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# Utilizing Pregnancy Associated Glycoproteins to improve reproductive efficiency in cattle

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To the Graduate Council:

I am submitting herewith a thesis written by Sydney Taylor Reese entitled "Utilizing Pregnancy Associated Glycoproteins to improve reproductive efficiency in cattle." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Ky G. Pohler, Major Professor

We have read this thesis and recommend its acceptance:

J. Lannett Edwards, F. Neal Schrick

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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**Utilizing Pregnancy Associated Glycoproteins to improve reproductive  
efficiency in cattle**

**A Thesis Presented for the  
Master of Science  
Degree  
The University of Tennessee, Knoxville**

**Sydney Taylor Reese  
May 2017**

## **DEDICATION**

To my parents who have given me the world.

## ACKNOWLEDGEMENTS

The greatest thank you must be extended to my mentor, Dr. Ky Pohler. I am privileged to have been your first of many graduate students and am so thankful that you took a position where the winters are mild. Gratitude must also be extended to my committee members, Dr. Edwards and Dr. Schrick.

To those who my projects would not have been possible without, thank you. In Montana, Dr. Tom Geary for allowing me to spend a summer in your lab and being a constant source of advice now and in the future. Abby Zezeski deserves thanks for introducing me to lab work, the word “aliquot” and sharing words of wisdom when things got hairy. Thank you to Dr. Vasconcelo’s lab group at UNESP in Brazil and Santa Luiza Farm for sample collections. I owe a great deal to those who helped hone my lab skills, pointed me in the right direction and were forever fixing my computer problems. Thank you to Dr. Rebecca Payton and Dr. Louisa Rispoli.

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## **ABSTRACT**

Diagnosing and maintaining successful pregnancies are one of the most important components to profitable and efficient management of beef and dairy cattle operations. Pregnancy loss is a major component of reproductive inefficiency and has been investigated less intensively in beef cattle than in dairy cattle. Pregnancy Associate Glycoproteins [PAGs] are placental products which have been identified as an accurate tool for pregnancy diagnosis and as substantial evidence indicates, markers of embryo and placental competence. The aim of these two studies is to further distinguish characteristics of pregnancies based on PAG concentration. Serial embryo transfer was used in beef heifers to assess PAG concentrations of heifers of varying fertility status. There was no difference in PAG concentration between high or subfertility heifers. However, heifers which maintained pregnancy until day 60 of gestation had an increased PAG concentration compared to those that lost pregnancy between day 28 and 60 ( $P=0.051$ ). The second study examined the use of PAG concentration at day 24 of gestation as a tool for early gestation pregnancy diagnosis in Holstein dairy heifers carrying in vitro produced embryos. Circulating PAG concentration at day 24 was increased in animals that were pregnant compared to animals that were not; however, pregnancy maintenance could not be determined based on day 24 PAG concentration. Early gestation pregnancy diagnosis using PAG may be a viable option with more data and possible assay refinement for specific PAGs.

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## LIST OF ABBREVIATIONS

BNC	Binucleated Trophoblast Cell
CIDR	Controlled Intravaginal Drug Release device
CL	Corpus Luteum
EM	Embryo Mortality
EEM	Early Embryo Mortality
ELISA	Enzyme Linked Immunosorbent Assay
EPF	Early Pregnancy Factor
ET	Embryo Transfer
EV	Extracellular Vesicles
GnRH	Gonadotropin Releasing Hormone
HF	High Fertility
IFNT	Interferon Tau
ISGs	Interferon Stimulated Genes
IVP	In Vitro Produced
LEM	Late Embryo Mortality
miRNA	MicroRNA
PAG	Pregnancy Associated Glycoprotein
PGF	Prostaglandin F2 alpha
SF	Subfertility
TAI	Timed Artificial Insemination
TET	Timed Embryo Transfer

## INTRODUCTION

The United States has one of the largest cattle industries in the world. With \$78.2 billion dollars in cash receipts in 2015, cattle production is the largest commodity contributor to total agricultural cash receipts. The foundation of this industry is the cow. Cow inventory for January 2017 consisted of 31.2 million beef cows in cow-calf operations and 9.35 million lactating dairy cows (USDA NASS). In order for these cows to be productive, it is important that they have a calf each year. A study by Giordano et al. (2013) determined that an increased interbreeding interval from 30 to 60 days will decrease a dairy cows' net present value by \$200. Most beef operations wean all calves from a single breeding season at one time, meaning that a cow which calves later in the calving season will have a smaller calf. It is paramount that cattle get pregnant early in the breeding season and maintain successful pregnancies.

Sustainability is becoming a greater priority for most industries. Increased reproductive efficiency can reduce resources wasted on unproductive animals. In dairy cattle, extended interbreeding intervals can result in decreased milk production by delaying the dry off phase. Increased feed costs negatively affect both beef and dairy cows that do not calve yearly. Reproductive performance is one of the most commonly measured outputs in all fields of animal science, yet pregnancy loss is not well understood throughout all periods of gestation. Reports of pregnancy loss are highly variable and lack consistency between beef and dairy cattle data. Understanding pregnancy loss is a crucial step in improving reproductive management and increasing reproductive efficiency.

**CHAPTER I:  
MARKERS OF PREGNANCY: HOW EARLY CAN WE DETECT  
PREGNANCIES IN CATTLE USING PREGNANCY-ASSOCIATED  
GLYCOPROTEINS (PAGS) AND MICRORNAS?**

## **Publication Statement**

A version of this chapter was originally published as:

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S.T. Reese was the main author and composed a majority of the writing. M.C. Pereira provided information on manual methods of pregnancy diagnosis. Research from the labs of Vasconcelos, Smith, Green, Geary, Peres, Perry were included in the manuscript. K.G. Pohler provided data and assisted with the editing process.

## **Abstract**

Pregnancy detection has evolved over the last few decades and the importance of early pregnancy detection is critical to minimize the amount of time a cow spends not pregnant. Embryonic mortality (EM) is generally considered to be the primary factor limiting pregnancy rates in cattle and occurs early (<day 28) or late ( $\geq$ day 28) during gestation (day 0 = estrus). In cattle, the incidence of early EM is approximately 20 to 40% and the incidence of late EM is approximately 3.2 to 42.7%. Significant effort has been directed toward understanding the mechanisms resulting in early EM up to day 17; however, relatively little is known about the causes or mechanisms associated with EM after day 17. Based on work in these areas, numerous investigators are pursuing methods of early pregnancy or EM detection after day 17 of gestation. This review will highlight some of the technology and markers being used for early pregnancy detection and provide evidence for just how early

pregnancy can be detected in the bovine. Advancements in early embryonic or pregnancy detection may lead to development of strategies to overcome early gestation losses.

## **Introduction**

Successful pregnancy is the most important factor to ensure an efficient and economically sound beef or dairy operation. In order to reach that end point, reproductive loss must be avoided. Early identification of pregnancy failure is key to determining the most effective management strategies and the ability to predict this loss offers greater opportunity to minimize its impact. Loss of pregnancy may occur at any time between conception and calving; however, some time points are more critical than others. Pregnancy failure affects all cattle; however, high producing dairy cattle are more susceptible to decreased pregnancy rates than dairy heifers and beef cows (Diskin et al., 2011; Pohler et al., 2015b; Pohler et al., 2016a; Pohler et al., 2016b). Although fertilization rate in cattle is often greater than 85% (Santos et al., 2004; Diskin and Morris, 2008) there have been reports of differences in fertilization rates in beef and dairy cattle that result in a large amount of reproductive failure (Breuel et al., 1993; Sartori et al., 2002; Santos et al., 2004; Santos et al., 2009); however, the focus of this review will be on post fertilization failure and detection. In addition, fetal losses (> day 45 of gestation) are low at approximately 3% (Inskeep and Dailey, 2005). Thus, reproductive loss during the embryo stage (day 0 to 44) of development is substantial. Early embryonic loss can be classified as loss that occurs before day 28 of gestation. Although the embryonic heartbeat can be detected by this time via real-time ultrasonography, the conceptus does not yet resemble a

calf. Causes of early embryonic mortality include lethal genetic mutations, uterine asynchrony, and maternal recognition failure (Ayalon, 1978; Diskin and Morris, 2008). Early embryonic loss is generally accepted to account for 20 to 40% of pregnancy failure (Sreenan and Diskin, 1986; Santos et al., 2004; Inskeep and Dailey, 2005; Santos et al., 2009). During the late embryonic period, through day 44, growth can be characterized by the development of limb buds, eye orbits and the formation of placentomes. Although late embryo mortality accounts for less than 10% of pregnancy loss, it has significant implications for the producer and has been suggested to cause greater financial burden than early EM (Diskin and Morris, 2008). By day 45, the conceptus takes the true form of a fetus with split hooves, ribs and displays limited movement (Curran et al., 1986). After days 45 to 60, pregnancy loss decreases and is less than 5% through the second and third trimester of pregnancy.

Pregnancy failure is extremely costly to the producer. Lost revenue can be attributed to cost of feeding and managing nonpregnant cows, decreased weight of late born calves at sale time and a decreased calving percentage due to cows that lost pregnancy. In a study involving lactating dairy cows, pregnancy loss after 1 month of gestation cost producers an average of \$555 (US) due to repeat breeding expenses, increased calving interval and increased probability of involuntary culling (De Vries, 2006). In beef cattle that have had pregnancy loss and manage to become pregnant to a subsequent breeding, there is still a significant amount of lost revenue from reduced weight of late born calves and decreased uniformity of the calf crop. Pregnancy diagnosis is a very important management tool that is underutilized in the United States. According to the USDA's 2008 National Animal Health Monitoring System (NAHMS) Beef survey, only 20% of

operations utilize pregnancy diagnosis via palpation or ultrasound, a number that has remained virtually unchanged since the 1997 survey (USDA, 2010). In comparison, the 2007 NAHMS Dairy survey reported 93% of operations perform pregnancy detection (USDA, 2009). Pregnancy diagnosis can identify open cows, help estimate calving dates, and help producers make culling decisions. This review will describe various methods of pregnancy diagnosis in cattle and research advancements that may allow for earlier detection of pregnancy.

## **Established Methods of Pregnancy Diagnosis**

### ***Rectal Palpation and Ultrasonography***

As the conceptus develops during gestation, fluid accumulates, and placentation advances, methods of pregnancy detection allow for manual transrectal palpation of the uterus per rectum and its contents. Transrectal palpation of the uterus, starting as early as day 35 of gestation allows for detection of a pregnancy by palpation of fluid and the amniotic vesicle within the uterus. Palpation of the uterus and its contents is traditionally practiced from 40 to 60 days after insemination with the earliest detection limit being approximately 30 to 35 days post insemination. Additional sensitivity can be achieved during this time point by using transrectal ultrasound for pregnancy detection (Lucy et al., 2011). Ultrasound is the gold standard for determining pregnancy and confirming the presence of a viable embryo. Transrectal ultrasonography can be accurate as early as day 26 to 29 to diagnose pregnancy and visualize a discernable heartbeat (Pierson and Ginther, 1984; Kastelic et al., 1988; Beal et al., 1992). Doppler ultrasound may provide additional information based on visualization of blood flow to the placenta/conceptus; however, data

supporting its use as a pregnancy diagnosis method has been mixed. Today, ultrasound is considered the only visual indicator of pregnancy in cattle and is used for comparison with all recent attempts at diagnosing earlier pregnancy in this review. With all palpation and ultrasound techniques, a highly experienced individual is required to complete these test.

### ***Chemical Based Pregnancy Tests***

Earlier and more effective means of pregnancy diagnosis are constantly being sought and evaluated. Numerous chemical and biochemical based pregnancy tests have been developed and tested for use in cattle. Each has strengths and shortcomings that have led to their adoption or lack of use in various production schemes. One important consideration in evaluating pregnancy diagnosis tests is the difference between pregnancy specific and non-pregnancy specific methods. Pregnancy specific markers are physiologically present only in pregnant animals and produced specifically from the pregnancy; whereas non-pregnancy specific markers, while elevated during pregnancy, may be produced under other physiological conditions as well.

Progesterone is one example of a non-pregnancy specific diagnosis method. Progesterone is one of the more common chemical based pregnancy tests commercially available although overall a very small percentage of producers use it (USDA, 2009). Produced by the corpus luteum (CL), progesterone is a steroid hormone that is crucial for maintaining pregnancy; however, the cyclic profile of progesterone mandates that pregnancy detection must occur between luteolysis and the formation of a new CL. During this time period, non-pregnant cows should exhibit low progesterone levels, whereas progesterone concentrations in pregnant cows should remain elevated. Significant

differences in progesterone concentration appear between pregnant and non-pregnant cows between day 20-24 post insemination in both serum and milk. Accurate positive pregnancy diagnosis varied between 60 and 100% for milk progesterone, however detection of non-pregnant animals varied between 81 to 100% (Sasser and Ruder, 1986; Nebel et al., 1987; Nebel, 1988). Longer luteal phases in some cows, cysts or persistent follicles may play a role in elevated progesterone concentrations during the pregnancy test period that yield false positive results (Pohler et al., 2015b). Some discrepancies exist in evaluating the effectiveness of progesterone testing with regards to embryo loss. Research has shown that pregnant cows with progesterone concentration below 3.76 ng/ml at week 5 were more likely to experience embryonic mortality before week 9 than cows with a greater concentration of progesterone (Starbuck et al., 2004). However, it should be noted that a majority of cows (77%) in the low concentration group maintained pregnancy through week 9. A later study by Pohler *et al.* (2013), demonstrated that serum concentration of progesterone between day 28 to 30 in pregnant cows was not predictive of pregnancy loss between a positive pregnancy diagnosis by progesterone and final pregnancy confirmation at day 70 of gestation (Pohler et al., 2013b).

Another steroid hormone, estrone sulfate is produced by the conceptus and can be detected at day 100 of pregnancy in cattle (Holdsworth et al., 1982). Estrone sulfate is a pregnancy specific marker, though its' late period of detection limits its use in domestic cattle. Although steroid hormone pregnancy tests have largely been replaced in cattle, sheep and swine, use of estrone sulfate has been used for pregnancy detection of non-domestic animals in the wild and zoos through noninvasive fecal and urine samples.

Estrone sulfate has been successfully evaluated for use in pregnancy detection in hoofed stock, gorillas, orangutans, baboons and wild felids (Kumar et al., 2013).

Early pregnancy factor (EPF), also known as early conception factor, appears in maternal circulation shortly after fertilization. In cattle, EPF is observed within 48 hours after breeding and seems to contribute to maternal immune suppression and implantation preparation (Morton, 1998; Cordoba et al., 2001). A study by Athanasas-Platsis, *et al.* (1989) demonstrated a critical role of EPF when mice that had been immunized against EPF had decreased embryo viability and an increased incidence of pregnancy failure (Athanasas-Platsis et al., 1989). Action of EPF is not confined to pregnancy as growth factor-like properties have been seen in tumors. A large percentage of embryo loss occurs after the recommended sampling time of commercial EPF assays (48 h to 7 d). Studies have indicated that commercial EPF tests have a sensitivity (or detection of pregnant animals) of 45 to 86% and a specificity (or detection of non-pregnant animals) of 4 to 28.8% (Cordoba et al., 2001; Gandy et al., 2001). In regards to EPF as a pregnancy detection method, commercial tests are unreliable at identifying non-pregnant animals that limits its use in this capacity.

The maternal recognition of pregnancy signal in cattle and other ruminants is interferon tau (IFNT). Interferon tau stimulates CL maintenance through endocrine-like actions, blocks estrogen receptors and paracrine mechanisms on the endometrium prompt IFNT stimulated gene production. Due to the difficulty associated with detecting small concentrations of IFNT directly, research has focused on measuring IFNT- stimulated genes (ISGs) that have been upregulated in peripheral mononuclear blood cells (PBL). Conceptus IFNT mRNA concentration peaks in cattle at day 20 post-conception (Han et

al., 2006; Spencer and Hansen, 2015). Genes including ISG15, Mx1 and Mx2 are more highly expressed in peripheral blood leukocytes (PBL) of pregnant cows than non-pregnant cows (Han et al., 2006; Gifford et al., 2007). A study by Green, *et al.* (2010), showed that pregnant heifers had greater IFNT- stimulated gene expression than cows (Green et al., 2010). They concluded that a IFNT-stimulated gene based pregnancy test would be possible for heifers at day 18 but not for cows whose response is more limited based on the current sensitivity of available assays (Green et al., 2010). Unfortunately, ISGs are not unique to pregnancy which limits their use as a pregnancy detection tool to identifying non-pregnant animals (Han et al., 2006; Gifford et al., 2007; Pohler et al., 2015b); however, a resynchronization protocol can be implemented in cows that are identified as non-pregnant which improves operation efficiency (Lucy et al., 2011).

### **MiRNAs: Potential Biomarkers for Pregnancy Diagnosis**

The search for easily accessible biomarkers of various diseases and physiological states has recently focused on circulating microRNAs (miRNA). Between 18 and 22 nucleotides in length, miRNAs play important roles in regulation of gene expression and have been found in biological fluids ranging from serum and amniotic fluid to urine and milk (Reid et al., 2011; Pohler et al., 2015b). MicroRNAs are released from cells of most tissue types in plasma membrane bound extracellular vesicles (EV), especially exosomes. The packaging of miRNA in EVs or exosomes is important from a detection standpoint as RNA-ases are unable to penetrate and breakdown the miRNA allowing them to be extracted from blood and serum (Reid et al., 2011). Exosomes and EVs play a crucial role in intercellular communication, including promotion of sperm maturation, regulation of

immune function, release of miRNA for a wide array of regulatory functions, as well as other roles currently being studied (Raposo and Stoorvogel, 2013). Serum and whole blood have proved an acceptable source of EV-derived miRNA profiles, thus providing a potential blood-borne biomarker candidate for various disease and physiological states (Häusler et al., 2010; Reid et al., 2011). Human based disease research has revealed significant differences in miRNA abundance for many cancers (Lawrie et al., 2008; Häusler et al., 2010), heart disease (Tijssen et al., 2010) and sepsis (Wang et al., 2010). In addition, circulating miRNAs in maternal serum have been observed as potential biomarkers of pregnancy status due to their significant impact on gene expression and regulation (Chim et al., 2008). A study by Gilad *et al.* (2008) identified miRNAs that are increased in abundance in pregnant humans but not in non-pregnant females (Gilad et al., 2008). This finding led to the rapid expansion of identifying miRNAs that were specific to pregnancy and across various species, although none have been thoroughly explained.

An initial study of pregnancy specific markers in mares identified 7 miRNAs that were only expressed in pregnant mares (Cameron et al., 2011) compared with the non-pregnant controls. In addition, work in the sheep has confirmed the presence of miRNA in uterine lumen fluid in pregnant and cyclic sheep (Burns et al., 2014). These data support the idea for a likely role of miRNA in conceptus-endometrial interactions during the establishment of pregnancy (Burns et al., 2014). In addition, a follow up study to the one described above, provides evidence that EVs are produced from the trophoderm and uterine epithelia in the pregnant ewe and are involved in intercellular communication (Burns et al., 2016).

Many groups are now looking into miRNAs as biomarkers for pregnancy detection

in the cow. There is increasing evidence that pregnancy specific miRNAs exist and may be potential markers for pregnancy diagnosis. In 2015, exosomal miRNAs were reported to be differentially expressed in pregnant versus non-pregnant cows and cows undergoing early embryonic mortality (Pohler et al., 2015a). A recent study by Fiandanese, *et al.* (2016) identified a potential miRNA, bta-mir 140, as an early biomarker for pregnancy detection. At day 19, bta-mir 140 was up regulated in all pregnant cows, and at day 13 onwards, it was upregulated in pregnant, non-lactating cows (Fiandanese et al., 2016). Similarly, Ioannidis and Donadeu (2016) identified 6 miRNA (Day 16: bta-miR-26a, bta-miR-29c, bta-miR-138, bta-miR-204. Day 24: bta-miR-1249 Day 16 & 24: hsa-miR-4532) that were differentially expressed in pregnant heifers (Ioannidis and Donadeu, 2016). Although refinement is necessary to pinpoint ideal miRNA for pregnancy diagnosis, results indicate that miRNAs have potential as an early pregnancy detection tool. Furthermore, miRNA may provide information to denote embryonic viability. Preliminary data from our laboratory indicate cows that experience embryo mortality compared to cows that have a successful pregnancy have a significantly increased abundance of specific miRNAs at days 17 and 24 of gestation. Future studies are needed to assess the repeatability of these findings and to determine precise miRNA most applicable for embryo viability analysis.

## **Developing Early Pregnancy Diagnosis Method: Pregnancy Associated Glycoproteins**

### ***The Placenta***

Proper placentation is crucial for pregnancy development and ultimately pregnancy success. Active placentation in the cow occurs between day 28 and 40 of gestation (Aires et al., 2014). Bovine placentation involves adhesion between the maternal-caruncle

structures and fetal cotyledonary tissues to form placentomes. Superficial interdigitation begins around day 20 in cattle between microvilli of the trophoderm and uterine epithelium. True placentomes, marked by increased villi length and raised tissue in caruncular endometrium are distinct by days 31 to 33 of gestation. By day 39, placentomes are easily discernable and have long, occasionally branching villi (King et al., 1979).

Binucleated trophoblast cells (BNCs) appear in the fetal chorion of ruminants at days 18 to 19 of gestation and comprise 15 to 20% of the trophoderm throughout pregnancy. Binucleated trophoblast cells migrate to the maternal epithelium from the fetal chorion after maturation but do not penetrate past the basement membrane (King and Atkinson, 1987). Contact between the maternal and fetal interface at the microvilli junction allows migration of BNC's towards the basement membrane to begin (Wooding and Wathes, 1980; Wooding and Burton, 2008). Products of BNC's, including hormones, placental lactogen and pregnancy associated glycoproteins (PAGs), are packaged in secretory granules and enter maternal circulation across the basement membrane (Pohler et al., 2015b).

### ***PAG Production***

Pregnancy associated glycoproteins were identified in the 1980's during early attempts to develop pregnancy-specific markers that could be used for pregnancy diagnosis. Although PAGs are often thought of to be produced by BNCs, Green et al. (2000) reported that PAGs can be sorted into two separate families based on their expression in trophoblast cells (Green et al., 2000). Some PAGs are expressed in both BNCs and mononucleated trophoblast cells while others are solely produced in BNCs (Green et al., 2000). Although

their physiological role is unknown, a large number of distinct PAGs and more than two dozen specific PAG genes have been described. Based on accumulation of PAGs at the junction between uterus and placenta and known proteolytic activity of certain PAGs, it has been hypothesized that PAGs may help process growth factors or may have adhesion actions (Wallace et al., 2015). Based on evidence that PAGs may inhibit certain immune cells, they may also play a role in disguising antigens from the maternal immune system (Perry et al., 2005). After appearance of BNCs and epithelial adhesion of trophoblast, the first sizable increase in PAG concentration occurs between days 22 to 24 of gestation. Concentrations of PAG continue to increase through day 36, followed by subsequent decrease in concentration until day 60 of pregnancy followed by a steady increase through the second and third trimesters of pregnancy. In the weeks preceding parturition, a substantial increase in circulating concentrations of PAG occurs that peaks at calving. This may be attributed to significant placental growth at the end of gestation or the release of stored PAG from other tissues (Green et al., 2005; Pohler et al., 2013b). Eight weeks post parturition, PAGs are not detectable in maternal circulation (Green et al., 2005).

### ***PAGs and Pregnancy Diagnosis***

Since their discovery, PAGs have been a target for pregnancy diagnosis. Pregnancy-specific protein B was the first identified PAG of interest by scientists looking for pregnancy specific markers that could be detected early in gestation in the 1980's (Butler et al., 1982; Sasser et al., 1986). Using early assays, PAGs were detectable at day 24 of gestation in cattle; however, the physiological function was unknown which is still the case today. Discovery of multiple PAG families and genes has contributed to understanding the

increasing complexity of PAGs. The radioimmunoassay (RIA) first developed shortly after discovery of pregnancy specific protein B was the standard for PAG detection for many years (Zoli et al., 1992). This assay was highly specific and the validating study concluded that PAGs were secreted into the maternal system and were unique to pregnant animals. A study by Green et al (2005) validated an ELISA that specifically targeted PAGs secreted early in gestation that had a shorter half-life (4.3 days vs 8.4 days) than the previous targets to reduce the potential for false positives in postpartum cows (Zoli et al., 1992; de Sousa et al., 2003; Green et al., 2005). The ELISA was demonstrated to accurately detect pregnant cows via serum concentrations of PAGs at day 28 post insemination. Studies comparing the efficacy of the PAG ELISA, PAG RIA and transrectal ultrasonography revealed comparable results for the diagnosis of pregnancy in cattle at day 28 of gestation although some differences were identified in the ability of certain assays to detect non-pregnant animals (Szenci et al., 1998; Karen et al., 2015).

Commercial PAG tests are currently available using both milk and blood samples, and include BioPRYN (BioTracking LLC. Moscow, ID USA), IDEXX Bovine pregnancy test (IDEXX Laboratories Inc. Westbrook, ME USA) and DG29 pregnancy test (Genex Cooperative Inc. Shawano, WI USA). BioPRYN accepts blood samples from heifers 25 days post breeding and cows 28 days post breeding, IDEXX recommends day 28 blood or milk samples and DG29 has been validated using day 29 blood samples. At the recommended sampling time, all commercial tests provide 98-99% true positive (pregnant) reading and false positive (reported as pregnant but open) rates range from 1-5% however, some variation may be due to late embryonic mortality.

### ***Early Pregnancy Diagnosis***

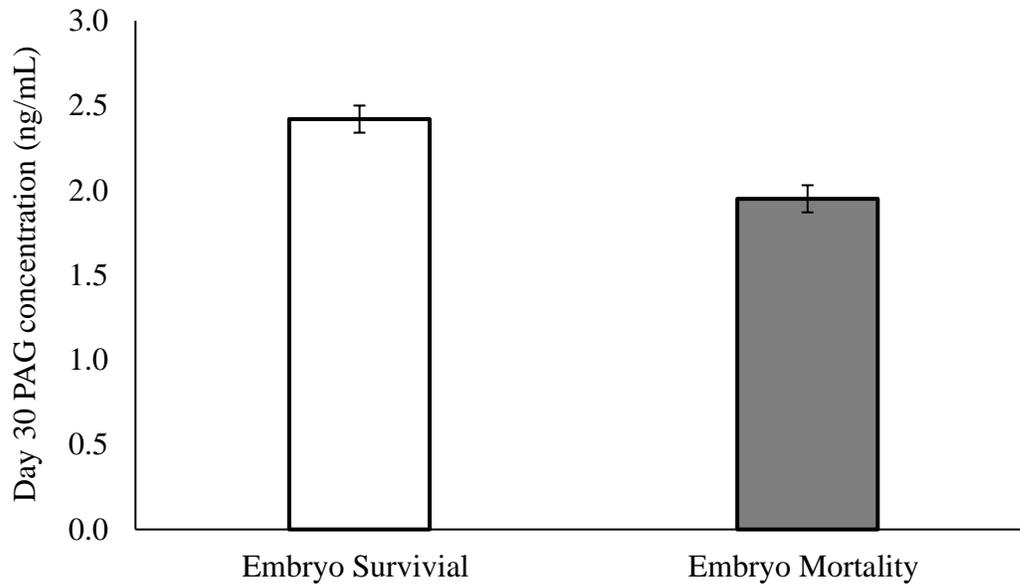
Current research is focused on finding markers and increasing sensitivity to identify pregnant cows before day 28 of gestation. Although diagnosis is limited to the time frame following the introduction of PAGs to maternal circulation at days 19 to 20, preliminary research indicates that PAGs may be effective at diagnosing pregnancy as early as day 24. Currently, research is focused on heifers, which exhibit greater PAG concentrations earlier in gestation compared with cows. A recent study in Brazil evaluated the accuracy of pregnancy diagnosis at day 24 in predominantly Holstein heifers following timed embryo transfer (Reese et al., 2016b). Serum PAG concentrations at day 24 differed between pregnant (2.98 ng/mL) and non-pregnant (0.69 ng/mL) heifers. Using receiver operating curve analysis, PAG concentrations greater than 1.39 ng/mL were 95% accurate in diagnosing a pregnant heifer at day 24 of gestation. Using a day 17 baseline sample, the difference between day 24 and day 17 samples predicted 79% of pregnancies. This, early pregnancy diagnosis using PAG is possible; however, more work is needed in this area. It is realistic to assume that day 24 PAG concentrations can diagnosis pregnancy, but high rates embryonic loss before the standard day 30 tests may decrease the efficiency and benefits of testing early. In a recent study, embryonic mortality between day 24 and 31 of gestation was 20.8% in lactating dairy cows, thus pregnancy loss following maternal recognition of pregnancy may be more prevalent than previously thought making early pregnancy diagnosis less useful (Pohler et al., 2016a).

### ***PAGs as Indicators of Embryo Success***

Recent studies have demonstrated a strong correlation between successful pregnancies and PAG concentrations during early gestation. Increased circulating PAG

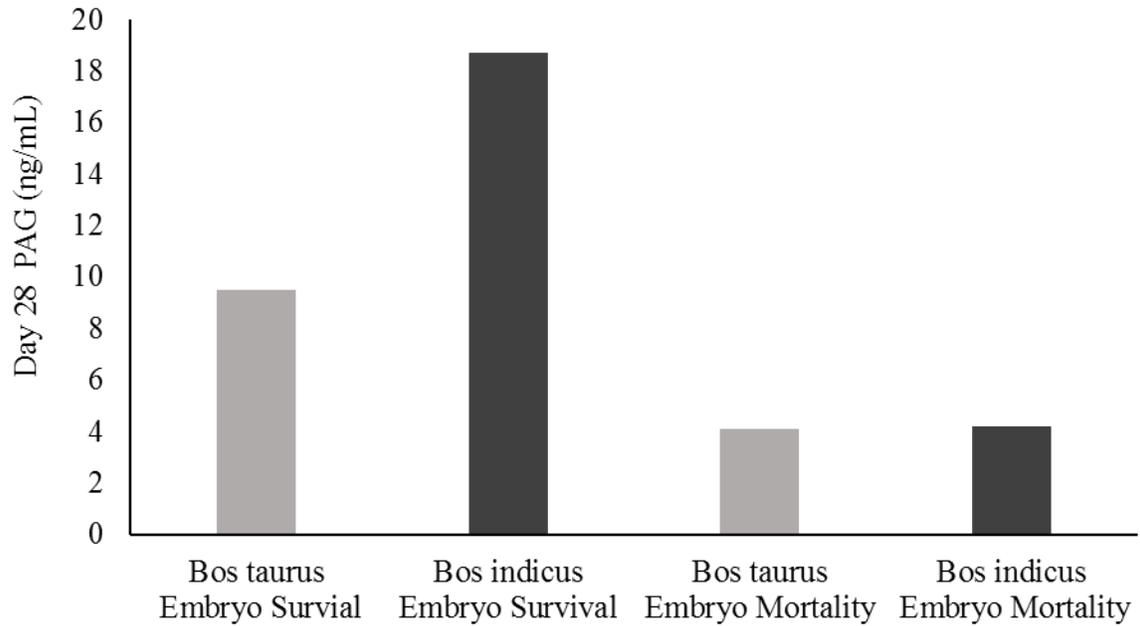
concentrations approximately day 28 of gestation are generally predictive of increased embryo survival, making PAG a likely marker for evaluating embryo viability and placental competence. In comparison to progesterone, which exhibit no difference between heifers or cows that undergo embryo mortality and those that maintain pregnancy, PAG concentrations are significantly different between heifers and cows (Figure 1; (Kill et al., 2013)). Serum PAG concentrations in cows that maintained pregnancy ( $4.53 \pm 0.34$  ng/ml) were significantly higher than cows that underwent pregnancy loss ( $3.14 \pm 0.72$  ng/ml) after fetal heartbeat detection at day 28 (Pohler et al., 2013b). All cows had a pregnancy with a fetal heartbeat at day 28, indicating a viable pregnancy at that time. Perhaps more importantly, PAGs seem to be particularly effective at identifying cows that will undergo late embryonic or early fetal mortality. As serum concentrations increased, the probability of embryo mortality significantly decreased. Late embryo mortality between days 31 and 59 was predicted with 95% accuracy if PAG concentrations were  $< 1.4$  ng/ml at day 31 after timed artificial insemination (Pohler et al., 2016a). Pohler *et al.* (2016a) demonstrated that both *Bos indicus* and *Bos taurus* cows that experienced embryo mortality had similar and lower PAG concentration at day 28 despite the significant differences between PAG concentrations of successful pregnancy in *Bos taurus* compared with *Bos indicus* cows (Figure 2).

A multitude of factors have may affect circulating PAG concentrations including subspecies, parity and sire. Despite a correlation between embryonic size and placental size, results indicate no significant relationship between PAG concentration and embryo size during early gestation (Pohler et al., 2014). Lack of correlation indicates that the decrease in PAG concentration in cows experiencing late embryonic mortality is indicative



**Figure 1: PAG Concentration from heifers expected to lose or maintain pregnancy.**

Serum concentrations of PAGs for heifers that had an embryonic heartbeat on day 30 and maintained pregnancy (Embryo survival; n= 406) or did not maintain pregnancy (Embryo mortality; n=21). Heifers that experienced embryo mortality between day 30 and 65 had significantly lower PAG serum concentrations than heifers that maintained pregnancy. Adapted from Kill et al., 2013.



**Figure 2: PAG concentration by subspecies on pregnancy outcome.**

Serum concentrations of PAGs in samples collected on day 28 of gestation from pregnant *Bos taurus* cows and *Bos indicus* cows with a viable embryo based on fetal heartbeat. Cows were then categorized into whether they maintained pregnancy until day 72 (*Bos Taurus*; n=1416) or day 100 (*Bos indicus*; n=1365) of gestation (Embryonic survival) or embryonic mortality (between days 29 to 72 or 100; *Bos Taurus*; n=171; *Bos indicus*; n=213). Beef cows that experienced late embryonic mortality had decreased ( $P<0.05$ ) circulating concentrations of PAGs on day 28 compared to cows that maintained an embryo. Adapted from Franco et al., 2016.

of impaired placental or endometrial function, not slow embryonic growth. In addition, *Bos indicus* cattle tend to have greater circulating PAG concentrations compared to *Bos taurus* cattle (Pohler et al., 2016b). Any *Bos indicus* influence in the genetic base will increase PAG concentrations over a straightbred *Bos taurus* cow (Mercadante et al., 2013). Others have reported that profiles of circulating PAG are similar between subspecies although *Bos indicus* cows may have a smaller relative increase in the weeks preceding parturition (de Sousa et al., 2003). High producing dairy cows exhibit a significant negative correlation between milk production and PAG concentration.

In a study by Lopez-Gatius *et al.* (2007), each 1 kg increase in milk resulted in a decrease in PAG of 0.08 to 0.1 ng/mL (Lopez-Gatius et al., 2007). Perhaps even more interesting are the effects of parity and sire on PAG concentrations. Heifers have consistently been reported to have the greatest PAG concentration and as parity increases, mean PAG concentration subsequently decreased in a somewhat linear fashion until the 3 or 4<sup>th</sup> parity (Lobago et al., 2009; Ricci et al., 2015; Pohler et al., 2016b). Recent work has examined sire differences on PAG concentration due to the paternal influence over trophoblast and placental development. Preliminary evidence suggests that pregnancies produced by bulls accounting for decreased rates of late EM may exhibit increased PAG concentrations compared to bulls that result in increased rates of late EM (Pohler et al., 2016b).

Estrus expression at the time of insemination or prior to embryo transfer has been directly correlated with pregnancy success in both beef and dairy cattle (Perry et al., 2005; Pereira et al., 2016). In a recent study by Pereira *et al.* (2016), lactating dairy cows undergoing TAI or embryo transfer had increased fertility and decreased embryonic

morality if they exhibited estrus versus those that did not exhibit estrus (Pereira et al., 2016). Furthermore, lactating dairy cows with pregnancy loss had decreased circulating concentrations of PAGs early in gestation (Pohler et al., 2016a), similar to the current study. In a study with postpartum beef cows, there was an increase in PAG concentrations on day 28 of gestation when comparing estrotech patch scores at TAI (day 0). Surprisingly, previous work has not demonstrated an association with pre/postovulatory estradiol or progesterone production with PAG production early in gestation (Pohler et al., 2013b). Thus, these data indicate that cows which exhibit estrus and conceive have increased circulating concentrations of PAGs on d 28 and increased likelihood of pregnancy success compared with pregnant cows that did not express estrus at TAI. Future experiments are needed in this area to truly understand this relationship and potential mechanism that is underlying this increase in PAG production.

### **Conclusion**

Although numerous detection methods can accurately diagnosis pregnancy in cattle, PAGs and pregnancy specific miRNA are biomarkers that can be used before day 30 of gestation and may be useful in predicting embryonic mortality. Although pregnancy diagnosis may be possible earlier in gestation, benefits may be mitigated by high incidence of embryonic mortality after day 24.

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**CHAPTER II:  
PREGNANCY ASSOCIATED GLYCOPROTEINS (PAGS) IN HIGH  
VERSUS SUBFERTILITY BEEF HEIFERS**

## Abstract

Reproductive inefficiency and infertility are a major financial burden to domestic livestock. Variables associated with these reproductive losses during early gestation are multifaceted including contributions from the oocyte, uterus, sperm, embryo and the placenta. This research is focused on potential placental inefficiencies that may be associated with reproductive failure. Bovine pregnancy associated glycoproteins (PAGs) produced by the binucleate cells of the ruminant placenta are used to diagnosis pregnancy and may serve as a potential marker of embryonic mortality in cattle. Increased circulating concentrations of PAGs at day 28-31 of gestation have been correlated with pregnancy and predictive of impending embryonic mortality in both beef and dairy cattle. The objective of the current study was to 1) determine whether heifer fertility level is associated with circulating concentrations of PAGs and 2) whether PAG concentrations within the same animal are repeatable across multiple pregnancies. Our hypothesis was that maternal PAG concentrations would be increased in high fertility compared to subfertile heifers but not maternally driven to be repeatable in subsequent pregnancies. Serial embryo transfer (ET;  $n=4$  rounds) was used to classify commercial Angus heifers ( $n=95$ ) as highly fertile (HF; 100% pregnancy success) or subfertile (SF; 25%-33% pregnancy success) based on day 28 ultrasound diagnosis. A single in vitro-produced embryo of high quality was transferred into synchronized heifers ( $n=265$ ) that exhibited a CL during each round of ET. Pregnancy diagnosis was repeated at day 44-45 and blood was collected at both day 28 and 44 for measurement of PAG concentrations. Pregnancy was terminated at day 44 and heifers allowed 30 days recovery before synchronization for the next ET. Only heifers that were diagnosed pregnant by ultrasound (HF:  $n=30$ , SF:  $n=53$ ) were used in this study. Serum

concentrations of PAG were quantified using an in house PAG ELISA with antibodies raised against PAGs expressed early in gestation (Green et al., 2005, Pohler et al., 2016). Serum concentrations of PAG were not different ( $P= 0.4119$ ) for HF ( $5.101\pm 0.29$  ng/mL) compared to SF ( $4.76\pm 0.27$  ng/mL) heifers at day 28 of gestation. Nor were concentrations at day 44 different ( $P=0.987$ ) between HF ( $4.41\pm 0.41$  ng/mL) and SF ( $4.40\pm 0.49$  ng/mL) heifers that remained pregnant. There was no correlation ( $P > 0.05$ ) in maternal PAG concentrations between pregnancies on day 28 or day 44 of gestation in samples obtained from HF heifers. In summary, high and subfertility heifers have similar PAG concentrations at day 28 and 44 and increases in circulating concentrations of PAG early in gestation seem to be predictive of the developing embryo's success. Thus, maternal PAG concentrations appear to be embryo rather than dam dependent since they were not repeatable over successive pregnancies within dam and were not indicative of fertility status in the dam.

## **Introduction**

In the United States, most beef heifers have a genetic predisposition and ability to reach puberty before the recommended age for first breeding (13- 15 months) due to selection for maternal characteristics and milk production in cow calf operations (Martin et al., 1992). Despite achieving puberty, a significant portion of heifers do not conceive or fail to maintain pregnancy from the first breeding attempt. Day 30 pregnancy rates in beef heifers bred by artificial insemination are often reported between 55-70% (Lamb et al., 2006; Pessoa et al., 2012; Kill et al., 2013; Mercadante et al., 2015). Reproductive failure and loss may be associated with multifaceted variables that include contributions from the

oocyte, uterus, sperm, embryo and the placenta. Research to distinguish characteristics associated with fertility have identified trends in reproductive tract score, antral follicle count and uterine environment (Bridges et al., 2013; Gutierrez et al., 2014; McNeel and Cushman, 2015). This research is focused on inherent potential placental inefficiencies that may be associated with decreased fertility in virgin beef heifers. Placental abnormalities have been regarded as a major cause of pregnancy loss, especially in pregnancies from assisted reproductive technologies including in vitro produced (IVP) embryos and somatic cell nuclear transfer cloning (Hill et al., 2000; Farin et al., 2006). Placentas of IVP embryos have more abnormalities in placentome formation, blood vessel establishment genetic expression and allantoic membrane development which may contribute to the increased abortion rates between day 30 and 90 of gestation compared to in vivo produced embryos (Hasler et al., 1995; Agca et al., 1998; Miles et al., 2004, 2005; Farin et al., 2006; Oishi et al., 2006). Pregnancy loss in the late embryonic and early fetal period is low compared to other stages of development. The major transition during this stage of pregnancy is development and growth of the placenta, anomalies during this phase may decrease the probability of a successful outcome.

Bovine pregnancy associated glycoproteins (PAGs) produced by the binucleate cells of the ruminant placenta are used to diagnosis pregnancy and have been associated with placental function (Sasser et al., 1986; Zoli et al., 1992; Green et al., 2000). Concentration of PAGs at day 28-31 of gestation are reliable for pregnancy diagnosis but have also been linked with pregnancy outcome and predictive of embryonic mortality in cattle (García-Ispuerto et al., 2013; Pohler et al., 2013b; Pohler et al., 2016a). The objective of the current study was to determine whether heifer fertility level is associated with

circulating concentrations of PAG and whether PAG concentrations within the same animal are repeatable across multiple pregnancies. Embryonic mortality data were also used to compare PAG concentrations in heifers of different fertility classifications. Our hypothesis was that maternal PAG concentrations would be increased in high fertility compared to low fertility heifers. Furthermore, we hypothesized that concentration of PAGs would be different between multiple pregnancies in a single animal.

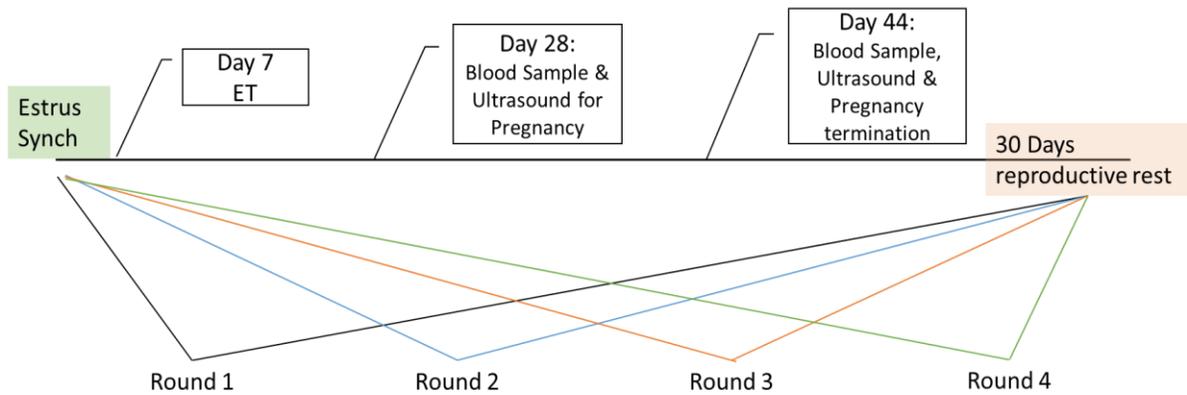
## **Materials and Methods**

### ***Animals***

All protocols and procedures were approved by Fort Keogh Livestock and Range Research Laboratory (LARRL) Animal Care and Use Committee. Study 1 of Geary et al. (2016) describes the experimental design, embryo production and animal management. Crossbred Angus × Polled Hereford beef heifers (n=265) were enrolled in a serial embryo transfer (ET) study at the LARRL in Miles City, MT.

### ***Embryo Transfer***

Estrus synchronization protocol began when the heifers were approximately 14 months old and weighed  $368 \pm 2.8$  kg. The experiment involved 4 rounds of ET occurring between August 2012 and May 2013 (Figure 3). During each round of ET, ovulation was synchronized in heifers using PG- 6d- CIDR protocol (Prostaglandin F2 alpha- 6 days-controlled intravaginal drug releasing device) where prostaglandin F2 alpha (PGF) (Lutalyse, Zoetis Animal Health) was administered on day -12. At day -9, gonadotropin releasing hormone (GnRH) (100 µg intramuscular, Factrel; Zoetis Animal Health) was administered and a CIDR inserted, followed by CIDR removal, estrus detection patch



**Figure 3: Experimental design of serial embryo transfer**

Heifers were synchronized using a prostaglandin and 6 day CIDR protocol. Embryos were transferred on day 7 after heat detection or GnRH injection. Blood samples and pregnancy diagnosis occurred on day 28 and 44. Serial ET was completed by repeating the process 4 times with 30 days of rest between pregnancy termination and initiation of the next estrus synchronization protocol.

(Estroject; Rockway, Inc.) application and another injection of PGF on day -2. Expected estrus was day 0. At day -1, heifers were evaluated 3 times daily for estrus activity. Animals that did not show signs of estrus received a dose of GnRH. Heifers were evaluated 7 days after expressed estrus or GnRH injection using a transrectal ultrasound (Aloka SSD 3500V with a 7.5 MHz convex transducer) to determine presence and location of a CL. Heifers with a CL were implanted with a high quality fresh in vitro produced morula or early blastocyst stage embryo. Embryos were produced at the University of Florida as described in Geary et al. (2016) using undefined, abattoir derived ovaries and high fertility semen from 3 pooled bulls was used to fertilize oocytes from a field of 20 bulls of various breeds. Ultrasound pregnancy diagnosis occurred on day 28 of gestation with a confirmation of pregnancy on day 44-45 via visible fetal heartbeat to detect embryonic loss. Blood samples were collected from the coccygeal vein via 10 mL vacutainer on day 28 and 44 of gestation for PAG concentration analysis. Blood was allowed to clot at room temperature for one hour for before being stored at 4°C for 24 hours. Following centrifugation of clotted blood samples at 1,200 x g for 25 minutes, serum was collected and stored at -20°C. At day 44, remaining pregnancies were terminated with PGF and heifers were allowed a minimum 30 days for recovery before resynchronization began.

Heifers were classified as highly fertile (HF; 100% pregnancy success) if they had a CL in at least 3 rounds, received an embryo each time they exhibited a CL and maintained each pregnancy until the first pregnancy diagnosis. Heifers were designated subfertile (SF; 25- 33% pregnancy success) if they had a CL in 3 or 4 rounds and received an embryo but were only diagnosed pregnant by ultrasound at day 28 one time. Heifers that did not maintain any pregnancy until day 28 after receiving embryos in each

round were classified as infertile (IF; 0% pregnancy success). Only heifers that were diagnosed pregnant by ultrasound at least one time at day 28 (HF: n=30, SF: n=53) were used in this study.

### ***Assay***

Serum concentrations of PAG were quantified using an in house PAG ELISA with antibodies (Ab63) raised against PAGs expressed early in gestation (Green et al., 2005, Pohler et al., 2016). All samples were ran in duplicates. Each plate had a serial dilution protein standard, non-pregnant pooled cow serum and third trimester pregnancy pooled cow serum control.

### ***Statistical Analysis***

Chi square test in SAS “Proc Freq” was used for analyzing pregnancy rates between rounds (SAS 9.4, SAS Institute). One way ANOVA using “Proc GLM” was used to determine differences between serum PAG concentrations in day 28 and day 44 samples. Outlier analysis was also used in some comparisons. Samples were considered to be outliers if they deviated more than 2 standard deviations away from the mean. Least significant difference test was used to compare PAG concentrations.

## **Results**

### ***Pregnancy Diagnosis***

Estrous behavior was seen in 74.9% of heifers that received an embryo after confirmation of a present CL. Overall day 28 pregnancy rates from ET was 55%. While pregnancy rates in individual rounds of ET ranged between 51% (Round 4) and 59% (Round 1), there was no difference in pregnancy rate between rounds ( $P= 0.6760$ ).

Subfertile heifers had a decreased pregnancy rate compared to high fertility heifers which had positive pregnancy diagnosis at day 28 during all 4 rounds of ET (HF= 100% pregnancy rate). Pregnancy rate of subfertile heifers ( $n=53$ ) reached a high of 42% in Round 1 and a low of 30% in Round 4; however, there was no difference in sub fertility pregnancy rate by round ( $P= 0.4608$ ). Day 44 pregnancy rate averaged 48%.

### ***Late Embryonic Loss***

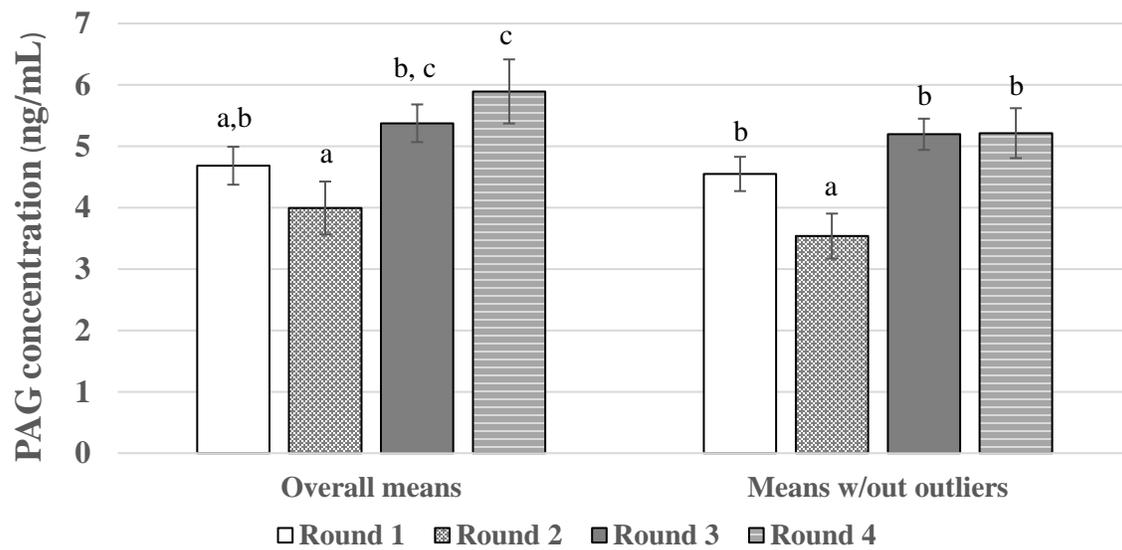
Late embryonic mortality (LEM), confirmed by absence of a viable heartbeat, between day 28 pregnancy diagnosis and day 44 pregnancy diagnosis differed between high and sub fertility groups ( $P= 0.0056$ ). Embryonic loss ranged from 10-65% in four rounds with 24.24% of all pregnancies ending in LEM. High fertility heifers experienced less late embryonic loss (15.76%) than sub fertility heifers (32.71%); however pregnancy loss from round 2 was abnormally high. Table 1 summarizes late embryonic loss.

### ***PAG Concentration***

The average PAG concentration at day 28 was  $4.913 \pm 0.202$  ng/mL. There were 10 samples identified as outliers due to being more than 2 standard deviations from the mean that were removed in stated analyses. Serum concentrations of PAG were elevated but not different ( $P= 0.4119$ ; without outliers,  $P=0.3496$ ) for HF ( $5.1035 \pm 0.2885$  ng/mL) compared to SF ( $4.761 \pm 0.272$  ng/mL) heifers at day 28 of gestation. Concentration of PAGs differed by round, specifically Round 2 had decreased circulating PAG concentration at day 28 ( $P= 0.0069$ ; w/out outliers  $P= 0.0025$ ) (Figure 4). There was no difference between the mean concentration of pregnant HF and SF heifer in any round

**Table 1: Late embryonic loss between days 28-45 of gestation by fertility**

	Embryonic loss rate	
	High Fertility	Subfertility
Round 1	10.34% (3/29)	11.54% (3/26)
Round 2	24.14% (7/29)	65.00% (14/20)
Round 3	17.86% (5/28)	28.00% (7/25)
Round 4	10.71%(3/28)	26.32% (5/19)
<b>Overall</b>	<b>15.76%</b>	<b>32.71%</b>



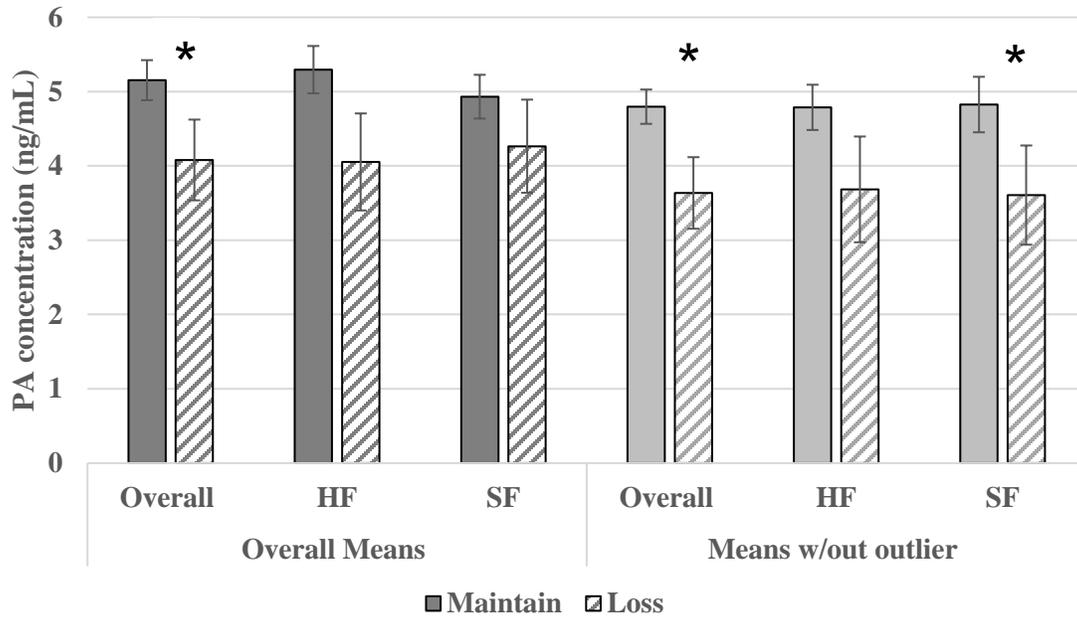
**Figure 4: PAG concentration by round**

Circulating PAG concentration of pregnant heifers varied by round ( $P= 0.0069$ ; without including outliers,  $P= 0.0025$ ). Round 2 had significantly lower PAG concentration than round 3 and round 4, as well as round 1 when outliers were removed.

( $P= 0.2878$ ). Concentrations at day 44 were not different ( $P= 0.9869$ ) between HF ( $4.406\pm 0.412$  ng/mL) and SF ( $4.395\pm 0.494$  ng/mL) heifers that remained pregnant.

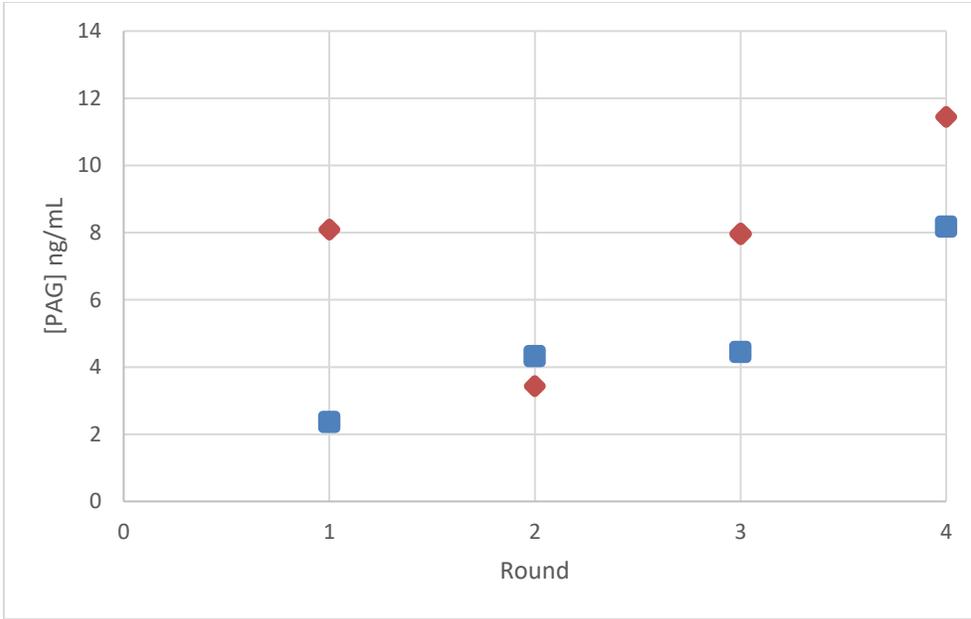
Heifers that experienced embryonic loss between day 28 and 44 had a lower PAG concentration ( $4.166\pm 0.447$  ng/mL) at day 28 compared to heifers that maintained pregnancy ( $5.154 \pm 0.269$  ng/mL;  $P=0.0519$ ) (Figure 5). When outliers were removed, the effect was more prominent ( $P= 0.005$ ). The difference was more pronounced in high fertility heifers where LEM pregnancies had  $4.053\pm 0.653$  ng/mL and animals that maintained pregnancy had a PAG concentration of  $5.295 \pm 0.319$  ng/mL ( $P=0.1295$ ; without outliers,  $P= 0.0693$ ). There was no difference between SF heifers that lost or maintained pregnancy ( $p=0.281$ ); however, the difference became significant when outliers were removed. It may be interesting to note that in round 2 where there was a high incident of late embryonic failure in the SF group, SF animals that had a successful pregnancy had an increased average PAG concentration ( $5.479 \pm 1.63$  ng/mL) over the HF group which maintained pregnancy ( $3.683 \pm 0.52$  ng/mL). Pregnancies maintained in round 2 had the lowest PAG concentration among the high fertility group.

In order to assess the repeatability of a PAG concentration from a maternal perspective, PAG concentrations of successive pregnancies from HF heifers of each round were compared. There was no correlation ( $P > 0.05$ ) in maternal PAG concentrations between pregnancies on day 28 or day 44 of gestation in samples obtained from a single HF heifers (Figure 6). Variability was seen between pregnancies in each round to a single high fertility heifer. Thus, concentration variation seen across a single heifer did not affect



**Figure 5: Circulating PAG concentration of fertility groups by pregnancy outcome**

Circulating PAG concentration (ng/mL) was significantly higher ( $P= 0.0519$ ; without including outliers,  $P= 0.005$ ) in heifers (combined SF and HF) that maintained pregnancy ( $5.1539 \pm 0.269$  ng/mL; without including outliers,  $4.797 \pm 0.2308$  ng/mL) compared to the group suffered pregnancy loss ( $4.080 \pm 0.544$  ng/mL; without including outliers,  $3.636 \pm 0.4815$  ng/mL). After removing outliers, maintained pregnancies in SF heifers had an increased PAG concentration compared to lost pregnancies from SF heifers ( $P= 0.03$ ). HF heifer pregnancies which were maintained had a tendency to be increased over HF pregnancies which were lost ( $P=0.0693$ ) when outliers were removed.



**Figure 6: PAG concentration of individuals across rounds**

Individuals subjected to for rounds of ET had day 31 PAG concentrations that were not repeatable across pregnancies. Two representative animals' (A, square marks; B, diamond shaped marks) PAG concentrations are depicted in each of 4 rounds of ET in which they maintained pregnancy.

pregnancy success eluding to serum PAG concentration not being exclusively driven maternally.

### **Discussion**

In the current study, concentration of PAGs did not differ between high and subfertility heifers. Individual merit is not easily identified until multiple pregnancies have either failed or been successful. Heifers that were repeatedly able to get pregnant did not have higher circulating PAG concentration compared to heifers which were not able to establish consecutive pregnancies. Although factors related to the production of the embryos including sire were not able to be analyzed, inconsistencies among PAG concentrations of multiple pregnancies in a single animal reveal drivers of PAG concentration to be outside of maternal contributions.

Uterine deficiencies have been identified that impact fertility of a single ovulation including regulation of steroid hormone receptors in the endometrium (Okumu et al., 2010; Bridges et al., 2013) and concentrations of estradiol and progesterone at critical time periods (Forde et al., 2009; Forde et al., 2010; Jinks et al., 2013). The physiological characteristics which differentiate reproductively successful, high fertility from less successful, subfertile animals are complex and only partially understood. Reproductive traits are not highly heritable based on phenotype based factors and potential differences based on hormone concentration have not been distinguished, both of which were substantiated in this study. Heifers in both HF and SF groups came from similar genetic backgrounds as they all originated from the same cowherd. In previously published data of this specific model, there was no difference in progesterone concentration of pregnant

individuals between HF and SF groups (Geary et al., 2016). However, genotyping, DNA/RNA sequencing and SNP identification may provide greater clarity to describing the fertility of an individual animal (Moore et al., 2015; Khatib, 2016; Moran et al., 2017). In the present set of animals, Geary et al. (2016) identified differences in endometrial gene expression through RNA sequencing between the HF and SF groups, especially related to immune system factors. Heifers in this study are similar in age, raised in the same environment and developed under the same management limiting the influence of environment to best express genetic fertility potential of the individual. Pregnancy rates from ET tend to be decreased compared to natural service or AI. In this study, pregnancy rate at day 28 was 55% which is comparable to other studies utilizing ET in beef cattle (Nasser et al., 2004; Jinks et al., 2013; Pohler et al., 2013b).

Using circulating PAG concentration as a predictive measure of embryo success has been reported by multiple groups (Chavatte-Palmer et al., 2006; Gábor et al., 2007; Breukelman et al., 2012; Mercadante et al., 2016; Pohler et al., 2016a). Pohler et al. (2013) correlated this relationship substantiated in a number of studies in heifers and cows of both *Bos indicus* and *Bos taurus* origin. (Pohler et al., 2013b; Pohler et al., 2016a; Pohler et al., 2016b). In the current study, low PAG was associated with pregnancy loss ( $p=0.0519$ ). Concentrations of PAG greater than 1 ng/mL detected 94.4% of day 28 pregnancies in this study. Of the pregnant heifers that had less than 1 ng/mL circulating PAG at day 28, 36.6% lost pregnancy between day 28 and 44. This set of heifers contributes to the growing evidence that in a group of cows, animals that experience embryo mortality will have lower PAG concentration than animals that have a successful pregnancy.

Average PAG concentration at day 44 was lower than day 28 which is expected based on PAG production curves. Concentrations of PAG increase in maternal circulation from approximately day 20 of gestation until day 36 to 38, coinciding with active placentation and fusing of the fetal and maternal tissues. A steady decline in circulating PAG concentration is observed from day 40 to day 56 of gestation (Pohler et al., 2013b). Ranking the rounds by percent of LEM were the same for both HF and SF groups, with round 1 having the lowest LEM followed by rounds 4, 3 and finally round 2 having the highest amount of LEM for both groups. Although embryos were of high quality and produced in the same way, this may also indicate an embryo Round 2 had the greatest LEM, highest SF PAG concentration and the lowest HF and overall PAG concentration. The cause for this increase in pregnancy loss is unknown; however, HF heifers were less effected and all embryos throughout the study were produced according to consistent protocols.

Circulating concentration of PAGs from one pregnancy to the next in a single animal has not yet been reported. In the current study, PAG concentration was not repeatable across multiple pregnancies in a single HF heifer ( $P>0.05$ ) (Figure 12.) Embryo based factors appear to have a strong influence on PAG concentrations of a specific pregnancy. Data from our lab also indicates that sire of a pregnancy plays a role in PAG production (Franco et al., 2016; Pohler et al., 2016b). Embryos produced for this study may be sired by as many as 20 different bulls that were represented in the pooled semen that was used to fertilize oocytes. Franco et al. (2016) reported higher PAG concentration in *Bos taurus* sired pregnancies compared to *Bos indicus* sired pregnancies in Nelore cows which provides further evidence for embryo derived factors driving PAG concentration.

Additionally, some studies report differences in PAG concentration between pregnancies from IVP produced embryos compared to in vivo produced pregnancies (Pohler et al., 2013b), while others find no differences (Breukelman et al., 2005; Breukelman et al., 2012). As products of trophoblast cells, differences in PAG concentration from pregnancies of in vitro produced embryos may be supported by the higher frequency of abnormalities which accompany the placental development of these pregnancies (Miles et al., 2005; Farin et al., 2006). Mean circulating PAG concentration was similar between HF heifers and SF heifers, especially in heifers that maintained pregnancy (HF, maintain  $5.295 \pm 0.319$  ng/mL; SF, maintain  $4.932 \pm 0.544$  ng/mL). This data indicates that PAG concentration is not indicative of fertility status, thus provides support for embryo driven factors driving PAG concentration rather than maternal contributions. This combination of factors may describe why PAG concentration cannot be solely driven through maternal influence.

### **Conclusion**

In summary, high fertility heifers and subfertility heifers have similar PAG concentrations at day 28 and day 44 of gestation. Successful pregnancies had a greater PAG concentration at day 28 than pregnancies which suffered embryo mortality. Therefore, increases in circulating concentrations of PAG early in gestation seem to be predictive of the developing embryo's success but not indicative of the fertility status of the dam. Maternal PAG concentrations appear to be embryo rather than dam dependent since they were not repeatable over successive pregnancies within dam and similar across fertility groups.

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**CHAPTER III:  
PREGNANCY DIAGNOSIS IN DAIRY CATTLE USING  
PREGNANCY ASSOCIATED GLYCOPROTEIN (PAG)  
CONCENTRATION AT DAY 24 OF GESTATION**

## Abstract

The ability to diagnose and identify successful pregnancies early in gestation has important economic and management applications for dairy cattle producers. Currently, producers are essentially limited to day 28 to 30 of gestation as the earliest time point for pregnancy diagnosis due to the effectiveness of most ultrasound and chemical based methods, including pregnancy associated glycoproteins. Pregnancy associated glycoproteins (PAGs) are produced by the ruminant placenta and can be used to accurately detect pregnancy as early as day 28 of gestation using commercially available tests. Recent data from our lab suggest pregnancy status can be determined by PAG as early as day 24. The objective of the current study was to determine if early gestation circulating PAG levels could be used to diagnose pregnancy in dairy cattle undergoing embryo transfer and predict an animal's likelihood to maintain pregnancy. In vitro produced embryos were transferred into estrus synchronized Holstein x Gir crossbred cows and heifers on day 7 following ovulation. Experiment 1 utilized only cows ( $n=101$ ) determined to be pregnant on day 24 of gestation following timed embryo transfer (TET). Heifers ( $n= 111$ ) and cows ( $n=242$ ) were used in experiment 2. In both experiments, blood was collected at day 24 for PAG analysis as well as day 31 for confirmation of pregnancy. Final pregnancy confirmation occurred on day 60 via transrectal ultrasonography. Serum concentrations of PAG were quantified using an in house PAG ELISA with polyclonal antibodies (experiment 1, Ab45; experiment 2, Ab63) raised against PAGs expressed early in gestation. Following TET in experiment 1 of the 101 cows diagnosed as pregnant on day 24, 77 cows were identified as still pregnant on day 31 of gestation (77%) using ultrasound

and PAG testing. Experiment 2 had an overall pregnancy rate at day 31 of 33.7% of total embryos transferred. Mean circulating PAG concentration at day 24 differed between pregnant and non-pregnant animals in both experiments (experiment 1,  $2.9635 \pm 0.262$  ng/mL vs  $0.94619 \pm 0.168$  ng/mL and experiment 2,  $1.962 \pm 0.261$  ng/mL vs  $0.707 \pm 0.114$  ng/mL). Concentration of PAG between pregnant and non-pregnant cows in experiment 1 and 2 was significant ( $p \leq 0.0001$ ), however, pregnant heifers in experiment 2 ( $1.562 \pm 0.266$  ng/mL) had concentration of PAGs that were not statistically different than non-pregnant heifers (non-pregnant,  $0.799 \pm 0.29$  ng /mL) ( $p= 0.0669$ ). A receiver operating characteristic (ROC) curve identified a predictive cutoff value for diagnosing pregnancy at 90% confidence at 2.5 ng/mL in experiment 2. Only animals that were pregnant at day 31 were analyzed in late embryo mortality analysis (experiment heifers,  $n= 54$ ; cows,  $n=159$ ), defined as pregnancy loss between day 31 and 60. Between day 31 and 60, 39 (11 in experiment 1 and 28 in experiment 2) animals experienced late embryo mortality. Circulating concentrations of PAG were not significantly different at day 24 of gestation in animals that maintained pregnancy until day 60 than animals that lost pregnancy between day 31 and 60 (late embryo mortality, LEM) ( $P> 0.05$ ); however, in experiment 2 the means of pregnant animals were numerically higher than animals that experienced LEM. In summary, early gestation circulating PAG concentration may have application in diagnosing pregnancy at day 24 gestation and more work is needed to determine the potential of early gestation PAGs predicting embryo loss in dairy.

## **Introduction**

Poor reproductive efficiency in the dairy industry has been marked by decreasing cow fertility associated with selection for increased milk production over the last 40 years (Lucy, 2001). Some measures of reproductive efficiency, such as days to first breeding after calving, have improved over the last 20 years due to increased adoption of technology such as estrus synchronization and advancements in timed breeding protocols (Norman et al., 2009). However, number of breedings per lactation (NB) has increased from 2.0 NB in the 1980's to 2.5 NB in 2006 (Norman et al., 2009). The number of days between calving and last breeding have stabilized over the last 10 years; however, 60 days exist between first breeding and last breeding in an average lactating cow which could be reduced (Norman et al., 2009). Identifying cost effective and accurate methods of detecting non-pregnant cows is a priority for decreasing the interbreeding interval and increasing reproductive efficiency of dairy cattle.

Pregnancy detection by palpation, ultrasound or chemical based pregnancy diagnosis tests is utilized by 93% of dairy producers in the United States (USDA, 2009). Pregnancy diagnosis is currently limited to day 28 of gestation by commercial Pregnancy Associated Glycoprotein (PAG) pregnancy diagnosis assays are effective or when a heartbeat can be seen via ultrasound. Palpation is further restricted, even when performed by an experienced technician, between day 35 and 40 for accurate diagnosis (Youngquist, 1997; Fricke, 2002). Chemical based pregnancy tests are increasing in popularity. Three commercial platforms offer pregnancy diagnosis assays by detection of PAGs in blood and milk samples beginning at day 28 gestation. Pregnancy associated glycoproteins are placental products secreted into maternal circulation beginning around day 22 of gestation. A study

by Pohler et al. (2013) pinpoints day 24 as the first significant increase in detectable PAG concentration in beef cows undergoing timed artificial insemination (TAI) (Pohler et al., 2013b). Early pregnancy diagnosis would reduce the interbreeding interval and allow for early resynchronization for breeding.

Late embryonic mortality poses a significant economic challenge and increases the interbreeding interval of animals that experience it compared to early embryonic loss before day 30 of gestation (Giordano et al., 2013). Late embryonic mortality in lactating dairy cattle is around 12%, however variation between farms, parity and seasonality results in a range from 10-20% (Wiltbank et al., 2016). Research indicates that PAGs have potential to be strong indicators of embryonic success in all types of cattle (Thompson et al., 2010; Breukelman et al., 2012; Pohler et al., 2013b; Pohler et al., 2016b). Lactating dairy cows with increased concentration of PAGs at day 31 have a higher likelihood of pregnancy success while cows with decreased concentration of PAGs have a greater likelihood of embryo mortality (Pohler et al., 2016a). Combining the potential of PAGs as an early gestation pregnancy diagnosis marker and indicator of embryo/pregnancy success, the objective of this study was to determine if 1) circulating day 24 PAG concentrations could be used to diagnosis pregnancy in dairy cattle and 2) pregnancy maintenance could be predicted via day 24 PAG concentration until day 60 of gestation.

## **Materials and Methods**

### ***Animals***

All protocols followed the guidelines recommended in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*. Animals (n=456) were housed at a commercial dairy in Minas Gerais, Brazil.

### ***Embryo Transfer***

All animals were synchronized with the following protocol; an intravaginal progesterone (P4) insert containing 1.9 g of P4 (Zoetis, São Paulo, Brazil) and 2.0 mg (i.m.) of estradiol benzoate (2.0 mL of Estrogen, Farmavet, São Paulo, SP, Brazil) on day -11, 25 mg (i.m.) of dino-prost (PGF; 5.0 mL of Lutalyse, Zoetis) on day -4, intravaginal-P4-insert withdrawal and 1.0 mg (i.m.) of estradiol cypionate (0.5 mL of E.C.P., Zoetis) on day -2 and ovulation was assumed on day 0. High quality morula or early blastocyst fresh in vitro produced embryos from Holstein donors and high fertility, frozen thawed Holstein semen were transferred on day 7 via TET.

### ***Experiment 1***

This study used 101 lactating Girolando cows averaging  $109 \pm 10.6$  days in milk as described in Pohler et al. (2016a) experiment 3. In order to be included, all 101 cows were considered pregnant at day 24 based on the following criteria (1) a well vascularized CL detected by Doppler ultrasound was present ipsilateral to where the embryo was deposited, (2) circulating progesterone concentration was greater than 1 ng/mL on day 24 and (3) PAGs were detected in day 24 serum samples (concentration  $>0.28$  ng/mL). Number of embryos transferred at day 7 to identify 101 pregnant cows at day 24 is unknown. Blood samples were collected via venipuncture in 10 mL red top vacutainer tubes on day 24 and

31. Transrectal ultrasound confirmed pregnancy via embryo/fetal heartbeat on day 31 and 59 of gestation.

Serum concentrations of PAG were quantified using an in house sandwich ELISA protocol established by Green et al (2005) modified using a polyclonal antibody raised against early secreted PAGs (Ab 45) that is identical to the assay used in Pohler et al. (2013a). All samples were ran in duplicates. Each plate had a serial dilution protein standard, non-pregnant pooled cow serum and third trimester pregnancy pooled cow serum control.

### ***Experiment 2***

The second experiment used Gir × Holstein cows (n= 242) and pubertal heifers (n= 111) ranging from ½ to 7/8 Holstein. Blood samples were collected at day 17 to provide a baseline PAG concentration, day 24 for early pregnancy diagnosis and day 31. All samples were processed according to the same protocol as experiment 1. Transrectal ultrasound was used to determine pregnancy status on day 31 and day 60 of gestation via fetal heartbeat. Pregnancy loss was defined as the absence of an embryo/fetus or an embryonic/fetal heartbeat.

Concentrations of PAG were quantified using the same in house sandwich ELISA protocol established by Green et al (2005) as experiment 1; however it was modified using a different, recently validated polyclonal antibody raised against early secreted PAGs (Ab 63) (Pohler et al., 2016b). Plates were laid out the same as experiment 1 with all samples run in duplicates, a serial dilution protein standard, a non-pregnant pooled cow serum and a third trimester pregnancy pooled cow serum control.

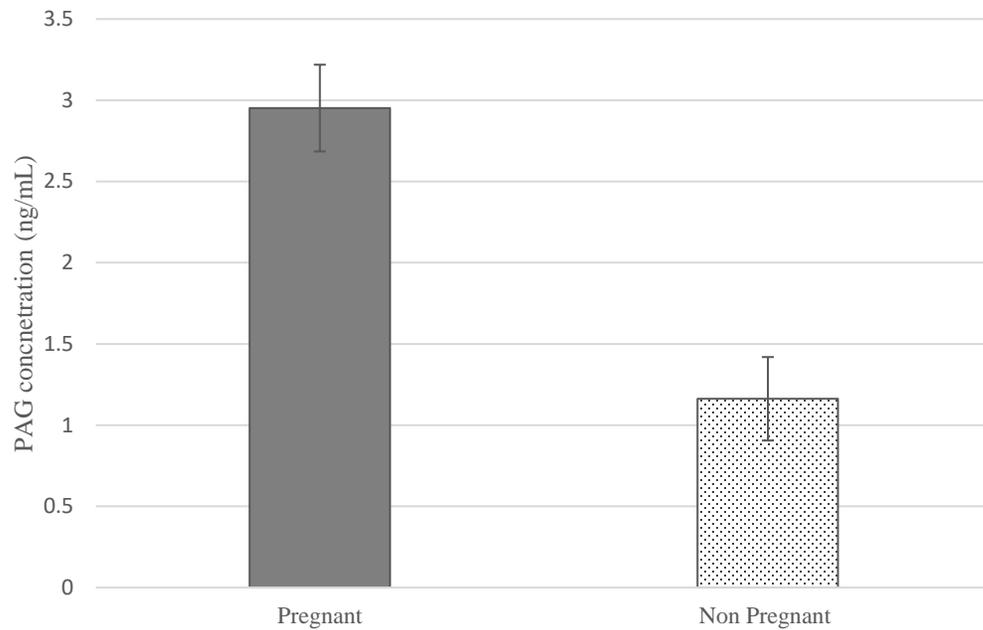
### ***Statistical Analysis***

Statistical analysis was conducted using a one way ANOVA in “PROC GLM” (SAS Institute Inc., Cary, NC) to test for differences in circulating PAG concentration. MedCalc software was used to create Receiver Operating Characteristic (ROC) Curve where pregnancy was designated as the true positive. Predictive value analysis based on the ROC curve was used to determine a PAG concentration where a cow could be diagnosed as pregnant with 90% confidence.

## **Results**

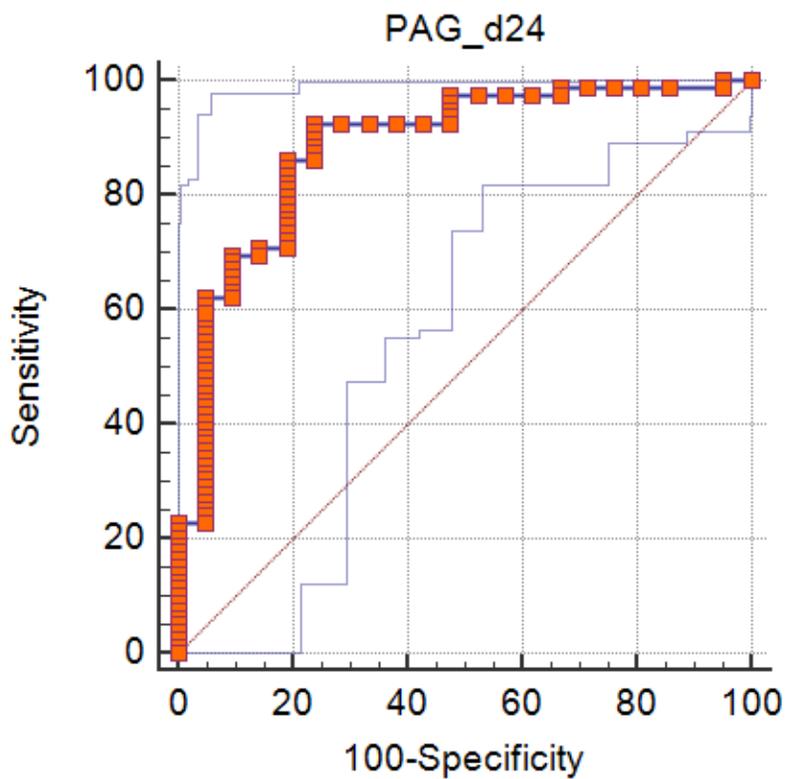
### ***Experiment 1***

Of 101 cows which were diagnosed pregnant on day 24, 80 were pregnant on day 31 via embryonic heartbeat. Raw transfer numbers were not available experiment 1; thus, pregnancy rates were not analyzed. Loss between day 24 and 31 was 20.8%. Mean circulating PAG concentration at day 24 of cows diagnosed as pregnant on day 31 of gestation was  $2.9635 \pm 0.262$  ng/mL (mean  $\pm$  SEM, range 0.445 to 14.995 ng/mL). Cows identified as non-pregnant at day 31 had a significantly lower ( $P < 0.001$ ) day 24 circulating PAG concentration ( $0.94619 \pm 0.168$  ng/mL, range 0.365 to 3.750 ng/mL) compared to pregnant animals (Figure 7). A ROC curve was used to test the potential effectiveness of day 24 PAG concentration and had an area under the curve of 88.4% (Figure 8). In this set of samples using polyclonal antibody Ab45, a positive (pregnant) predictive value was identified at 1.635 ng/mL for 90% confidence and 1.865 ng/mL for 95% confidence. There were too few negative (non-pregnant) outcomes to establish a predictive value to identify non pregnant animals.



**Figure 7: Day 24 PAG concentration in experiment 1**

Serum PAG concentration of samples collected at day 24 from cows diagnosed as pregnant at day 24. Cows were subsequently grouped by pregnancy status at day 31 of gestation as Pregnant ( $n= 80$ ) or Non Pregnant ( $n= 21$ ). Pregnant cows had a significantly higher ( $P < 0.0001$ ) circulating PAG concentration at day 24 ( $2.9635 \pm 0.262$  ng/mL) than Non-Pregnant cows ( $0.94619 \pm 0.168$  ng/mL).



**Figure 8: Day 24 ROC curve- Ab45**

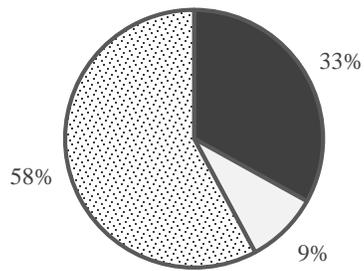
Receiver operating characteristic (ROC) curve was used for predictive value analysis. The area under the curve was 88.4% and a positive predictive value at 95% confidence to diagnosis pregnancy at day 24 of 1.865 ng/mL.

Pregnancy loss between day 31 and 60 occurred in 12 cows, resulting in a LEM rate of 11.8%. Circulating PAG concentration at day 24 was  $2.829 \pm 0.241$  for cows that maintained pregnancy (n=68) and  $3.715 \pm 1.098$  ng/mL for cows (n=12) experienced embryo mortality.

### ***Experiment 2***

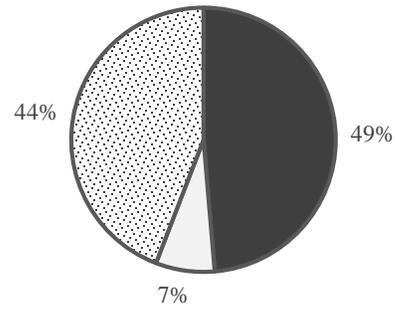
A total of 134 of the 353 animals were diagnosed pregnant at day 31 gestation based on presence of embryonic heartbeat, presenting a pregnancy rate of 33.7%. Pregnancy rates were lower in cows (33.1%) than in heifers (48.6%) (Figure 9). Animals were identified as have likely lost a pregnancy between day 24 and day 31 if the day 24 samples had a PAG concentration increase of at least 1 ng/mL compared to the day 17 sample but were not pregnant at day 31. A total of 8 heifers and 22 cows were not pregnant after a PAG increase from day 17 to 24. Animals diagnosed at day 31 as pregnant had a higher day 24 circulating PAG concentration ( $1.962 \pm 0.261$  ng/mL) than animals diagnosed as non-pregnant ( $0.731 \pm .109$  ng/mL;  $P < 0.0001$ ). Concentrations of PAG at day 24 in pregnant cows was  $2.232 \pm 0.369$  ng/mL; while non-pregnant cows had a decreased circulating concentration of  $0.707 \pm 0.114$  ng/mL ( $P < 0.0001$ ) (Figure 10). Pregnant heifers had a tendency for higher PAG concentration ( $1.562 \pm 0.266$  ng/mL,  $P = 0.0669$ ) than non-pregnant heifers ( $0.799 \pm 0.290$  ng/ mL); however, it was not significantly different than pregnant cows ( $P = 0.1309$ ). There was no difference at day 24 between cows with breed variation ( $P > 0.05$ ). At day 31, pregnant animals had a mean circulating PAG concentration of  $8.605 \pm 0.439$  ng/mL whereas non-pregnant animals have a concentration of  $1.243 \pm 0.248$  ng/mL ( $P < 0.0001$ ).

Cows



■ Pregnant   ■ Likely EM   ■ Non Pregnant

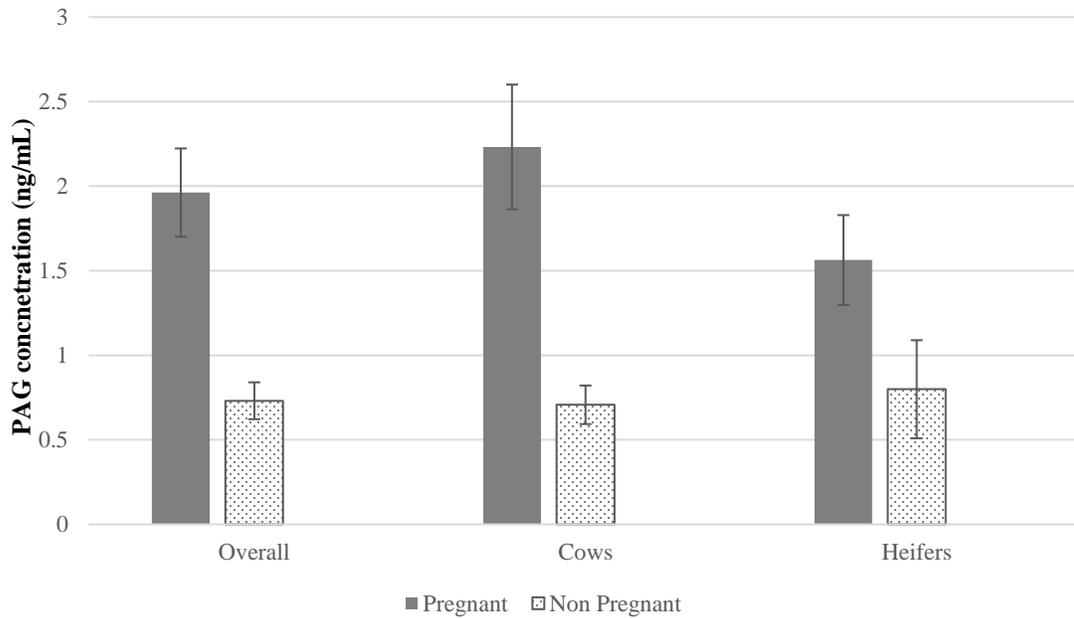
Heifers



■ Pregnant   ■ Likely EM   ■ Non pregnant

**Figure 9: Experiment 2 pregnancy diagnosis results**

Overall pregnancy rate for experiment 2 was 33.7% at day 31. Pregnant animals at day 31 are indicated by the solid portion of the pie charts (54 heifer and 80 cows). Cows ( $n=22$ ) and heifers ( $n= 8$ ) that had an increased PAG concentration at day 24 compared to day 17 but were not pregnant at day 31 were identified as likely EM (white portion). Non pregnant animals (light shaded portion) made up a larger proportion of cows ( $n= 140$ ) than heifers ( $n=49$ ).

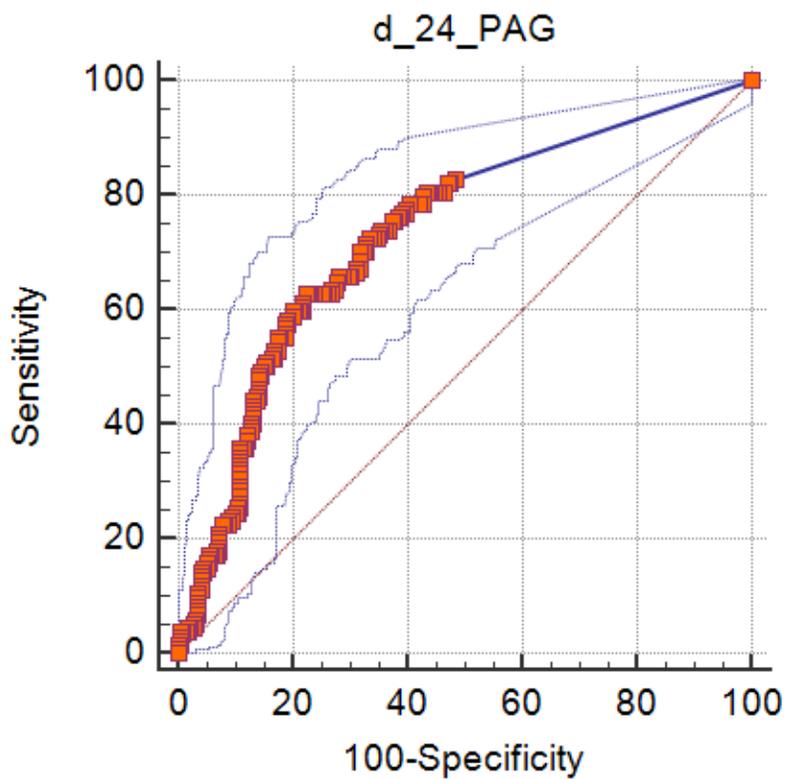


**Figure 10: Day 24 PAG concentration by parity**

Cattle diagnosed as pregnant on day 31 of gestation had a significantly higher ( $P < 0.0001$ ) PAG concentration ( $1.962 \pm 0.261$  ng/mL) at day 24 than non-pregnant cows ( $0.731 \pm .109$  ng/mL). By parity, pregnant and non-pregnant cows had different PAG concentrations ( $P < 0.0001$ ) but heifers showed only a tendency for different PAG concentrations ( $P = 0.0669$ ).

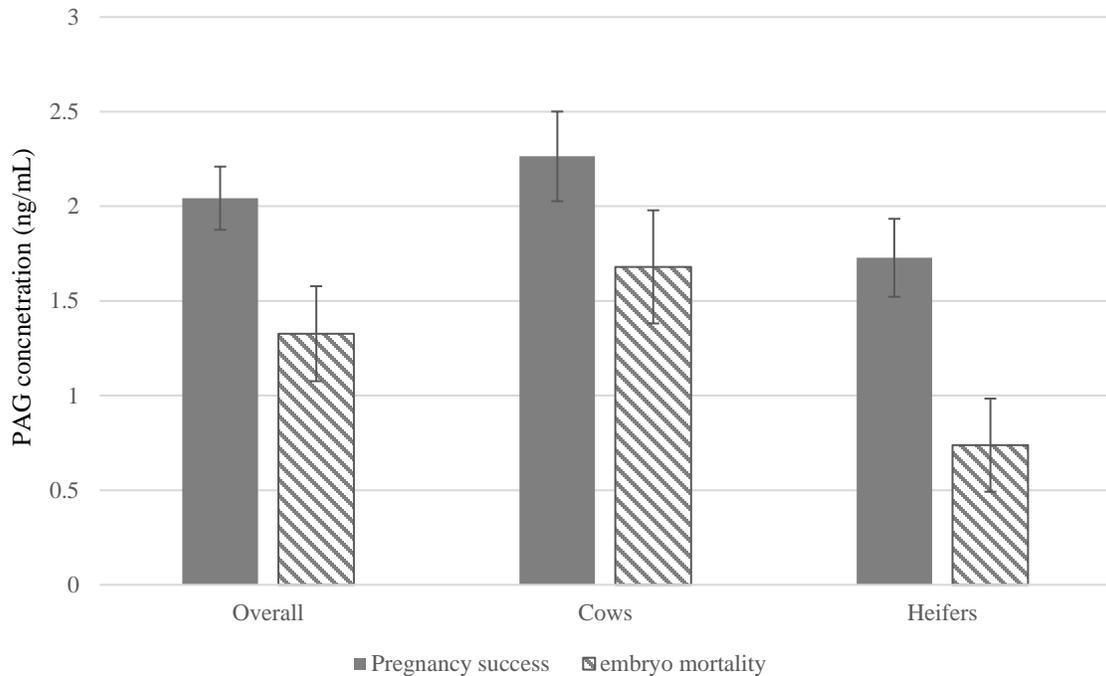
For pregnancy diagnosis at day 24, a ROC curve was used to test the potential effectiveness of day 24 PAG concentration and had an area under the curve of 73.4% (Figure 11). Based on predictive value analysis, there was not a clear cutoff value for identification of non-pregnant animals. Positive predictive value analysis concluded a concentration of 2.50 ng/mL resulted in a 90% confidence that the animal was pregnant, 95% confidence requires a concentration of 3.71 ng/mL.

Using 2.50 ng/mL, 56 animals (cows,  $n= 42$ ; heifers,  $n= 14$ ) were identified with 33 (cows,  $n= 24$ ; heifers,  $n =9$ ) of these animals pregnant on day 31 via embryonic heartbeat. In addition, 90.1% of animals with a circulating PAG concentration of at least 2.5 ng/mL had an increased PAG concentration of at least 0.75 ng/mL compared to the day 17 baseline sample. However, the day 17 baseline was not effective for pregnancy diagnosis. Increased circulating PAG concentration of at least 0.75 ng/mL at day 24 compared to the day 17 baseline sample accurately diagnosed pregnancy in only 51% (69/134) of animals that were pregnant at day 31. Of these 69 animals, 30 were heifers that had a 0 ng/mL baseline sample. Late embryonic mortality (LEM) after day 31 but before the final pregnancy diagnosis at day 60 occurred in 28 animals (7.93%). Heifers experienced LEM at a rate of 8.11% (9/111) while 7.85% of cows (19/242) suffered late embryo loss. Only animals diagnosed as pregnant at day 31 were included in the LEM analysis (heifers,  $n= 54$ ; cows,  $n=80$ ). There was no difference ( $P= 0.2284$ ) between animals that maintained ( $n= 106$ ,  $2.043\pm 0.167$  ng/mL) or lost pregnancy ( $n= 28$ ,  $1.327\pm 0.251$  ng/mL) (Figure 12). Day 24 concentrations of cows that lost pregnancy between day 31 and 60 ( $n= 19$ ,  $1.680\pm 0.299$  ng/mL) had a slight decrease but was not different ( $P= 0.4687$ ) from cows that maintained pregnancy ( $n= 61$ ,  $2.264\pm 0.237$ ng/mL). Although not



**Figure 11: Day 24 ROC curve- Ab63**

Receiver operating characteristic (ROC) curve was used for predictive value analysis. The area under the curve was 73.4% and a positive predictive value at 90% confidence to diagnosis pregnancy at day 24 of 2.50 ng/mL, a higher confidence level was not realistic as too few samples crossed the threshold.



**Figure 12: Day 24 PAG concentration of pregnancy outcome by parity**

Animals that maintained pregnancy had no difference ( $P= 0.2284$ ) in day 24 PAG concentration from animals that suffered LEM between days 31 and 60 of gestation. However, the means of both cows and heifers that maintained pregnancy were increased over the LEM groups.

significant ( $p=0.3430$ ), heifers which maintained pregnancy also had an increased mean day 24 PAG concentration ( $n= 45, 1.728 \pm 0.206$  ng/mL) over heifers which lost a pregnancy ( $n= 9, 0.738 \pm 0.246$  ng/mL).

## Discussion

Our results confirm that day 24 circulating PAG concentrations are elevated in pregnant compared to non-pregnant cattle. In this preliminary study, variation exists between assays and animals. The antibody used in experiment 1 was more accurate in diagnosing pregnancy at a lower concentration compared to the assay antibody used in experiment 2. In contrast to day 31 PAG concentrations, pregnant heifers had a lower PAG concentration compared to pregnant cows. The full extent of PAGs use to diagnose early gestation pregnancy is just beginning to be explored based on data collected in this study.

Pregnancy rates in dairy cattle are highly variable and dependent on genetic, production and environmental factors (Diskin et al., 2011; Wiltbank et al., 2016). A review of pastured dairy cattle by Diskin et al. (2006) indicated that average loss to day 2 is 53%, however early embryonic loss in TET pregnancies is often higher. A Brazilian study of TET in dairy cattle reported loss of 58% by day 32, a large study looking at TET using embryos produced with sex sorted semen reported loss of 62%, and experiment 2 had a day 31 pregnancy loss of 66% (Mikkola et al., 2015; Pereira et al., 2016). Influence of *Bos indicus* breeds may have reduced impact of heat stress associated with a tropical climate which severely undermines reproduction efficiency in lactating Holsteins (De Rensis et al., 2015; De Rensis et al., 2016); however, a study by Pontes et al. (2010) indicated pregnancy

rates from in vitro produced embryos was not different between Holstein and Gir influenced crossbred dairy cattle after timed embryo transfer.

Following migration of binucleated trophoblast cells and interdigitation of microvilli on fetal and maternal membranes, PAGs enter the maternal circulation (Wooding and Wathes, 1980; Wooding and Burton, 2008). Pregnancy diagnosis through detection of PAG is well established and available commercially in cattle and other ruminants beginning at day 28 of gestation (Reese et al., 2016a). Pregnancy associated glycoproteins may be detected in circulation of the pregnant cow as early as day 21 of gestation, although pregnancy detection using serum PAG concentration earlier than day 28 has been variable in its accuracy (Perényi et al., 2002; Sousa et al., 2006; Silva et al., 2007). Pohler et al. (2016) reported the first detectable, significant increase in circulating PAG concentration at day 24 of gestation in Nelore/cross bred beef cattle utilizing a similar polyclonal antibody used in the current study, thus the day of interest (Pohler et al., 2016b). As expected, mean PAG concentration was increased in pregnant animals compared to non-pregnant animals (2.32 ng/mL vs 0.7791 ng/mL). Day 24 PAG concentrations will likely fluctuate based on a number of embryo specific factors. Evidence suggests that pregnancies resulting from in vitro produced embryos may differ in circulating PAG concentration compared to AI or natural mating pregnancies and may be due to the irregularities that may accompany IVP embryo development (Constant et al., 2011; Pohler et al., 2016a). Contradicting day 28-31 trends, PAG concentration at day 24 was lower in pregnant heifers than in pregnant cows (Green et al., 2005; Pohler et al., 2013b). Experiment 2 is the first known study to measure PAGs at day 24 in heifers. The depressed PAG concentration at day 24 in heifers is not significantly different ( $P= 0.1309$ )

than cows and is based on a small sample size; however, more data are needed to confirm the presence or absence of a true difference. There are many reports that PAG concentrations differ between *Bos taurus* and *Bos indicus* breeds of cattle throughout gestation (de Sousa et al., 2003; Lobago et al., 2009; Mercadante et al., 2013). In a study by Mercadante et al. (2013), cows with an increasing percentage of *Bos indicus* genetics ( $\geq 80\%$  Brahman, 11.5 ng/mL; 41-60% Brahman, 8.4 ng/mL) had higher PAG concentration at day 35 of gestation compared to predominately *Bos taurus* cows ( $\geq 80\%$  Angus, 6.0 ng/mL). In the current study at day 24 gestation, however, there was no difference in PAG concentration based on breed ranging from  $\frac{1}{4}$  to  $\frac{7}{8}$  Holstein. There is a negative correlation between milk production and circulating PAG concentration at day 32 of gestation (Ricci et al., 2015).

At day 24 gestation, accurate pregnancy diagnosis using PAGs was variable between parities and assay. Other studies have investigated the used of early gestation PAG testing with similar results. Ricci et al. (2015) used a commercial based PAG ELISA with day 25 samples and concluded that accuracy of the test was too low to be used for effective pregnancy diagnosis. A study in Nelore beef cattle, identified a significant increase in PAG concentration in all cows at day 24; however, a previous study was more variable in detection of the increase (Pohler et al., 2016b). One potential cause of this variation may be attributed to dissimilarity between PAGs secreted at or before day 24 of gestation and PAGs detected by the assay (Green et al., 2000; Green et al., 2005). Based on ROC curve analysis from the data, only 32.1% of pregnant animals had a circulating PAG concentration greater than or equal to the positive predictive cut off value of 2.5 ng/mL. Although only 74% of the animals with a 2.5 ng/mL concentration were pregnant

on day 31, only one animal did not have an increased day 31 PAG concentration compared to the day 24 sample indicating that embryo mortality may have occurred just prior to day 31 ultrasound diagnosis. Additionally, 34.3% of open cows had a PAG concentration greater than 0.5 ng/mL which exceeds the expected embryonic loss between day 24-31 during time of active placentation and exponential placentome development (Leiser, 1975; King et al., 1979). A study by Pohler et al. reported embryo loss between day 24 and 31 as 20.8% in lactating dairy cattle (Pohler et al., 2016a). Time periods with significant change to the embryo and maternal environment can be associated with increased loss; however, an application for identification of non-pregnant cows needs to be both specific and sensitive. An increase in PAG concentration at day 24 compared to a baseline sample was not helpful in determining pregnancy status as only 71% of animals with a low baseline ( $\leq 0.25$  ng/ mL) and day 24 concentration of  $\geq 0.75$  ng/mL were pregnant on day 31. Baseline day 17 samples could theoretically be useful for cows with an unknown calving date due to pregnancy specificity associated with PAG and known clearance rate. Assay refinement using antibodies targeted for PAGs present on day 24 may provide more accurate results for pregnancy diagnosis.

Early gestation pregnancy diagnosis has important management and economic ramifications, especially with tests that may have the capacity to determine pregnancy success. Any potential pregnancy diagnosis method must be compared to the gold standard which is transrectal ultrasound. Limits of both must be considered. Pregnancy diagnosis based on day 31 PAG concentration has been shown to be comparable to accuracy from day 31 ultrasound (Karen et al., 2015; Ricci et al., 2015; Reese et al., 2016a). Based on this set of data, the ability to detect non-pregnant animals by PAG concentration is variable at

day 24 as evident by the inconclusive predictive cutoff value in both experiment 1 and 2. The role of day 24 PAG concentration as an early non pregnant detection tool given the current assay platform seems unlikely due to high variability in both pregnant and non-pregnant samples at day 24 of gestation.

Incidences of late embryonic loss contribute a significant economic detriment to dairy producers by extending the days to last breeding, increasing feed costs and decreasing yearly milk production. In lactating dairy herds, LEM contributes an average 12% loss with farm to farm variation from 3.5% to over 25% (Santos et al., 2004; Wiltbank et al., 2016). Significant evidence has been collected indicating increased day 28-32 PAG concentration has a strong correlation with greater embryo success in lactating dairy cattle and beef cattle (Pohler et al., 2013a; Pohler et al., 2016a; Pohler et al., 2016b). Commercial assays that only report S-N (sample- negative) values for pregnancy diagnosis are not accurate for predicting pregnancy maintenance in lactating dairy cows (Ricci et al., 2015). Thus, predictive pregnancy outcome determination may require particular antibodies which present a greater sensitivity to PAG concentrations. Day 31 samples collected from a subset of animals in this study contribute to this body of evidence. Animals that maintained pregnancy through day 60 of gestation had a PAG concentration at day 31 of 9.26 ng/mL whereas animals that lost pregnancy between day 31 and 60 had a PAG concentration of 7.04 ng/mL. There was no difference in day 24 PAG concentration between animals that lost or maintained pregnancy between days 31 -60. However at day 24, heifers which experienced LEM (experiment 2) had a numerically decreased PAG concentration (0.738 ng/mL) compared to heifers which maintained pregnancy (1.728 ng/mL), although it was not significant likely contributed to the low power of few LEM pregnancies. With further

assay refinement for day 24 PAGs, it may be possible to detect differences in embryo success in heifers.

## **Conclusion**

PAG concentrations at day 24 gestation are higher in pregnant compared to non-pregnant dairy cattle. Ability to diagnose pregnancy using predictive value analysis cut off concentrations identified in experiment 1 and 2 does not detect all pregnant animals and no value was found to determine non-pregnant status. Embryo success could not be predicted in the current study based on day 24 PAG concentration. However, refining antibodies for assay specification aimed towards day 24 gestation PAGs may provide greater accuracy for pregnancy diagnosis and predictive success.

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## CONCLUSION

Reproductive failure is a significant problem for dairy and beef operations. Timing and causes of pregnancy loss are varied and complex. Fortunately, increased information through research and novel advancements in reproductive management suggests that pregnancy loss and reproductive inefficiency can be controlled to acceptable levels. Establishing a baseline of pregnancy loss through meta-analysis provides a statistically grounded standard rate of loss during multiple time periods. Pregnancy associated glycoproteins tell an increasingly complex story. Work in fertility groups using serial ET has contributed evidence that embryonic factors are increasingly important to placental development and pregnancy success. Challenges associated with early pregnancy diagnosis are considerable, however PAGs may provide means for day 24 pregnancy diagnosis.

## **VITA**

Sydney T. Reese was born March 1993 to parents, Thomas and Stacy Reese. She was raised in Pleasant Grove, California and graduated from East Nicolaus High School in 2011. Following high school, Sydney enrolled at Texas A&M University in College Station, Texas and earned her B.S. in Animal Science. In 2015, she joined the lab of Dr. Ky Pohler at University of Tennessee, Knoxville to pursue her M.S. in Animal Science.