and interferon-tau synthesis by the conceptus in cattle. Theriogenology 47(3):703-714.

Ketchum, J. N. 2020. Investigation of mechanisms associated with establishment and maintenance of pregnancy in beef cattle, Doctoral dissertation, University of Missouri--Columbia.

King, G., B. Atkinson, and H. Robertson. 1979. Development of the bovine placentome during the second month of gestation. Reproduction 55(1):173-180.

King, G., B. Atkinson, and H. Robertson. 1980. Development of the bovine placentome from days 20 to 29 of gestation. Reproduction 59(1):95-100.

King, G., B. Atkinson, and H. Robertson. 1981. Development of the intercaruncular areas during early gestation and establishment of the bovine placenta. Reproduction 61(2):469-474.

Kiracofe, G. 1980. Uterine involution: its role in regulating postpartum intervals. Journal of Animal Science 51(suppl_II):16-28.

Kiracofe, G., J. Wright, R. Schalles, C. Ruder, S. Parish, and R. Sasser. 1993. Pregnancy-specific protein B in serum of postpartum beef cows. Journal of Animal Science 71(8):2199-2205.

Kuijk, E. W., L. T. van Tol, H. Van de Velde, R. Wubbolts, M. Welling, N. Geijsen, and B. A. Roelen. 2012. The roles of FGF and MAP kinase signaling in the

segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. Development 139(5):871-882.

Laing, J., and R. Heap. 1971. The concentration of progesterone in the milk of cows during the reproductive cycle. British Veterinary Journal 127(8):19-22.

Leiser, R., C. Pfarrer, M. Abd-Elnaeim, and V. Dantzer. 1998. Feto-maternal anchorage in epitheliochorial and endotheliochorial placental types studied by histology and microvascular corrosion casts. Placenta 19:21-39.

Lobago, F., M. Bekana, H. Gustafsson, J. F. Beckers, G. Yohannes, Y. Aster, and H. Kindahl. 2009. Serum profiles of pregnancy-associated glycoprotein, oestrone sulphate and progesterone during gestation and some factors influencing the profiles in Ethiopian Borana and crossbred cattle. Reproduction in Domestic Animals 44(4):685-692.

López-Gatius, F. 2000. Site of semen deposition in cattle: a review. Theriogenology 53(7):1407-1414.

Lucy, M., J. Green, and S. Poock. 2011. Pregnancy determination in cattle: a review of available alternatives. Proceedings of Applied Reproductive Strategies in Beef Cattle:367-376.

Maddox-Hyttel, P., N. I. Alexopoulos, G. Vajta, I. Lewis, P. Rogers, L. Cann, H. Callesen, P. Tveden-Nyborg, and A. Trounson. 2003. Immunohistochemical and ultrastructural characterization of the initial post-hatching development of bovine embryos. Reproduction 125(4):607-623.

Mann, G., M. Fray, and G. Lamming. 2006. Effects of time of progesterone supplementation on embryo development and interferon-τ production in the cow. The Veterinary Journal 171(3):500-503.

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

Matsuyama, S., T. Kojima, S. Kato, and K. Kimura. 2012. Relationship between quantity of IFNT estimated by IFN-stimulated gene expression in peripheral blood mononuclear cells and bovine embryonic mortality after AI or ET. Reproductive Biology and Endocrinology 10(1):21.

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

Meyer, M., P. Hansen, W. Thatcher, M. Drost, L. Badinga, R. Roberts, J. Li, T. Ott, and F. Bazer. 1995. Extension of corpus luteum lifespan and reduction of uterine

secretion of prostaglandin F2α of cows in response to recombinant interferon-τ. Journal of Dairy Science 78(9):1921-1931.

Mialon, M., S. Camous, G. Renand, J. Martal, and F. Ménissier. 1993. Peripheral concentrations of a 60-kDa pregnancy serum protein during gestation and after calving and in relationship to embryonic mortality in cattle. Reproduction nutrition development 33(3):269-282.

Milvae, R., S. Hinckley, and J. Carlson. 1996. Luteotropic and luteolytic mechanisms in the bovine corpus luteum. Theriogenology 45(7):1327-1349.

Mortimer, R., and T. Hansen. 2006. The future of pregnancy testing in beef cattle. Proceedings, Applied Reproductive Strategies in Beef Cattle:281-288.

Nalbandov, A., and L. Casida. 1942. Ovulation and its relation to estrus in cows. Journal of Animal Science 1(3):189-198.

Nebel, R. 1988. On-farm milk progesterone tests. Journal of Dairy Science 71(6):1682-1690.

Negrón-Pérez, V. M. 2017. Differentiation and development of the bovine blastocyst. University of Florida. ProQuest Dissertations & Theses Global.

Northey, D., and L. French. 1980. Effect of embryo removal and intrauterine infusion of embryonic homogenates on the lifespan of the bovine corpus luteum. Journal of Animal Science 50(2):298-302.

Northrop, E. J., J. J. J. Rich, J. R. Rhoades, and G. A. Perry. 2019. Comparison of two bovine serum pregnancy tests in detection of artificial insemination pregnancies and pregnancy loss in beef cattle. PloS one 14(1):1-10.

Oliphant, G., A. B. Reynolds, and T. S. Thomas. 1985. Sperm surface components involved in the control of the acrosome reaction. American Journal of Anatomy 174(3):269-283.

Oltenacu, P., J. Ferguson, and A. Lednor. 1990. Economic evaluation of pregnancy diagnosis in dairy cattle: a decision analysis approach. Journal of Dairy Science 73(10):2826-2831.

Paschal, J., J. Sanders, and J. Kerr. 1991. Calving and weaning characteristics of Angus-, gray Brahman-, Gir-, Indu-Brazil-, Nellore-, and red Brahman-sired F1 calves. Journal of Animal Science 69(6):2395-2402.

Patel, O., J. Sulon, J. Beckers, T. Takahashi, M. Hirako, N. Sasaki, and I. Domeki. 1997. Plasma bovine pregnancy-associated glycoprotein concentrations throughout

gestation in relationship to fetal number in the cow. European Journal of Endocrinology 137(4):423-428.

Perry, G., and R. Cushman. 2016. Invited Review: Use of ultrasonography to make reproductive management decisions. The Professional Animal Scientist 32(2):154-161.

Piechotta, M., J. Bollwein, M. Friedrich, T. Heilkenbrinker, C. Passavant, J. Branen, G. Sasser, M. Hoedemaker, and H. Bollwein. 2011. Comparison of Commercial ELISA Blood Tests for Early Pregnancy Detection in Dairy Cows. Journal of Reproduction and Developement 57:72-75.

Pohler, K., G. Franco, S. Reese, F. Dantas, M. Ellis, and R. Payton. 2016. Past, present and future of pregnancy detection methods. Applied Reproductive Strategies in Beef Cattle:7-8.

Pohler, K., T. Geary, C. Johnson, J. Atkins, E. Jinks, D. Busch, J. Green, M. MacNeil, and M. Smith. 2013. Circulating bovine pregnancy associated glycoproteins are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows. Journal of Animal Science 91(9):4158-4167.

Purohit, G. 2010. Methods of pregnancy diagnosis in domestic animals: The current status. WebmedCentral Reproduction 1(12):2-26.

Rani, P., R. Dutt, G. Singh, and R. Chandolia. 2018. Embryonic mortality in cattle—A review. International journal of current microbiology and applied science 7(7):1501-1516.

Ricci, A., P. Carvalho, M. Amundson, R. Fourdraine, L. Vincenti, and P. Fricke. 2015. Factors associated with pregnancy-associated glycoprotein (PAG) levels in plasma and milk of Holstein cows during early pregnancy and their effect on the accuracy of pregnancy diagnosis. Journal of Dairy Science 98(4):2502-2514.

Robinson, R., G. Mann, G. Lamming, and D. Wathes. 1999. The effect of pregnancy on the expression of uterine oxytocin, oestrogen and progesterone receptors during early pregnancy in the cow. Journal of Endocrinology 160(1):21-34.

Robinson, R., G. Mann, G. Lamming, and D. Wathes. 2001. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. Reproduction 122(6):965-979.

Romano, J. E., and J. E. Larson. 2010. Accuracy of pregnancy specific protein-B test for early pregnancy diagnosis in dairy cattle. Theriogenology 74:932-939.

Ruder, C., and R. Sasser. 1986. Source of bovine pregnancy-specific protein B (bPSPB) during the postpartum period and estimation of half-life of bPSPB. Journal of Animal Science 63:335.

Santos, J., H. M. Rutigliano, and M. Sá Filho. 2009. Risk factors for resumption of postpartum estrous cycles and embryonic survival in lactating dairy cows. Animal Reproduction Science 110(3-4):207-221.

Santos, J., W. Thatcher, R. Chebel, R. Cerri, and K. Galvao. 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Animal Reproduction Science 82:513-535.

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

Senger, P. L. 2003. Pathways to Pregnancy and Parturition. 3rd ed. Current Conceptions, Inc., Pullman, WA.

Shea, B. F. 1981. Evaluating the bovine embryo. Theriogenology 15(1):31-42.

Sheldon, I. M. 2004. The postpartum uterus. Veterinary Clinics: Food Animal Practice 20(3):569-591.

Shimada, A., H. Nakano, T. Takahashi, K. Imai, and K. Hashizume. 2001. Isolation and characterization of a bovine blastocyst-derived trophoblastic cell line, BT-1: development of a culture system in the absence of feeder cell. Placenta 22(7):652-662.

Shirasuna, K., H. Asaoka, T. J. Acosta, M. P. Wijayagunawardane, M. Ohtani, K.-G. Hayashi, M. Matsui, and A. Miyamoto. 2004. Real-time dynamics of prostaglandin F2α release from uterus and corpus luteum during spontaneous luteolysis in the cow. Reproduction 128(2):189-195.

Shirasuna, K., H. Matsumoto, S. Matsuyama, K. Kimura, H. Bollwein, and A. Miyamoto. 2015. Possible role of interferon tau on the bovine corpus luteum and neutrophils during the early pregnancy. Reproduction 150(3):217-225.

Short, R., R. Bellows, R. Staigmiller, J. Berardinelli, and E. Custer. 1990. Physiological mechanisms controlling anestrus and infertility in postpartum beef cattle. Journal of Animal Science 68(3):799-816.

Short, R., R. Staigmiller, R. Bellows, and R. Greer. 1994. Breeding heifers at one year of age: biological and economic considerations. Factors Affecting Calf Crop:55-68.

Short, R. V. 1958. Progesterone in blood. I. The chemical determination of progesterone in peripheral blood. The Journal of Endocrinology 16(4):415.

Silva, E., R. Sterry, D. Kolb, N. Mathialagan, M. McGrath, J. Ballam, and P. Fricke. 2007. Accuracy of a pregnancy-associated glycoprotein ELISA to determine pregnancy status of lactating dairy cows twenty-seven days after timed artificial insemination. Journal of Dairy Science 90(10):4612-4622.

Smith, M., G. Perry, J. Atkins, D. Busch, and D. Patterson. 2005. Physiological principles underlying synchronization of estrus. Proceedings applied reproductive strategies in beef cattle. Texas, USA:21-45.

Sousa, N., A. Ayad, J. Beckers, and Z. Gajewski. 2006. Pregnancy-associated glycoproteins (PAG) as pregnancy markers in the ruminants. Journal Physiology Pharmacology 57(Suppl 8):153-171.

Sousa, N. M., M. Zongo, W. Pitala, H. Boly, L. Sawadogo, M. Sanon, J. R. de Figueiredo, P. B. D. Gonçalves, B. El Amiri, and Z. Perènyi. 2003. Pregnancy-associated glycoprotein concentrations during pregnancy and the postpartum period in Azawak Zebu cattle. Theriogenology 59(5-6):1131-1142.

Spencer, T. E., and F. W. Bazer. 1996. Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. Endocrinology 137(3):1144-1147.

Spencer, T. E., and F. W. Bazer. 2004. Conceptus signals for establishment and maintenance of pregnancy. Reproductive Biology and Endocrinology 2(1):1-15.

Spencer T.E., Hansen T.R. (2015) Implantation and Establishment of Pregnancy in Ruminants. In: Geisert R., Bazer F. (eds) Regulation of Implantation and Establishment of Pregnancy in Mammals. Advances in Anatomy, Embryology and Cell Biology, vol 216. Springer, Cham. https://doi.org/10.1007/978-3-319-15856-3_7

Spieler, J., R. Webb, R. Saldarini, and J. Coppola. 1972. A radioimmunoassay for progesterone. Steroids 19(6):751-762.

Sreenan, J., and M. Diskin. 1986. The extent and timing of embryonic mortality in the cow. Embryonic Mortality in Farm Animals:1-11.

Staigmiller, R. B., B. G. England, R. Webb, R. E. Short, and R. A. Bellows. 1982. Estrogen secretion and gonadotropin binding by individual bovine follicles during estrus. Journal of Animal Science 55(6):1473-1482.

Stewart, H., F. Guesdon, J. Payne, B. Charleston, J. Vallet, and A. Flint. 1992. Trophoblast interferons in early pregnancy of domestic ruminants. Journal of Reproduction and Fertility. Supplement 45:59-68.

Stice, S. L., N. S. Strelchenko, C. L. Keefer, and L. Matthews. 1996. Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. Biology of Reproduction 54(1):100-110.

Stroband, H., and T. Van der Lende. 1990. Embryonic and uterine development during early pregnancy in pigs. Journal of Reproduction and Fertility. Supplement 40:261-277.

Szenci, O., J.-F. Beckers, P. Humblot, J. Sulon, G. Sasser, M. Taverne, J. Varga, R. Baltusen, and G. Schekk. 1998. Comparison of ultrasonography, bovine pregnancy-specific protein B, and bovine pregnancy-associated glycoprotein 1 tests for pregnancy detection in dairy cows. Theriogenology 50(1):77-88.

Telugu, B. P. V., A. M. Walker, and J. A. Green. 2009. Characterization of the bovine pregnancy-associated glycoprotein gene family–analysis of gene sequences, regulatory regions within the promoter and expression of selected genes. BMC Genomics 10(1):185.

Thatcher, W., M. Meyer, and G. Danet-Desnoyers. 1995. Maternal recognition of pregnancy. Journal of Reproduction and Fertility-Supplements only (49):15-28.

Thomas, I., and H. Dobson. 1989. Oestrus during pregnancy in the cow. The Bovine Practitioner:160-164.

Trenkle, A., and R. Willham. 1977. Beef production efficiency. Science 198(4321):1009-1015.

Trimberger, G. W., and H. Davis. 1943. Conception rate in dairy cattle by artificial insemination at various stages of estrus. Research Bulletin: Bulletin of the Agricultural Experiment Station of Nebraska No. 129.

USDA, A. 2009. Beef 2007–08 Part II: Reference of Beef Cow-calf Management Practices in the United States, 2007–08. USDA: APHIS: VS, CEAH, National Animal Health Monitoring System Fort Collins, CO

USDA, A. 2017. Beef 2017 Report 2: Beef Cow-calf Health and Management Practices in the United States, 2017. USDA: APHIS: VS, CEAH, National Animal Health Monitoring System Fort Collins, CO

Valadão, L., H. M. da Silva, and F. M. da Silva. 2018. Bovine Embryonic Development to Implantation, Embryology-Theory and Practice: 77-86.

Vejlsted, M., Y. Du, G. Vajta, and P. Maddox-Hyttel. 2006. Post-hatching development of the porcine and bovine embryo—defining criteria for expected development in vivo and in vitro. Theriogenology 65(1):153-165.

Warnick, A., and P. Hansen. 2010. Comparison of ovulation, fertilization and embryonic survival in low-fertility beef cows compared to fertile females. Theriogenology 73(9):1306-1310.

Wassarman, P. M. 1988. Zona pellucida glycoproteins. Annual Review of Biochemistry 57(1):415-442.

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

Wijma, R., M. L. Stangaferro, M. M. Kamat, S. Vasudevan, T. L. Ott, and J. O. Giordano. 2016. Embryo mortality around the period of maintenance of the corpus luteum causes alterations to the ovarian function of lactating dairy cows. Biology of Reproduction 95(5):111-114.

Williamson, N., R. Morris, D. Blood, and C. M. Cannon. 1972. A study of oestrous behaviour and oestrus detection methods in a large commercial dairy herd. I. The relative efficiency of methods of oestrus detection. Veterinary Record 91(3):50-58.

Wilmut, I., and R. Hunter. 1984. Sperm transport into the oviducts of heifers mated early in oestrus. Reproduction Nutrition Development 24(4):461-468.

Wiltbank, J. 1970. Research needs in beef cattle reproduction. Journal of Animal Science 31(4):755-762.

Wiltbank, J., E. Warwick, E. Vernon, and B. Priode. 1961. Factors affecting net calf crop in beef cattle. Journal of Animal Science 20(3):409-415.

Wiltbank, M. C., G. M. Baez, A. Garcia-Guerra, M. Z. Toledo, P. L. Monteiro, L. F. Melo, J. C. Ochoa, J. E. Santos, and R. Sartori. 2016. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 86(1):239-253.

Winters, L. M., W. W. Green, and R. E. Comstock. 1942. Prenatal development of the bovine. Minnesota Technical Bulletin 151: 3-50.

Wisnicky, W., and L. Casida. 1948. A manual method for the diagnosis of pregnancy in cattle No. 113. p 451-452. American Veterinary Medical Association 113(860):451-452.

Wooding, F. 1992. The synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta 13(2):101-113.

Wooding, F., and D. C. Wathes. 1980. Binucleate cell migration in the bovine placentome. Reproduction 59(2):425-430.

Yanagimachi, R. 1988. Mammalian fertilization. The Physiology of Reproduction:135-185.

Zemjanis, R. 1962. Diagnostic and Therapeutic Techniques in Animal Reproduction. The Williams and Wilkins Company, Waverly Press, INC.

Zoli, A. P., L. A. Guilbault, P. Delahaut, W. B. Ortiz, and J.-F. Beckers. 1992. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application for pregnancy diagnosis. Biology of Reproduction 46(1):83-92.