ALTERED ENDOCRINE PROFILES CONTRIBUTING TO LATE EMBRYONIC MORTALITY IN CATTLE

A Dissertation

by

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ABSTRACT

Reproductive inefficiency is a critical barrier maximizing profitability and sustainability of cattle industries. While intensive management strategies have provided crucial information regarding the amount of pregnancy loss that occur in dairy cattle, beef cattle are less understood. Over 30 years of beef cattle research from around the globe was compiled to quantify pregnancy loss throughout different developmental stages of gestation. A clear gap in knowledge exists around the physiological mechanisms and endocrine profile contributing to pregnancy loss during late embryonic development when active placentation occurs. To study uterine-secreted products, a protocol was developed using a coccygeal vein catheter to sample blood at the site of uterine ovarian drainage in the vena cava of pregnant cows without negative consequences to the pregnancy. Cows with an increased likelihood of experiencing pregnancy loss have similar responses to oxytocin challenge as cows likely to maintain pregnancy at day 30 of gestation. Basal prostaglandin concentrations increased between day 30 and 40 of gestation without negative consequences to the pregnancy; however, late embryonic loss was affected by the pulsatility of prostaglandin $F_{2\alpha}$ and prostaglandin E_2 concentrations during this period. This foundational knowledge about the endocrine environment during active placentation lays the groundwork for future studies to understand the mechanisms of pregnancy loss and increase reproductive efficiency in both beef and dairy cattle herds.

DEDICATION

To those that believe in the future of agriculture.

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NOMENCLATURE

CL	corpus luteum
D	day
E2	estradiol 17-β
EEM	early embryonic mortality
INFT	interferon tau
IVF	*in vitro* fertilization
LEF	late embryonic /early fetal mortality
LEM	late embryonic mortality
OT	oxytocin
P4	progesterone
PAG	pregnancy associated glycoproteins
PGE_2	prostaglandin E_2
PGEM	prostaglandin E_2 metabolite
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGFM	prostaglandin $F_{2\alpha}$ metabolite

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1. INTRODUCTION

1.1. Pregnancy Loss

Reproductive failure is a biological process that affects all living organisms but has considerable economic and environmental implications reducing the efficiency of livestock species. Although seemingly inevitable, considerable and continuing research aims to quantify and reduce reproductive inefficiency associated with subfertility and pregnancy loss. The long generation intervals and gestation periods in cattle make this an especially significant problem compared to other livestock species. As in all species, reproductive failure can occur at any point, from gamete development and maturation to parturition, with the amount of losses decreasing as gestation progresses. A large majority of losses occur within the first trimester of gestation with less than 10% occurring during later developmental periods; however, pregnancy loss is significantly impacted by subspecies, parity, genetics, production stressors and nutrition [1-5]. Fertilization and initial embryonic development rate can be as high as 90% but impaired gamete quality decreases embryo efficiency significantly [5-8]. Following the initial cell divisions until the establishment of trophectoderm and inner cell mass lineages (i.e., blastocyst stage), most embryonic mortality can be attributed to chromosomal abnormalities, cell cycle failures, or genetically lethal mutations that prevent proper embryonic development [9]. In dairy cattle 20 - 50% of embryos will not develop past the blastocyst stage [5]. Degenerate or non-viable embryos collected following embryo flush in beef and dairy cattle at day 7 after insemination are consistent with these findings[10-12]. The period of

development beginning with a blastocyst stage embryo until recognition of the embryonic heartbeat around day 28 of gestation is considered the early embryonic period. Following this developmental milestone, pregnancy loss decreases in the late embryonic stage (until day 45 of gestion) [13]. Fetal losses from the second trimester until parturition are infrequent without infectious causes or environmental stressors. Each period consists of complex and coordinated physiological processes that are necessary for the continued development of a viable and healthy conceptus. Some of the mechanisms contributing to pregnancy loss are well understood, while others are not. In the following sections, we will discuss the mechanism that contribute to embryonic mortality and the influence that the endocrine environment may have on pregnancy success.

1.2. Understood mechanisms of pregnancy loss

1.2.1. Fertilization

The processes contributing to fertilization success are some of the most studied reproductive processes due to the ability to recreate them in an *in vitro* setting. Fertilization is reliant on the viability of both male gametes (sperm) and female gametes (oocytes). Prior to fertilization, follicular development and exposure to a precise endocrine/cellular environment ensures proper oocyte maturation. Developmental competency of the oocyte is achieved only after meiotic and cytoplasmic maturation. This process is dependent on production and storage of mRNA transcripts and proteins that requires specific cell to cell interactions within the follicle and carefully orchestrated concentrations of estradiol (E2) and luteinizing hormone (LH) [14]. Ovulation of small

or physiologically immature follicles, due to fixed-time synchronization protocols, result in pregnancy rates that are 16% to 34% lower than cows that ovulate a follicle greater than 12 mm [15-17]. Additionally, oocytes from small, physiologically immature follicles are less likely to develop into blastocysts in *in-vitro* fertilization (IVF) embryo production systems [18, 19]. Exposure to increasing concentrations of E2 are critical to regulating cross talk in the cumulus oocyte complex, modulating uterine pH and oviduct secretion, and increasing luteal cell progesterone (P4) secretion in the subsequent corpus luteum (CL) [20-23]. Without adequate E2 exposure the oocyte is immature, and fertilization and embryonic development rates decrease [24, 25]. On the male side, sperm maturation and capacitation problems may prevent fertilization and zygote formation. Young bulls generally have a greater incidence of morphological abnormalities compared to older bulls [26, 27]. Capacitation failure is difficult to identify in field conditions and may be generalized as idiopathic infertility; however, in an *in vitro* embryo production setting, it is hypothesized that a subpopulation of bulls with poor IVF fertilization results may have impaired response to *in vitro* capacitation stimulus [28-30]. Additionally, both male and females may be affected by environmental factors that decrease fertilization potential of the gametes, including heat stress [31, 32] , metabolic disorders [33, 34], and disease state [35, 36]. Although fertilization rates are often reported above 90% in cattle [7, 37], gamete quality is crucial to reproductive success during later stages of embryo development.

1.2.2. Early cell division failures

Fertilization rates following natural ovulation and estrus expression using semen that meets basic morphology and motility standards is often above 90%. Fresh embryo

recovery rates, however, are often much lower than 90% even when ovulation is confirmed [5, 38-40]. Both intrinsic and extrinsic factors play roles in initial embryonic cell division. Extrinsic factors include stressors and conditions that alter the maternal reproductive tract environment making it inhospitable for embryo development. In high producing dairy cows, metabolic stressors play a significant role in pre-blastocyst embryonic failure. Physiological concentrations of non-esterified fatty acids associated with negative energy balance, as observed in most dairy cows at the time of first insemination, decreases the developmental competence of embryos to the blastocyst stage in both bovine and murine models [33, 41]. *In vitro* systems utilizing oviduct epithelial cells in culture show benefits of oviduct secreted factors on development to the blastocyst stage [42, 43]. Embryo driven loss, or losses due to intrinsic factors, are the predominant cause of embryonic mortality during the first 2 weeks of development and are usually related to chromosomal abnormalities [44]. Certain populations under heavy selection pressures, such as Holstein dairy cattle, have hundreds of variants that are promoted through heterozygote animals for milk production that are also embryonic lethal in homozygote forms [45, 46]. In a study of genetic screening for embryonic lethal mutations in New Zealand dairy cattle, it was found almost 1% of conceptuses were positive for an embryonic lethal genotype which would cost farmers $NZ 14 million [47]. In populations of Belgian beef cattle, bulls that are known carriers of the most common embryonic lethal variants will have affected conceptuses at 3 times higher proportions than the general population of animals with similar genetic backgrounds [47]. Translocation of non-homologous chromosomes, polyploidy and haploidy are observed in karyotype studies of

early embryos; however, few of these abnormalities were observed after the blastocyst stage indicating that transition to embryonic control and cell lineage differentiation are vulnerable times of development [9, 44]. Most evidence suggests high fertilization rates in cattle; however, intrinsic factors contributing to pregnancy loss may be difficult to overcome.

1.2.3. Early embryonic mortality (EEM)

Major embryonic developmental milestones from day 7 to 28 of gestation include elongation, maternal recognition of pregnancy and the establishment of the embryonic heartbeat [13, 48, 49]. Early elongation depends on the maternal environment, especially adequate uterine gland secretions in ruminants as knockout uterine gland models have severely growth retarded conceptuses by day 15 of gestation [50]. Early conceptus regulation of the endometrial transcriptome stresses the importance of communication between the embryo and uterus for pregnancy establishment [51, 52]. Premature regression of the CL due to failure of maternal recognition of pregnancy (MRP) causes considerable pregnancy loss, especially in lactating dairy cattle [48, 53-55]. Additionally, there is evidence that the period of MRP is important for maternal immune system modulation for pregnancy acceptance [51, 55, 56]. Recent research, in both beef and dairy cattle, has illustrated the prevalence of embryonic mortality between days 24 and 30 of gestion ranges from 5 to 10% [57-59]. The mechanisms of pregnancy loss during this period between MRP and detection of an embryonic heartbeat is not well understood due to the challenges associated with early pregnancy diagnosis and characterization of embryonic development during this interval. Retarded embryo growth and development

leading to early embryonic mortality have also been associated with specific chromosomal abnormalities and other embryo driven factors [44, 60].

1.2.4. Late embryonic mortality (LEM)

The causes and mechanisms of late embryonic mortality are the least understood of the gestational periods. This period, defined as between day 24 and 42 of gestation, is often reported in the literature as the second month of gestation between day 30 and 60 due to common management protocols [13]. Decreased pregnancy associated glycoprotein (PAG) concentrations in cows that undergo LEM as early as day 24 of gestation indicate that abnormal placental development may not sufficiently provide for the developing embryo [57, 58, 61-63]. Additionally, nuclear transfer somatic cell clone pregnancies, which have increased likelihood of LEM, exhibit significant vascular deformities and poor chorioallantoic development [64]. In dairy cattle, premature CL regression and decreased P4 concentrations have been observed prior to incidences of LEM [65] but Pohler et al. [66] reported termination of the embryonic heartbeat prior to decreased concentrations of P4, indicating a conceptus driven loss rather than a maternal environment driven loss. The causes of these insufficiencies and/or abnormalities may stem from the individual gametes prior to fertilization. Metabolic stressors, usually implicated in poor follicular maturation or early embryonic loss, may also play a role in LEM as indicated by high advanced oxidative protein product levels observed in silage fed dairy cattle that experienced pregnancy loss after day 25 of gestation compared to animals that maintained pregnancy [67]. An increasing body of evidence indicates that a subpopulation of sires have greater portion of pregnancies that undergo LEM compared with a separate population that have

very little LEM; however, the identifying markers of these distinct phenotypes are unknown [68, 69]. Additionally, pregnancies derived from oocytes out of small follicles or in low E2 environments are more likely to undergo LEM prior to day 60 of gestation [14, 17]. In a recent study of high fertility and sub fertile heifers as classified by d 28 pregnancy rates, sub-fertile heifers were also 2.4x more likely to undergo pregnancy loss between day 28 and 44 compared to the high fertility group [3]. This finding of pregnancy success being established during early gestion is supported by findings of uterine transcriptome variations in day 18 somatic cell nuclear transfer (SCNT) clone pregnancies compared to IVF produced pregnancies [70]. Placental failure is a common cause of SCNT pregnancy loss at later stages of gestation; however, this study suggests that it originates as early as the third week of gestation due to abnormal embryo- maternal communication[70]. Most research regarding LEM has focus on identifying markers of LEM rather than the mechanistic causes of embryonic death or failure of the placenta to support the pregnancy. Research including findings reported in this dissertation aim to elucidate the controlling mechanisms to identify and decrease the impact of pregnancy loss.

1.3. Hormones of Pregnancy

1.3.1. Progesterone

Progesterone is the key hormone regulating pregnancy maintenance and mammary gland development by preventing estrous cyclicity through quiescence of hormone production and receptor expression [71]. The CL, which produces a majority of

P4, is maintained throughout gestation and undergoes luteolysis approximately 2 days prior to parturition [72]. While the main source of P4 in the pregnant cow is the CL, the placenta serves as a source of P4 during the second half of gestions [73, 74]. Unlike other species, where the placenta is the primary source of P4 during pregnancy, in the cow the importance of this redundant accessory P4 source has not been explained [75].

1.3.2. Estrogens

Progesterone is the primary steroid hormone of pregnancy; however, E2 and estrone-sulfate have critical roles in regulating pregnancy development. Concentrations of estrogens are low in the initial stages of pregnancy but rise dramatically in the second and third trimesters of gestation in cattle [76, 77]. The initial increase begins between day 80-120 with a second, more pronounced increase around day 250. The physiological role of the initial rise in estrogens around day 100 remains unclear; however, the second increase prepares the reproductive tract for parturition. Co-localization of estrogen receptors (ER) and proliferation markers in caruncular epithelial tissue suggests a potential role of placental growth regulators during the initial rise of estrogens [75]. Estrogens also modulates blood perfusion in the uterus and placental tissues, thickening of the myometrium and, at the time of parturition, strengthening of uterine contractions and softening of the cervix [78, 79]. Interestingly, estrone-3- sulfate, the main form of estrogen produced in pregnancy, is not active in nuclear estrogen receptors and is thought to have biological actions, although unknown, separate from ovarian estrogens [75].

1.3.3. Interferon tau (IFNT)

Interferon tau is the primary signal of maternal recognition of pregnancy in ruminants that blocks luteolysis of the CL to maintain P4 levels. Secreted by the trophectoderm, IFNT blocks transcription of estrogen receptors which prevents expression of oxytocin receptors needed to induce pulsatile release of Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) for luteolysis [80]. Additionally, INFT may stimulate the conversion of $PGF_{2\alpha}$ to PGE_2 through modulation of the enzymes required for prostaglandin production [81]. In cattle, IFNT protein and mRNA is detectable around day 15 of gestation and increases rapidly until day 21, but decreases to very low concentrations by day 24 when the trophectoderm has attached to uterine lining [82]. Secondary signals for CL maintenance during late gestation are less understood. Without INFT and expression of interferon stimulated genes, oxytocin receptors are present on the pregnant endometrium and pulsatile release of prostaglandin $F_{2\alpha}$ occurs [83].

1.3.4. Oxytocin (OT)

Oxytocin is a neuropeptide hormone that is primarily associated with bonding and lactation. Upon interaction with OT receptors on the endometrium, however, OT will induce a $PGF_{2\alpha}$ release. This OT mediated release is the driving factor of luteolysis in nonpregnant females, thus the presence of OT receptors in pregnant endometrium as early as day 28 of gestation is surprising [83, 84]. Multiple studies have examined the ability of the uterus to release prostaglandin in response to oxytocin administration during early pregnancy but the mechanism that protects the CL from regression following exposure to

$PGF_{2\alpha}$ is unclear [83-85]. Chapter 3 investigates the responsiveness of the endometrium to oxytocin during the period of active placentation between day 30 to 42 of gestation.

1.3.5. Pregnancy-associated glycoproteins (PAGs)

Pregnancy associated glycoproteins are products of binucleate trophoblast cells that appear in maternal circulation around day 24 of gestation and continue to increase until just prior to parturition [61, 62, 86]. Over 2 dozen individual PAG genes are present in the bovine genome with individual temporal and spatial patterns of expression during gestation [87]. The biological function of PAGs are unclear; however, PAG detected in maternal circulation is a positive indicator of pregnancy and has been commercialized for use in pregnancy diagnosis in blood and milk [87]. Correlations between circulating PAG concentrations and late embryonic mortality have provided a potential marker to evaluate pregnancy viability and placental function [17, 62, 86, 88].

1.4. Prostaglandins

1.4.1. Biological properties of prostaglandin

Prostaglandins (PG) are 20-carbon molecules synthesized from arachidonic acid through the cyclo-oxygenase (COX) pathways, found in almost every body tissue, and regulate key homeostatic functions including inflammation, muscle contraction, vasodilation and vasoconstriction [89]. Synthesis of PG, from arachidonic acid, occurs through both COX-1 (constitutive) and COX-2 (inducible) pathways. The COX-1 pathway provides basal levels PG synthesis, whereas the COX-2 pathway responds to factors such as cytokines and growth factors to increase PG production [90]. Various enzymes, including,

prostaglandin endoperoxide reductases and prostaglandin endoperoxide isomerases, are used to convert the primary PG-G_2 and PG-H_2 forms into more biologically active forms like thromboxane, $PGF_{2\alpha}$ and PGE_2 [91]. Most prostaglandins act in a paracrine fashion, due to a high metabolism rate [92]. Prostaglandins are primarily metabolized in the lungs by the enzymes, prostaglandin dehydrogenase and 13, 14- reductase [93]. In cattle, simple passage through the lungs can metabolize up to 90% of circulating $PGF_{2\alpha}$ [92]. Because of this rapid metabolism, the metabolite, 15-keto-13,14-dihydro-prostglandin $F_{2\alpha}$ (PGFM) has been validated as an accurate marker of endogenous $PGF_{2\alpha}$ production [94]. Similarly, metabolites have been used to quantify PGE_2 and PGI_2 where infrequent sampling (> hourly) makes it difficult to assess prostaglandin concentration due to pulsatile release patterns and rapid metabolism [92, 95].

1.4.2. Major reproductive functions of prostaglandin

Prostaglandins are the most ubiquitous hormone family, affecting almost every organ and tissue. The reproductive tract and many reproductive processes are no exception. Crucial for maintaining cyclicity, prostaglandins also have well defined functions for the establishment and maintenance of pregnancy [96]. The following sections will outline the roles of prostaglandins, primarily $PGF_{2\alpha}$ and PGE_2, in maintaining reproductive processes and potential causes of reproductive failure.

During the estrous cycle, prostaglandins have important roles in ovulation and drive the mechanism by which luteolysis occurs. Prior to the LH surge, PGE_2 increases pituitary responsiveness to LH [97]. Concentrations of $PGF_{2\alpha}$ and PGE_2 increase in the follicular fluid and follicle wall beginning 8 hours post LH surge[98]. Separation of mural

granulosa and cumulus cells are PGE_2 dependent, while $PGF_{2\alpha}$ activates collagenolysis [97]. Treatment with a COX inhibitor, such as indomethacin, can alter or prevent ovulation from occurring if administered directly to the ovarian stroma but not when administered intramuscularly or to the uterine lumen [99].

Luteolysis is initiated by $PGF_{2\alpha}$ around day 16-17 of the cow estrous cycle. Due to the rate of pulmonary metabolism, $PGF_{2\alpha}$ from the endometrium is transferred via countercurrent exchange from the uterine vein to the ovarian artery [100]. A pulsatile pattern of $PGF_{2\alpha}$ is required for luteolysis, which decreases cholesterol precursors and steroidogenic enzymes needed for P4 production; additionally, vasoconstriction properties may play a role in the reduction of ovarian blood flow during this period [100, 101]. During luteolysis, there is also an increase of PGE_2 conversion to $PGF_{2\alpha}$ by 9-keto- PGE-reductase. The use of $PGF_{2\alpha}$ for manipulation of the estrous cycle by controlling luteolysis is the most commonly used hormone for applied reproductive management [102].

1.4.3. Prostaglandins and first trimester pregnancy in the cow

Although pregnancy establishment and maintenance are reliant on preventing luteolysis by blocking pulsatile $PGF_{2\alpha}$ release, prostaglandins have a number of important roles during early embryonic and placental development. Embryonic cleavage rates are positively correlated with endogenous PGE_2 secretion. Moreover, addition of PGE_2 into IVF culture media increased cleavage rates of bovine embryos [103, 104]. In horses, PGE_2 plays a critical role for oviductal transport of the embryo and embryo prostaglandin secretion may be necessary for gamete and early embryo transport in other species [105-107].

Despite the role of $PGF_{2\alpha}$ in luteolysis, basal concentrations of $PGF_{2\alpha}$ in uterine venous blood and transport across the utero-ovarian vascular plexus are not decreased in early pregnancy (before day 16-18) [108-110]. The peaks of $PGF_{2\alpha}$ required for luteolysis, however, are suppressed by INFT secreted by the trophectoderm [111, 112]. Interferon tau prevents upregulation of oxytocin receptors that stimulate pulsatile $PGF_{2\alpha}$ secretion [113]. Despite increased basal $PGF_{2\alpha}$ metabolite concentrations compared to nonpregnant animals, no peaks were observed between days 16 and 21 of gestation in pregnant animals [91]. Additionally, INFT downregulates 9-keto-PGE_2 reductase, a key enzyme in the pathway to convert PGE_2 into $PGF_{2\alpha}$ [81, 114]. There is some evidence that treatment with PG inhibitors can increase fertility in embryos that fail to produce adequate INFT; however, others suggest that the increased handling stress associated with administration of the inhibitor may decrease pregnancy rates [115-117]. In addition to INFT, other molecules have been associated with luteal protective mechanisms including PGE_1 and PGE_2. Although structurally similar to $PGF_{2\alpha}$, PGE_1 and PGE_2 are vasodilators and have been shown to increase luteal P4 secretion *in vitro* and *in vivo* [118-120]. Increases of PGE_2 during early pregnancy alters the PGE_2: $PGF_{2\alpha}$ ratio compared to cyclic animals [121, 122]. Simultaneously, luteal PGE receptors and endometrial PGE-synthase mRNA are upregulated [123, 124]. Endometrial PG synthesis capacity is low until day 18 of gestation but increases from day 20 onward [91]. These processes are crucial to maintaining CL function and P4 production during the first weeks of pregnancy.

The roles of prostaglandins in the second month of gestation may play just as critical of a role although, less research has been directed during this period. Basal levels

of PGFM are increased during the initiation of active placentation around day 30 of gestation [84, 125, 126]. Bridges et al. [127] reported that cows with an increased $PGF_{2\alpha}$ concentrations between days 31 and 35 were less likely to experience pregnancy loss than cows with lower concentrations during the same period. Concentrations of $PGF_{2\alpha}$ were consistent and do not exhibit the pulsatile release patterns observed during luteolysis or in premature luteolysis [125, 127]. It is hypothesized that increased $PGF_{2\alpha}$ aids in placentation as exponential development of placentomes occurs between day 30 and 40 of gestation. In buffalo, PGF-synthase is upregulated between days 29 and 38 of pregnancy, but not at days 48-56 [128]. This is further supported by the inflammatory functions of $PGF_{2\alpha}$ and potential to modulate the immune environment [96]. The role of prostaglandins later in gestation is further supported by the production of prostaglandins by binucleated trophoblast cells. Binucleated cells that were isolated from mid to late gestation placentomes produced both PGE_2 and $PGF_{2\alpha}$ with the capability of converting $PGF_{2\alpha}$ into PGE_2 and other metabolites in culture treated with FBS [73, 129]. Receptors for PGE_2 and $PGF_{2\alpha}$ are upregulated in caruncular endometrial tissue during the second month of gestation and may play roles in increasing angiogenesis of the placenta and stimulating P4 production by the CL [128]. Oxytocin receptors are present and capable of stimulating considerable $PGF_{2\alpha}$ responses from day 30 to the end of gestation, rapid association and dissociation with its receptors causes pulsatile release of $PGF_{2\alpha}$ rather than a gradual fluctuation [83, 84]. Despite the capability to release significant $PGF_{2\alpha}$, CL function and the pregnancy is maintained [83, 84].

1.5. Conclusion

The role of prostaglandins during this period is not well understood. In combination with the unknown mechanisms of pregnancy loss during this period, our objectives in the following chapters were to 1) quantify pregnancy loss throughout gestation, 2) establish prostaglandin release potential in cows with high or low likelihood of pregnancy maintenance and 3) identify prostaglandin profiles associated with late embryonic mortality.

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2. PREGNACY LOSS IN BEEF CATTLE:A META ANALYSIS[1]

2.1. Introduction

A main principle for most profitable cowherd models is to maximize the number of cows that produce a marketable calf yearly; however, calf crop percentage often fall below the level of expectation due to reproductive failures. Many cow calf operations are less intensively managed than dairy herds resulting in minimal awareness of reproductive failure within a herd. Understanding the timing of reproductive failure can assist scientists and producers in making important management decisions; however, results from conducting studies aimed at quantifying pregnancy loss during specific periods of gestation in beef cattle have been somewhat inconsistent. It is generally accepted that fertilization rates in beef cattle are considerably greater than pregnancy rates due to embryonic mortality occurring within the first 30 days of gestation which accounts for the largest percentage of pregnancy loss. The amount of embryonic loss reported after day 30 until the early fetal period, however, is variable [2, 3]. Causes of embryonic and fetal mortality are wide ranging from genetic lethal mutations and uterine asynchrony to failure in maternal recognition of pregnancy, placental insufficiency and disease [3-8].

Within beef cattle production, type of cattle and management strategies can significantly affect the extent of reproductive failures. Ayalon [9] provided one of the

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earliest reviews of embryonic loss in cattle which is still commonly cited in recent publications. During the last 40 years, there have been few publications in which there has been a specific review or in which there has been a summary of pregnancy loss throughout gestation in beef cattle. Furthermore, there has been no systematic review or meta-analysis of pregnancy loss in beef cattle. This gap in knowledge has a fundamental impact in measuring reproductive success and obtaining an accurate estimate of when there are reproductive failures during the various reproductive processes that result in production of calves. The primary objective of this meta-analysis is to conduct a review of studies and data to predict accurate values for reproductive failures during multiple periods of gestation including fertilization, early embryonic, late embryonic/early fetal development in beef cattle using quantitative analyses procedures. Secondarily, there was use of moderator analyses procedures of subspecies and parity to evaluate the effect of these characteristics on reproductive failures during critical periods of gestation in beef cattle. While many factors, including disease, environmental condition and management strategy, can increase or decrease reproductive success, the aim with this meta-analysis is to identify an updated baseline value for critical periods of loss throughout gestation in beef cattle.

2.2. Materials and methods

2.2.1. Data collection

Relevant literature was identified through comprehensive searches of Web of Science, PubMed, Google Scholar, pertinent scientific journals and meeting proceedings.

In addition to articles accessed as a result of original searches, reference lists from these articles were used to identify additional articles in which there was relevant research reported. Search terms included “pregnancy loss”, “embryo mortality”, “embryo loss”, “fertilization”, “conception rate”, “pregnancy rate”, “early embryo”, “late embryo”, “beef cattle”, “beef cow”, and “beef heifer.” More than 1,000 articles were identified and were further examined to determine suitability for inclusion utilizing PRISMA guidelines for systematic reviews. Primary screening of every article was undertaken by S.T. Reese with secondary reviews by G.A. Franco and K.G. Pohler. Each reviewer recommended or excluded articles based on a series of criteria to avoid bias. Primary screening was based on title and abstract information to establish whether in the article there was reporting on original research, determination of pregnancy rates in beef cattle and in the study(ies) conducted that there were not treatments that were intended to be detrimental to pregnancy. Articles meeting these criteria were further evaluated for data extraction and, subsequently, appraisal by G.A. Franco and K.G. Pohler. Mandatory inclusion criteria included i) cows or heifers of beef breeds ii) published after adoption of ultrasonic technology for early pregnancy diagnosis to allow for accurate pregnancy determination between days 28 and 32 of gestation and iii) day of gestation of pregnancy diagnosis, subspecies, location, parity, and/or breeding method was listed. Studies with first pregnancy diagnosis after day 32 of pregnancy or that included dairy animals and trials with treatments that could bias pregnancy success, such as induced twinning, were excluded from the meta-analysis. Articles were sourced from countries with modern beef production systems, including North America, Europe, Brazil and Australia. In papers

where there was reporting on results from multiple treatment and/or control groups, and/or where there were detrimental losses as a result of treatment (induced disease states, severe nutrient restriction, etc.), there was exclusion of these data from the average analysis.

Each study was assigned a pregnancy loss time period (fertilization, early embryo, late embryo/early fetal) based on when pregnancy diagnoses occurred. Unfortunately, the physiological periods of pregnancy development do not coincide with common time points of pregnancy diagnosis in herd management protocols. Time of pregnancy diagnosis in many studies does not correspond to a single development period, therefore, some periods of this meta-analysis were extended beyond the usual physiological developmental period to include a greater number of studies (Figure 2- 1). Results from studies in which the pregnancy rate or embryo recovery and survival was determined before day 7 of gestation (approximately a blastocyst stage embryo) were included in the initial period subsequently referred to in this manuscript as the period of fertilization and pre-blastocyst loss (FERT; days 1-7 of gestation). This allowed for results of a more substantial number of studies to be included in the meta-analysis because actual fertilization data are difficult to collect in *in vivo* studies. Pregnancy status was most commonly diagnosed before day 7 by flushing of the uterus after uterine tissues were collected. With many studies there was reporting of individual stages of embryo development but with this particular meta-analysis there was utilization of data from the most advanced stage of embryo present on the day of collection (i.e., cleaved on day 4; blastocyst on day 7 of gestation). Studies in which there was flushing of the uterus strictly for evaluation of embryo transfer factors were not included due to large variability and

discrepancies on how data were reported and the potential basis for the techniques used. Data collected from days 27 to 32 of gestation were classified as being collected during a period when there is early embryonic mortality (EEM) for this meta-analysis, although the physiological period of early embryonic development is considered to have concluded by day 28 of gestation. Transrectal ultrasound was the primary method of pregnancy diagnosis; however, reproductive tract collections and pregnancy associated glycoprotein blood testing were utilized in some studies. Importantly, collected EEM data will be confounded by loss that occurs during FERT because the data related to losses cannot be separated in the studies reported in the original publications and are cumulative as the pregnancy progresses. While embryo developmental stage shifts to stage of fetal development between days 42 and 45 of gestation, most commonly reporting in these studies of a secondary pregnancy diagnosis between days 60 and 100 of gestation evaluated using transrectal ultrasonography. Data from all studies in which there was diagnosis of pregnancy between days 60 to 100 are combined to assess reproductive failures occurring from days 60 to 100 of gestation which is termed late embryo/early fetal loss (LEF) for purposes of this analysis. This meta-analysis included more than 56,000 diagnostic records in 159 studies reported in 48 papers with 12 FERT studies, 107 EEM studies, and 40 LEF studies. Classification of studies is reported in Table 1.

2.2.2. Effect size and moderator variables

A meta-analysis of reproductive failures during various reproductive processes was conducted to determine percentage of pregnancy losses and periods when there were significant reproductive failures. Although meta-analyses are generally conducted to

examine a relationship between two groups or treatments, pregnancy loss was the single group effect size for this analysis. Effect sizes were calculated from data provided within the publications as percent pregnancy loss during each developmental period. Fertilization and EEM classification effect sizes are reported as percent of cows diagnosed as being non-pregnant when there were uterine flushing or ultrasonic diagnosis and percentage determinations of reproductive failures relative to total cows inseminated. In studies where both conception rate based on ovulation or estrus expression and pregnancy rate based on total cows inseminated were reported, pregnancy rate was utilized for effect size calculations to maintain consistency across all studies. Effect sizes for LEF are reported as a percent of cows that were diagnosed pregnant between days 28 and 32 but not pregnant at a secondary diagnosis, not as a percent of total cows inseminated.

Variables that may have contributed to variation among pregnancy losses were collected to be used as moderators. Described as third variables, moderators are variables which may have an effect on the extent or direction of change in the dependent variable and is generally a subset of the independent variable [10]. Moderators that were subjected to analysis included country of study, subspecies, parity and breeding method. Other moderator variables collected, if available, included state/region, service sire, synchronization protocol, body condition score and objective of original paper. Availability of all moderators did not affect eligibility for inclusion in the analysis; however, all papers did include descriptions of parity, subspecies (or breed) and country of study. Acquisition of these data allowed for an adequate number of studies to be included in each group for moderator analysis of parity and subspecies. Country of study

was closely aligned with subspecies moderator analysis; therefore, it was not reported separately. In all studies, there was utilization of only cows with adequate body condition scores.

2.2.3. Meta-analysis

When conducting the meta-analysis, the methodology established by Borenstein et al [11] was utilized. Summary effects and associated statistics were computed using Comprehensive Meta-Analysis Version 3 (CMA) software (Biostat, Englewood, NJ, USA; 2014). Due to the high probability that true effects vary among studies, the random-effects model was used. A nonparametric variance was calculated using the following to weight studies within the meta-analysis as standard errors and standard deviations were not reported in a majority of papers

$$V = \frac{P \times (P-1)}{n} \times m^{0.5}$$

where *V* is the variance, *P* is the point estimate, *n* is the sample size for the specific period and *m* is the number of studies extracted from the individual paper. For some papers, there were results from numerous studies from a single cow herd reported; the *m* correction was used to decrease weight that may be given when there were multiple studies with one herd so as to decrease the bias. Heterogeneity was calculated to evaluate the variation of random true effects that exist in pregnancy loss populations across multiple studies. Heterogeneity was assessed using the Q test for which the formula is subsequently described. This is a chi-square statistic that can be used to evaluate total weighted variability by accounting for both true heterogeneity (variation among studies) and

expected sampling error (within study variation). The formula for this determination is as follows.

$$Q_t = Q_b + Q_w$$

Heterogeneity was quantified using the formula for calculation of I^2 as an index that provides the proportion of variation due to true effects if sampling error was removed:

$$I^2 = \frac{Q_t - df}{Q_t} \times 100$$

where *df* (degrees of freedom; number of trials – 1 for each period of loss) represents expected variation (Q_w) and $Q_t - df$ represents the excess variation (Q_b). Lesser I^2 values close to 0% indicate most variation is due to sampling error or no heterogeneity; whereas, I^2 values closer to 100% denote variation in true effect sizes and indicate there is heterogeneity with the data [12]. For heterogeneity analysis, the prediction intervals (PI) were reported. Prediction intervals are dispersion indexes based on standard deviation that indicates how the effect sizes vary among all populations (95% confidence that an individual study will fit), whereas, confidence intervals (CI) are more specific as it relies on the standard error and is dependent on the number of studies (essentially there is 95% confidence that the mean will fall in this range) (Borenstein, Higgins [13]. Heterogeneity *P* values are reported among moderator subgroups and denote the probability that all groups share a common effect size.

Although this meta-analysis was conducted to examine a single effect size rather than a treatment effect, publication bias analysis was conducted to ensure balance between the results of large and small studies for each of the periods when reproductive failures were assessed. Two separate tests were used to detect potential bias. Funnel plot analysis

can be used to provide a visual assessment to determine whether sample size affects the distribution of data around the mean [11]. A symmetrical funnel plot can be used to indicate large and small studies are equally represented on either side of the mean. Secondly, Duval and Tweedie's (2000) trim and fill test can be used to adjust the effect size by removing data from small studies with extreme effect sizes and imposing studies to make the funnel plot symmetrical on both sides of the found effect size [14]. Once the potentially missing studies are filled the possibility of exaggerated effect size can be assessed.

2.3. Results

2.3.1. Fertilization and pre-blastocyst failures

Due to the difficulty and cost associated with conducting fertility studies, a limited number of studies (n = 12) that examined pregnancy loss during the earliest periods of gestation were identified. Studies that determined outcomes through day 7 of gestation (approximately blastocyst developmental stage) were included in FERT analysis. It is recognized this does not accurately represent the actual percentage of zygote production but includes all loss during the initial stages of embryo development and cell division. Across 12 trials, the average pregnancy loss was 28.4% (CI, 19.4% - 37.4%) by day 7 after fertilization. Interestingly, in studies with data collected before day 4 (n = 6), reproductive failures were 23%, indicating that most losses during this time period are due to fertilization or initial cell division failures. Heterogeneity was low (I^2 = 18.5%). The prediction interval indicated that 95% of pregnancy failures by day 7 of gestation will be

in the range of 9.3% to 47.5%. For the limited number of trials in fertilization analysis, publication bias did not affect the analysis based on funnel plot and trim and fill analysis publication bias tests. There were inadequate numbers of *Bos indicus* studies to provide a subspecies comparison and all but one study was conducted using heifers, thus, there was not moderator analysis for the FERT period.

2.3.2. Early embryo loss

In most studies the end of the early embryonic period was defined as ending on day 28 of gestation from a physiological perspective, therefore, the initial pregnancy diagnosis in beef cattle usually occurs after this timepoint, around day 30 to 32 of gestation. To utilize data from the maximum number of studies possible, EEM analysis included studies of data collected using pregnancy diagnosis occurring between days 27 and 32 of gestation (n = 107). Pregnancy loss during the EEM period was 47.9% (CI, 45.8% - 50.0%) for more than 53,000 individual cows. Additionally, 11 separate studies were identified in which there was diagnosis of pregnancy between days 12 and 16 of gestation using data collected at the time of detection of an embryo following collection of uterine tissues (slaughter) and reported a pregnancy loss point estimate of 32.3% (CI, 24.9% - 37.8%). The 47.9% reproductive failure rate that occurs during the first month of gestation in beef cattle as detected using the meta-analysis can be refined: 28.4% by day 7 of gestation, 3.9% between days 7 and 16, and 15.6% between days 16 and 32.

Reproductive failures during the EEM period was highly variable and moderator factors were more easily evaluated than fertilization data (Figure 2- 2). Moderator analysis of subspecies indicated a point estimate of 50.4% reproductive failure during the first

month of gestation for cattle of *Bos indicus* breeds while *Bos taurus* counterparts had a lesser reproductive failure (44%; P = 0.001). Fewer data were available for crossbred cattle with both *Bos indicus* and *Bos taurus* genetic influence (n = 9) and data were highly variable (52.3%, CI, 44.1% - 60.4%). Parity also affected early embryonic mortality (P = 0.002). For parity moderator analysis, average early embryonic mortality for nulliparous heifers (n = 39) was 44.3%, and for primiparous cows (n = 17) was 54.7% and multiparous cows (n = 49) was 48.0%. Breeding method affected early embryonic mortality (P = 0.001), with reproductive failures in cows bred using AI after natural estrous expression being 32.2% (n = 10), fixed time AI (FTAI) 49.5% (n = 83) and embryo transfer (ET) 54.6% (n = 13).

Heterogeneity of the EMM data set and by moderator sub level analysis was low as indicated by overall I^2 value equaling 13.1%. Based on PI calculations, about 95% of populations will have an overall EEM effect size in the range of 40.9% to 54.9%. There was no indication of publication bias contributing to the effect size of early embryo loss.

2.3.3. Late embryo and early fetal loss

The late embryonic period has been defined as day 29 to approximately day 45 of gestation [15]. Due to limited number of trials in which there was diagnosis of pregnancy at day 45, day 60 was considered as the last day of the late embryonic period. Additionally, there were a significant number of studies in which pregnancy diagnosis was conducted at day 30 and again around day 100 of gestation. Other than when there are infectious causes, there is little late fetal mortality in beef cattle and data for losses after day 100 were not included in the meta-analysis. After including data from studies in which there

was a final pregnancy diagnosis between days 60 and 100, there was identification of 40 studies including 30,500 individual animals that were classified as LEF. Reproductive failures during the LEF period averaged 5.8% (CI, 4.8% - 6.9%). There was no subspecies affect on the frequency of pregnancy loss during this period (*Bos indicus* 5.0% and *Bos taurus* 5.9%, $P = 0.389$, Figure 2-3). Moderator analysis of parity indicated there were differences ($P = 0.048$) between nulliparous heifers ($n = 10$; 8.1%), primiparous cows ($n = 4$; 5.4%), and multiparous cows ($n = 14$; 5.1%) (Figure 2-3). When there were pregnancies resulting from ET, there was a greater ($P = 0.001$) LEF ($n = 7$; 10.2%) compared with pregnancies resulting from FTAI ($n = 26$; 4.9%). Consistent with other periods, results from heterogeneity analysis indicated there was a significant sampling variation compared to actual variation with an I^2 value of 8.7%. Late embryonic/early fetal loss data were not affected by publication bias.

2.3.4. Pregnancy loss through gestation

Reproductive failures during the various developmental periods can be combined to determine the overall losses from the time of fertilization to the end of gestation (Figure 2-4). In beef cattle, more than 50% of the total reproductive failures occur prior to day 16 after insemination. Between day 16 and 32, there will be reproductive failures (pregnancy losses) in an additional 15.5% of cows. Reproductive failures after the first month of gestation, on average, occurs in less than 6% of beef cows; however, this is primarily affected by moderators and environmental factors.

2.4. Discussion

Meta-analysis results indicate that incidence of reproductive failure in beef cattle has not drastically changed since the first scientific reports [9]; although a detailed description of periods during which pregnancy losses occur has potential impacts for research advancements and modified industry recommendations. Collecting large quantities of accurate reproductive data from beef cattle is more difficult when compared with dairy cattle, as less intensive management routines limit collection of large quantities of field data. This has led to limited information regarding timing of pregnancy loss in beef cattle which have different patterns of fertility and reproductive failure compared to dairy cattle.

Pregnancy loss periods as reported in this meta-analysis differ in terms of days of gestation compared to developmental period definitions based on physiological events. Although overlap may occur between physiological periods, the main objective of the present meta-analysis was to identify and report a summary of the pregnancy loss based on available reports in research articles. While fertilization is generally thought of as a singular event at the initiation of pregnancy, results from all studies were included in which there was identification of pregnancies before day 7 accounting for fertilization and initial embryo development failure. Embryonic period, when strictly classified according to the physiological events during gestation, should refer to the period from conception to the end of embryonic differentiation stage, which is around day 42 to 45 of gestation (Hubbert, et al., 1972). It is commonly subdivided into early embryonic period (conception to day 28) and late embryonic period (days 28-42) marked by placental attachment and

delineation of the fetal shape; however, pregnancy diagnoses are often reported at days 30 to 32 and later at days 60 to 100. The timeline used for the present meta-analysis maximizes the number of trials included in the analysis to obtain a more accurate prediction of reproductive efficiency data.

Fertilization and blastocyst formation are the initial processes for any pregnancy to occur. In early reviews of reproductive failure, there is reports indicating fertilization rates in beef cattle are approximately 90% which is consistent with findings of structures collected at day 7 of gestation with embryo transfer [1, 16]. Unfortunately, significant embryo failure occurs between fertilization and day 7. Furthermore, collecting fertilization data is difficult and often requires uterine flushing after collection of uterine tissues. In the current meta-analysis, fertility and pre-blastocyst development failures during the FERT period averaged 28.4%, with a range from 2.9% to 44.4%. In comparison, embryo mortality during the first week of gestation in lactating dairy cattle can average 50% when there is no evidence of excess stressors [17]. Although beef cattle have limited production stress compared to dairy cattle, there are physiological factors that may have important functions in pregnancy success during the first week of gestation. Data suggest beef cows with large (>15.7 mm) or persistent dominant follicles are less fertile, likely due to decreased concentrations of P4 and E2 during follicular development [18-20]. Body condition score (BCS) and effects of nutrient restriction also impact initial embryo development. Cows and heifers with decreasing BCS or body weight post-AI not only have greater pregnancy losses but specifically have embryos with lesser quality grades and a greater percentage of immature staged embryos when collected at day 7 of gestation

[21-23]. Results from studies support that these failures are not due to fertilization failure or less than optimal P4 concentration, but some other developmental incompetency related to the maternal environment [21, 24]. Animals in studies included in this meta-analysis were bred after observation of estrus using semen of acceptable fertility or by natural service. Sire effects could not be assessed but paternal genetics can contribute significantly to early embryonic mortality [25]. Samples sizes in studies were small and that may contribute to the variation in pregnancy loss. Furthermore, the absence of studies in which there was comparison of different factors such as parities and subspecies, indicate that there is a gap in current knowledge of pregnancy development during the first week of gestation in beef cattle. More research could result in enhanced knowledge about how factors, including parity and breeding method, contribute to pregnancy loss in the first week of gestation in beef cattle. The current meta-analysis is one of the more homogeneous; however, limitations of sample population diversity may mask differences between subspecies or parities, as only *Bos taurus* animals were represented and most studies conducted with heifers.

A significant amount of pregnancy loss in cattle occurs during the first month of gestation in beef cattle. There is, however, some debate on when this loss is most significant: during initial embryo elongation (days 7 -14) or during maternal recognition of pregnancy and beyond (days 15 - 28). There are reports indicating the greatest single period of pregnancy loss is the second week of gestation when there is hatching of the blastocyst and initiation of elongation of the embryo [26-28]. Alternatively, other recent evaluations of available data, including this meta-analysis, may indicate otherwise [17,

29]. It is important to note that reports of increased pregnancy loss during the second week of gestation may be heavily influenced by data collected from lactating dairy cattle. Only 11 studies in beef cattle were identified in which there was measurement of pregnancy loss before day 16, likely due to inconsistencies in identifying pregnancies at this early stage of development. Of the 11 studies, in one there was reporting of data collected from *Bos indicus* cattle which warrants further research to establish potential subspecies differences. The results from this meta-analysis suggest increased pregnancy loss after the second week of gestation whereas the traditional assumption is there are greater pregnancy losses during the second week of gestation prior to maternal recognition of pregnancy. During the second half embryonic development between days 15 and 28 of gestation, for successful pregnancy maintenance there is reliance on proper maternal recognition of pregnancy and important processes protecting the embryo from the maternal immune system [30-32]. Losses during this period provide significant challenges to the adoption of early pregnancy diagnosis methods including the use of information related to interferon stimulated genes [33, 34].

This meta-analysis provides a baseline value based on large quantities of data in current research that model beef production systems utilizing assisted reproductive technologies. Based on the consistency in results from this meta-analysis, there is approximately a 50% pregnancy rate at day 30 of gestation when utilizing estrous synchronization, regardless of moderator combinations. Although there are a limited number of trials available, results from the current meta-analysis indicate there is a 15% increase in pregnancy rate in cattle bred following estrus expression compared to those

bred using a FTAI protocol following synchronization of estrus. This may be confounded by results from studies where there is the requirement for controlled data collection and use of FTAI protocols, especially in large *Bos indicus* trials conducted in South America. In a meta-analysis of expression of estrus in FTAI protocols, heifers exhibiting estrus before AI had a 27% greater conception rate compared to heifers that did not express estrus [35]. Estrous expression with use of FTAI protocols is highly variable with there being reports of between 20% to 80% of animals not exhibiting estrus prior to AI in both *Bos indicus* and *Bos taurus* subspecies [35-37]. Additionally, *Bos indicus* cattle are generally located in regions where the severe climatic conditions result in greater physiological stress, particularly as a result of nutritional factors, as compared with *Bos taurus* beef cows which contribute to trends of decreased fertility [38, 39]. Cows with a lesser BCS or that are anestrous will have decreased estrous expression which is a variable that is correlated with pregnancy rates [35]. A less than optimal BCS is a critical factor in reduced fertility of primiparous cows [40, 41]; however, data were not variable enough to utilize BCS as a moderator in the current meta-analysis. Additionally, results from studies in which there was examination of the combination of growth, lactation and reproduction stressors indicate there is an increased pregnancy loss in primiparous cows compared to heifers and multiparous cows [42-44].

Hormone manipulation, nutritional management, health protocols and other factors that may increase day 30 pregnancy rates have been studied extensively. Late embryonic and fetal mortality is the focus of less research and, thus, how these factors affect fertility failures is less understood than other areas of pregnancy loss. Late

embryonic/early fetal mortality has significant negative impacts on reproductive efficiency and economic consequences because cows may be retained in the herd for an entire season without producing a marketable product. Based on the current study, overall LEF in beef cattle is 5.8% which is significantly less compared to what occurs in dairy cattle. In most reports, there is an estimation of late embryonic mortality of lactating dairy cows between 10% and 20% [17, 45, 46], although in some studies results indicate there is about a 7% late embryonic loss [47]. With beef cattle herd management, there is more crossbreeding utilized than occurs in dairy cattle, thus, there is less inbreeding and expression of recessive genetically lethal traits which are known contributors to increased late embryonic mortality in dairy cattle [3, 48]. Additionally, use of advanced reproductive technologies, such as *in vitro* produced embryos, result in increased LEF; however, these technologies are not widely used in beef production [6]. With the current meta-analysis, the studies included were where there was a confirmed pregnancy on either day 60 or 100 of gestation. Interestingly, results from studies with pregnancy diagnosis on day 60 indicated there was no difference ($P = 0.39$) in pregnancy loss compared to studies where there was pregnancy diagnosis at day 100 when initial diagnosis occurred around day 30. This indicates that fetal loss during the third month of gestation between days 60 and 100 is limited. Most studies in which there is pregnancy diagnosis on day 100 are conducted with *Bos indicus* cows and most day 60 studies were conducted in *Bos taurus* cows. It, therefore, may be interesting to analyze data from *Bos indicus* cows at day 60 and *Bos taurus* cows at day 100 to ascertain if a subspecies effect exists. Heifers had a greater late embryonic/early fetal mortality rate (8.1%) than cows (5.1%) but there were inadequate

numbers of trials included in the meta-analysis for detection of a difference between *Bos indicus* heifers and *Bos taurus* heifers. From a management perspective, it is unknown if there is a physiologic factor affecting parity differences or if animals more susceptible to LEF are culled as heifers before being retained for placement in the mature cow population.

While the results from available studies only provide enough data for moderator analysis of subspecies, breeding method and parity, other factors may have important effects when quantifying embryo loss. Using results from available studies, there was no identification of other moderators or additional variables that significantly affected the results from the meta-analysis, therefore, estimates for pregnancy loss during multiple periods are both statistically and biologically sound. Optimal reproductive management strategies are dependent on numerous factors and with future analyses there should be comparisons of the impact of estrous synchronization protocols, sire effects, and nutritional status on overall reproductive performance to make recommendations for field use.

2.5. Summary

Gestational loss during the early stages of pregnancy can be detrimental to calving rates in beef cattle. The results from the current meta-analysis and further heterogeneity analysis indicates early fertilization failures are variable among cattle types and ages providing opportunity for both research and improved production strategies. Fertilization rates may be as high as 95% in some scenarios; however, current research is limited to a few studies in beef cattle. Loss during the early embryonic period is dependent on many

factors, the most impactful being parity with primiparous heifers where there are large amounts of reproductive failure early in gestation. Approximately 48% of cows will not be pregnant at day 30 of gestation following a single insemination. Late embryonic mortality is variable among beef cattle and significantly less than what is reported in dairy cattle. Further reporting of pregnancy loss data is of great interest to identify other factors that may positively or negatively affect pregnancy loss at different points in gestation.

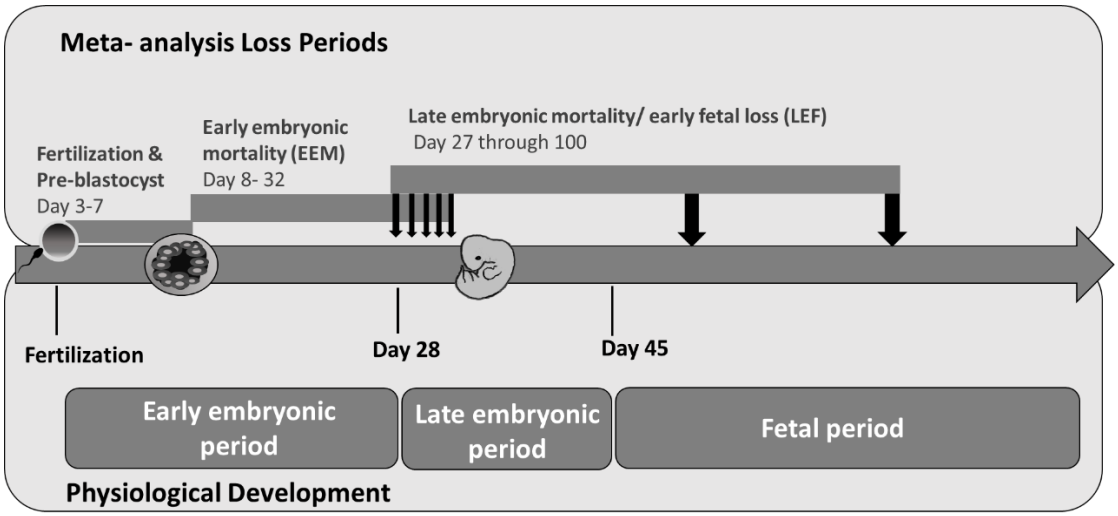

Figure 2-1. Loss periods and physiological development of pregnancy.
Divisions between periods used to classify studies in the meta-analysis did not align with physiological development periods. Effort was made to best utilize the most possible trials within a logical distance from the true periods. The black arrows represent the most common times for pregnancy diagnosis in beef cattle: an initial diagnosis between days 27 and 32 of gestation, and a second diagnosis around day 60 or day 100 of gestation.

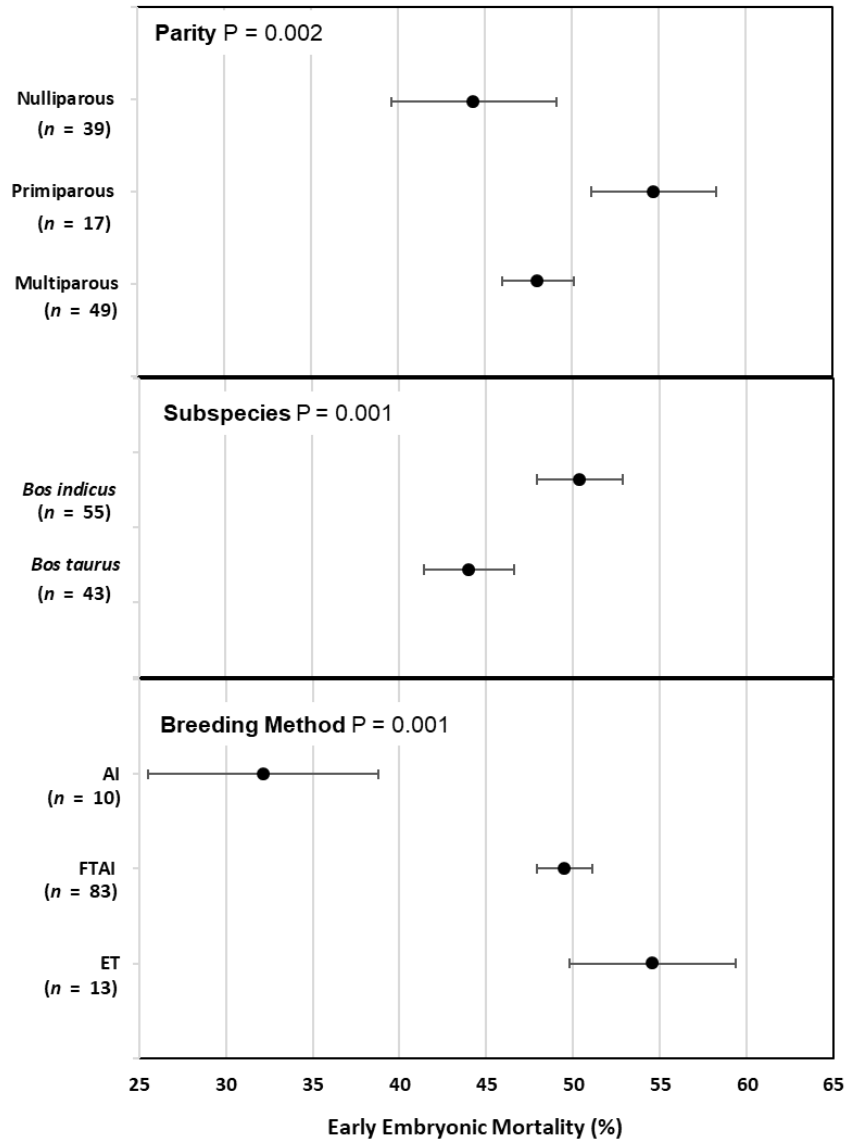

Figure 2-2. Moderator plots of early embryonic mortality

Point estimates and 95% CI for moderators explaining early embryonic mortality during the first month of gestation. *n* = number of trials ; heterogeneity *P* denotes the probability all trials share a common point estimate. Some publications that utilized multiple moderators in a single trial and could not be separated were excluded from moderator analysis.

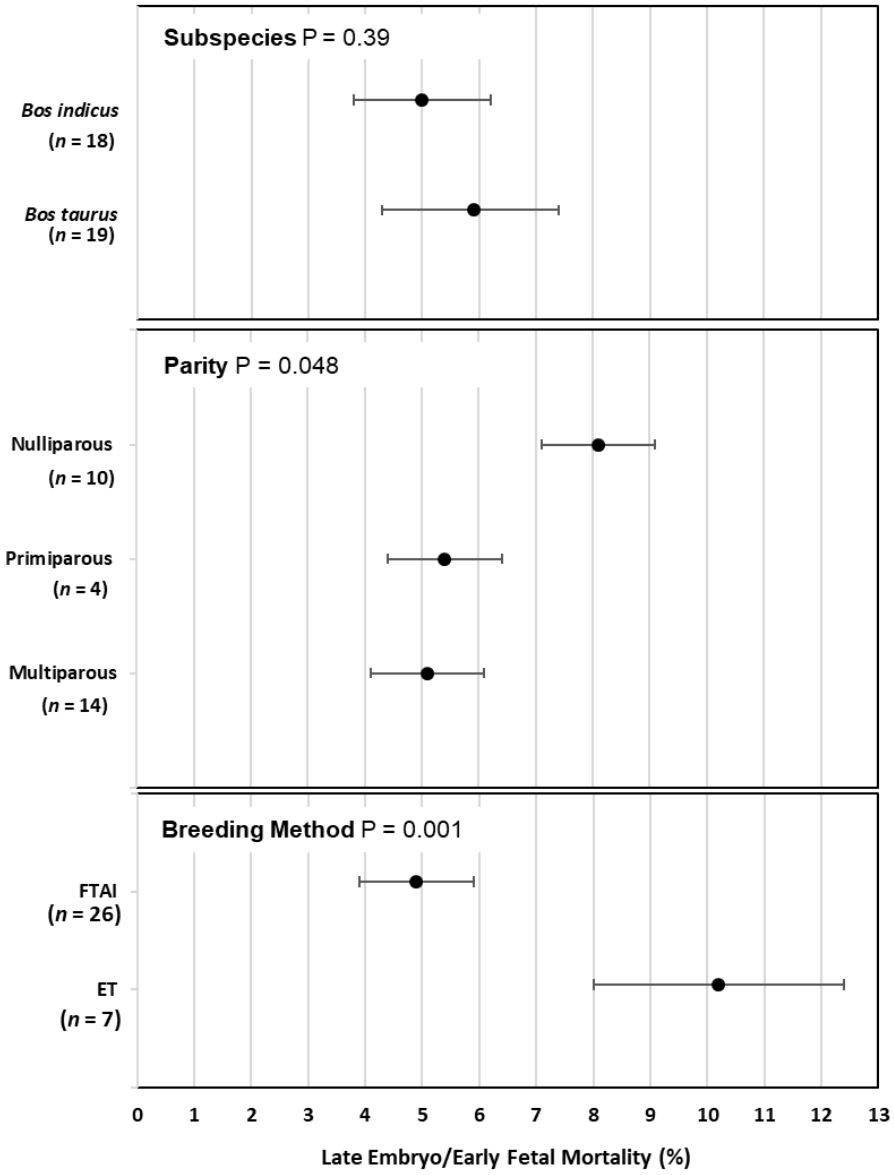

Figure 2-3. Moderator plots of late embryonic/ early fetal mortality.
Point estimates and 95% CI for moderators explaining late embryonic/ early fetal mortality. *n* = number of studies; heterogeneity *P* denotes the probability all studies share a common point estimate. Some publications that utilized multiple moderators in a single trial and could not be separated were excluded from moderator analysis.

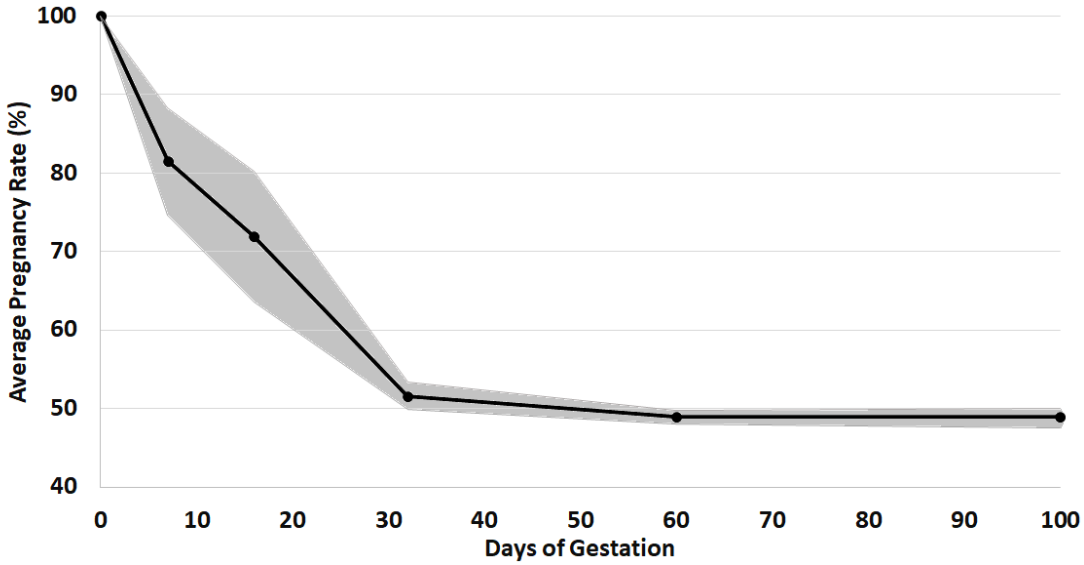

Figure 2-4. Average predicted pregnancy rate by day of gestation in beef cattle. Grey area indicates area of 95% confidence interval.

Table 1. Period and moderator classification

Reference	Country	Period[1]	Subspecies[2]	Parity[3]	Breeding Method[4]	No. of Animals
Aono et al. 2013	Brazil	E, L	I	P, M	FTAI	12,357
Beal et al. 1992	USA	L	T	M	AI	205
Breuel et al. 1993	USA	F	T	M	N, AI	50
Burns et al. 2008	USA	E, L	T	P, M	FTAI	676
Carter et al. 2008	Ireland	F, E	T	N	AI	125
Colazo et al. 2004	Canada	E	T	N, M	FTAI	363
Cooke et al. 2017	Brazil	E, L	I	M	FTAI,	1,209
Cordeiro et al. 2015	Brazil	E	I	N, M	FTAI, ET	350
Diskin and Sreenan, 1980	Ireland	F, E	T	N	AI	145
Dobbins et al. 2009	USA	E, L	T	P, M	FTAI	605
Dunne et al. 2000	USA	E	T	N	AI	158
Ferreira et al. 2016	Brazil	E, L	I	M	FTAI	604
Franco et al. 2018	Brazil	E, L	I	M	FTAI	1,228
Garrett et al. 1988	USA	F, E	T	M	N	31
Jinks et al. 2013	USA	E, L	T	M	ET	350
Kill et al. 2013	USA	E, L	T	N	FTAI	679
Lamb et al. 2001	USA	E	T	P, M	AI, FTAI	365
Lamb et al. 2006	USA	E	T	N	AI, FTAI	1,019
Larson et al. 2006	USA	E, L	T	P, M	AI, FTAI	2,417
Lopes et al. 2009	Brazil	E	I	P, M	FTAI, ET	2,667
Martinez et al. 2002a	Canada	E	T	N	FTAI	503
Martinez et al. 2002b	Canada	E	T	N, M	FTAI	622
Meneghetti et al. 2009	Brazil	E	I	P, M	FTAI	3,260
Mercadante et al. 2015	USA	E	T, X	N, M	FTAI	2,370
Mialon et al. 1993	France	L	T	N	AI	1,102

Table 1: Continued

Reference	Country	Period[1]	Subspecies[2]	Parity[3]	Breeding Method[4]	No. of Animals
O'hara et al. 2014	Ireland	E	T	N	AI	33
Parr et al. 2017	Ireland	E	T	N	AI	83
Peres et al. 2009	Brazil	E	I	N, M	FTAI	1,855
Perry et al. 2003	USA	E, L	T	P, M	FTAI	174
Perry et al. 2007	USA	E, L	T	N	AI, FTAI	208
Pessoa et al. 2012	Brazil	E, L	I	N, P, M	FTAI	658
Pfeifer et al. 2017	Brazil	E	I	P, M	FTAI	253
Pohler et al. 2013	USA	E, L	T	M	FTAI, ET	354
Pohler et al. 2016	Brazil	E, L	I	P, M	FTAI	2,205
Pontes et al. 2009	Brazil	E, L	I	N	ET	1,199
Pontes et al. 2011	Brazil	E, L	X	N	ET	5,938
Pradebon et al. 2017	Brazil	E	T	N	FTAI	414
Radigonda et al. 2017	Brazil	E	X	M	FTAI	150
Roche et al. 1981	England	F	T	N	AI	131
Sá Filho et al. 2010	Brazil	E	I, X	M	AI, FTAI	2,388
Sa Filho et al. 2009	Brazil	E	I	M	FTAI	2,491
Sa Filho et al. 2014	Brazil	E, L	I	P, M	FTAI	1,538
Sales et al. 2011	Brazil	E, L	X	N	ET	495
Smith et al. 1982	USA	F, E	I	N	AI	101
Spitzer et al. 1978	USA	F	T	N	AI	30
Starbuck et al. 2006	USA	E, L	T	M	AI, FTAI	267
Stevenson et al. 2003	USA	E, L	T	M	FTAI	1,048
Unpublished Pohler Lab	USA	E, L	T	P, M	FTAI	229

Table 1. Continued

[1]F = Fertilization (diagnosed before day 7 of gestation), E = early embryo mortality (loss prior to day 32 of gestation), L = late embryo/ early fetal mortality (pregnancy loss between initial pregnancy diagnosis at days 28 to 32 of gestation and second pregnancy diagnosis between day 60 and 100)

[2]Subspecies evaluated: T = *Bos taurus* , I = *Bos indicus*, X = cross breed of *Bos taurus* x *Bos indicus*

[3]N = nulliparous, P = primiparous, M = multiparous

[4]AI = artificial insemination based on estrus expression, FTAI = fixed time artificial insemination based on protocol specifications, N = natural service, ET = embryo transfer 7 days post predicted ovulation

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3. INDUCED PROSTAGLANDIN RELEASE ALTERS STEROID CONCENTRATIONS BUT NOT PREGNANCY SURVIVAL IN COWS[1]

3.1. Introduction

Late embryonic mortality, occurring between days 28 and 45 of gestation in cattle, has been identified as a significant economic problem within the cattle industry [1, 2]; however, causes of embryonic loss during this period are relatively unknown [3]. It is hypothesized that inadequate or defective placentation may play a significant role in pregnancy loss during this pivotal period. Pregnancy- associated glycoproteins (PAGs) may be a marker of placental competence because cows with greater circulating PAG concentration between days 28 and 32 of gestation have an increased likelihood for pregnancy success compared to cows with lower PAG concentrations [4-7]. Circulating concentrations of PAG measured as early as day 24 of gestation differ between animals that experience late embryonic mortality and those that maintain pregnancy [8, 9]. Thus, using circulating PAG concentration early in gestation has allowed for investigation of pregnant cows that have a high likelihood for late embryonic/early fetal mortality, a population that has been previously difficult to identify.

Late embryonic mortality coincides with the period of active placentation in cattle. Although prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) have a well-established

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role during early gestation, their role during active placentation (days 31 - 35 of gestation) have received only minor attention [10, 11]. Schallenberger et al. [10] and Bridges et al. [11] both observed elevated concentrations of PGE_2 and $PGF_{2\alpha}$ in pregnant cows during this period. Ginther et al. [12] reported increased basal concentrations but lower pulse frequency of $PGF_{2\alpha}$ during placentation compared to the period prior to luteolysis. In addition, Bridges et al. [11] reported increased concentrations of $PGF_{2\alpha}$ in cows that maintained pregnancy compared to cows that underwent embryonic mortality. More recently, Prostaglandin F Synthase (PGFS) mRNA has been shown to be upregulated in *Bubalus bubalis* endometrium between days 29 and 38 of gestation compared to non-pregnant animals, which contributes to previous findings of increased PGFS mRNA in bovine caruncular tissue during gestation [13, 14]. Therefore, an increase in $PGF_{2\alpha}$ may have an important role in proper placentation and placentome development. Elucidating the role that prostaglandins play during this critical time point is central to bridging this gap in knowledge, specifically around late embryonic mortality.

Uterine secretion of $PGF_{2\alpha}$ during the estrous cycle is dependent upon specifically timed events during which the uterus is primed and responsive to luteolytic signals. Oxytocin is commonly used to test the ability of the uterus to secrete $PGF_{2\alpha}$ [15-17]. In both sheep and cattle, $PGF_{2\alpha}$ release in response to exogenous treatment with oxytocin is increased between days 16 to 19 (days 13-14 in the sheep) of the estrous cycle, but is significantly less responsive earlier in the cycle [16, 18, 19]. Cows exhibiting a subnormal luteal lifespan after parturition respond to oxytocin on day 5 of the estrous cycle with $PGF_{2\alpha}$ release and premature luteal regression [15], but pregnant cows at day 17-19 of

gestation have a low oxytocin-induced prostaglandin release [16]. A hallmark of pregnancy establishment and imperative signal for maternal recognition of pregnancy is the downregulation of oxytocin receptor expression by day 16 of gestation to suppress $PGF_{2\alpha}$ when on the same day of the estrous cycle it would be upregulated to allow luteolysis to occur [20]. By day 31 of pregnancy, oxytocin receptors are present on the endometrium and exogenous oxytocin administration induces prostaglandin release [21, 22]. Despite an ability for the uterus to release prostaglandin, the CL does not regress following oxytocin challenge around day 30 of gestation; however, exogenous $PGF_{2\alpha}$ in the sheep during mid gestation alters placental E2 production and data show a potential mediatory role for protection of the CL and P4 production[23, 24]. The mechanisms of late embryonic loss are unclear; however, prostaglandins and the increase in basal $PGF_{2\alpha}$ around day 30 of gestation suggests a role during the period of active placentation. During this same period, circulating PAG concentrations are indicative of the likelihood of late embryonic mortality and offer a model to evaluate potential differences in cows with successful pregnancies and those that undergo pregnancy loss. Thus, the hypothesis of this study was that uterine $PGF_{2\alpha}$ release would differ in cows with a high likelihood of undergoing late embryonic mortality, marked by low circulating PAG concentration compared to cows with increased PAG concentration. The objectives were to evaluate the concentrations of $PGF_{2\alpha}$ metabolite (PGFM), P4 and E2, as well as pregnancy outcomes in cows with varying levels of PAG at day 30 of gestation as an indicator of potential pregnancy success.

3.2. Materials and Methods

3.2.1. Oxytocin Challenge

All animal procedures were approved and conducted in accordance with Texas A&M IACUC guidelines. Mature multiparous Brangus and Braford cows (*n* = 60) were subjected to the *Bos indicus* PG 5 day + CIDR estrous synchronization protocol as described by Williams and Stanko [25]. On day 0, cows received gonadotropin releasing hormone (GnRH) and were inseminated with semen from one of two sires. At day 29, pregnancy status was evaluated by transrectal ultrasonography and confirmed via presence of an embryonic heartbeat (n = 32). Pregnant cows (*n* = 25) meeting the PAG group criteria as described below were subjected to oxytocin challenge on day 30 of gestation. Blood samples were collected every 30 minutes beginning 1 hour before the initiation of the challenge to establish baseline concentrations. At hour 1, cows received either saline injection (control; *n* = 12) or 100 I. U. of oxytocin intramuscularly (OT; *n* = 13) based on previously established doses used in oxytocin challenges in mature cows [15, 26]. Sampling continued every 30 minutes for 4 hours after treatment administration. Samples were collected via the coccygeal vein into EDTA K2 blood collection tubes (BD Vacutainer, Franklin Lakes, NJ) containing 10 µM/mL indomethacin and placed on ice. Plasma was separated by centrifugation for 15 minutes at 2500 g within 30 minutes of sample collection and stored at -20 °C until hormone analysis. A final pregnancy diagnosis via ultrasound occurred at day 100 to confirm pregnancy maintenance.

3.2.2. Assays

Concentrations of PAG were quantified using an in-house ELISA established by Green et al. [27] using antibodies produced against early secreted PAGs as validated by Reese et al [9]. Each assay was run with a standard curve, positive controls from a pool of 2nd trimester pregnant cow serum and negative pooled steer serum controls. The inter-assay and intra- assay CV's were 5.15% and 7.23%, respectively. An ELISA described by Mezera et al. [28] was used to quantify PGFM using a 1: 16,000 dilution of primary antibody (gift from Dr. William Thatcher, University of Florida) and PGFM- HRP conjugate (gift from Dr. Milo Wiltbank, University of Wisconsin). The intra-assay and inter- assay CV's were 5.76% and 15.12%, respectively. Estradiol concentrations were evaluated using an RIA protocol described in Kirby et al. [29] with antibody and 3-Ido-Estradiol-17β Tracer from MP Biomedicals (Santa Ana, CA). Standard curves and high/low control serum samples were run at the beginning and end of the assays. The inter-assay and intra- assay CV's were 4.24% and 6.81% respectively. Progesterone concentrations were quantified using a commercial RIA kit (MP Biomedicals, Santa Ana, CA) previously validated in our lab in a single assay with high and low P4 controls and standard curves at the beginning and end. The intra-assay CV was 3.23%.

3.2.3. Data and statistical analysis

Cows subjected to oxytocin challenge were classified into PAG groups using the day 29 PAG samples collected at pregnancy diagnosis. Cows with circulating PAG concentrations greater than 8 ng/mL were classified as high PAG (High PAG OT) and

those with less than 4 ng/mL were classified as low PAG (Low PAG OT) and used in further analysis. Cows with intermediate PAG concentrations were removed and were not included in study numbers (n = 7). Cows receiving saline were analyzed as a single control (CON) group due to limited variation in circulating PAG concentrations among individual animals (Figure 3-1). Hormone concentrations, except for peak concentrations, were analyzed by hour where 2 samples were combined to give an hour average. In cases where there was no difference in response between High PAG OT and Low PAG OT groups, data was reported combined. Concentrations are reported as average ± SEM. Data for PGFM, E2, and P4 were analyzed using PROC MIXED to account for repeated measures (hour) using SAS 9.4 with first baseline sample concentration as a covariate. Area under the curve (AUC) analysis was conducted using the trapezoidal method [30]. For simple correlations, PROC CORR was used in SAS 9.4. Significance was set at $P \leq 0.05$, and tendencies were determined if $P > 0.05$ and $P \leq 0.10$.

3.3. Results

Cows in the High PAG OT (n = 7) group had increased ($P <$ 0.001) average circulating PAG concentrations (10.22 ± 0.34 ng/mL; range: 8.16 - 13.89 ng/mL) compared to CON cows (n = 12; 5.77 ±0.33 ng/mL; range: 2.35 - 10.65 ng/mL) which had increased ($P <$ 0.001) concentrations compared to the Low PAG OT group (n = 6; 3.26 ± 0.17 ng/mL; range: 1.65 - 3.94) (Figure 3-2). Circulating PAG concentration did not change from the baseline during the sampling period in any group ($P > 0.05$). Despite

differences in circulating PAG concentration, all cows maintained their pregnancy until the final pregnancy diagnosis at day 100 of gestation.

There was no correlation between PAG concentrations and basal ($P = 0.86$) or peak ($P = 0.64$) PGFM concentrations across all animals. Baseline concentrations of PGFM did not differ between OT and CON groups. Following oxytocin challenge, there was a time by treatment interaction detected ($P < 0.001$) when comparing PGFM. Concentrations of PGFM peaked 2 hours after administration in High PAG OT and Low PAG OT groups (Figure 3-3). There was no difference ($P = 0.58$) in peak PGFM concentration between High PAG OT (345.6 ± 73.6 pg/mL) and Low PAG OT (326.4 ± 61.4 pg/mL) groups. Additionally, there was no difference ($P = 0.52$) in AUC between High PAG OT (638 ± 105 pg/mL·hr) and Low PAG OT (592 ± 144 pg/mL·hr) groups. Concentrations of PGFM in both High PAG OT and Low PAG OT groups had returned to basal concentrations by hour 4. Significant variation in basal (range: 22.17 - 219.23 pg/mL) and peak concentrations (range: 124.25 - 668.44 pg/mL) existed between cows. Circulating concentrations of PGFM were not correlated with P4 ($P = 0.79$) or E2 ($P = 0.92$) concentrations among cows across treatment groups.

Basal concentrations of P4 were included as a covariate in the model and were similar in all groups of cows (12.65 ± 0.26; $P = 0.59$). There was a treatment by time interaction detected ($P = 0.006$) resulting in a decrease in P4 concentrations in both OT groups at hour 2 compared to basal concentrations (13.47 ± 0.39 vs 9.19 ± 0.34 ng/mL; $P < 0.01$). By hour 4, P4 concentrations returned to basal levels in the Low OT group but

not the High OT group (Figure 3-4). Progesterone concentrations did not change in CON cows over the sampling period ($P > 0.05$).

Basal concentrations of E2 were included as a covariate in the model and were similar between OT and CON cows ($P = 0.31$; Figure 3-5). Although there was no treatment by hour interaction detected ($P > 0.05$), E2 concentrations decreased ($P = 0.04$) in OT cows from baseline (3.24 ± 0.54 pg/mL) to hour 4 (1.51 ± 0.26 pg/mL) but there was no difference between High PAG OT and Low PAG OT groups at any time point ($P = 0.43$).

3.4. Discussion

In this study, PAG concentration had no influence on hormone responses following oxytocin challenge in pregnant *Bos indicus*-influenced cows at day 30 of gestation. Cows with low circulating PAG concentrations at day 30 of gestation have been shown to have a greater risk of undergoing late embryonic mortality during the second month of gestation [5, 6, 31]. Additionally, pregnancies with abnormal placentas, such as those found in somatic nuclear transfer clone pregnancies, have severe deviations from normal PAG profiles when monitored throughout gestation [32] and PAG may have a positive impact on P4 production [33]. Despite this predictive factor, little is known about the causes and mechanisms contributing to pregnancy loss during this period. Clearly, there is an increase in basal concentrations of $PGF_{2\alpha}$ during active placentation [9,10] which may be critical for placental interdigitation or development. Excessive synthesis and secretion of $PGF_{2\alpha}$ during this period, however, could potentially lead to late embryonic loss. In the current

study, all cows had a circulating PAG concentration greater than the 95% confidence cutoff (1.4 ng/mL) for late embryonic mortality identified in using a similar antibody as described by Pohler et. al. [6]. Cows in the Low PAG OT group, however, had similar or lower concentrations of PAG compared to cows that underwent late embryonic pregnancy loss in previous studies (average range 3.14 - 6.25 ng/mL) [4-6]. Our hypothesis was cows with substandard placental function and an increased likelihood of pregnancy loss, as predicted by decreased PAG concentration, would have alterations in prostaglandin release following oxytocin administration. The functions of prostaglandins throughout the body, including modulation of immune cell populations, regulation of growth factors and vascular modification, are congruent with the changes that accompany placental development [34-36]. It has been established that dynamic changes of $PGF_{2\alpha}$ profiles occur throughout pregnancy. During early pregnancy, interferon-tau suppresses $PGF_{2\alpha}$ pulsatility to prevent luteolysis [37] and, during parturition, peak concentrations of $PGF_{2\alpha}$ are required for proper placentome detachment and placental expulsion [38]. It has also been reported that administration of oxytocin during the second month of gestation has the capacity to induce $PGF_{2\alpha}$ secretion [28]. In addition, Bridges et al. [11] reported that pregnant cows with greater $PGF_{2\alpha}$ concentrations were more likely to maintain pregnancy following induction of a replacement CL following regression of the primary CL. Increased basal concentrations of PGFM during this period are evident, in both cattle and sheep [10, 28, 39]; however, the physiological reasoning for the increased responsiveness to oxytocin and the luteal protective mechanisms that surround the CL are unknown.

In this study, concentrations of P4 significantly decreased at hour 2 after oxytocin administration compared to basal concentrations; however, concentrations returned to basal levels by the completion of the sampling period in Low PAG OT cows but not High PAG OT cows. In previous studies from our lab, oxytocin challenge did not negatively influence blood P4 concentrations around day 30 of gestation in non-lactating cows [21]. Interestingly, Drum et al. [40] observed a tendency for P4 to increase after oxytocin challenge in pregnant, lactating dairy cows. Changes in blood flow and potential second signals of pregnancy are hypothesized as mechanisms that protect the functionality of the CL from luteolytic effects of $PGF_{2\alpha}$ during the second month of gestation [41]. Compared to the current study, previous studies have been conducted in *Bos taurus* cows and the sampling period after oxytocin administration was shorter [22]. *Bos indicus* and *Bos indicus*-influenced cattle have different reproductive physiology compared to *Bos taurus* cattle [42], including greater sensitivity to gonadotropins and steroid hormones [43, 44]. Oxytocin induced a significant pulse of $PGF_{2\alpha}$ in all treated cows and the physiological sensitivity of *Bos indicus* subspecies may explain the P4 decrease observed in this study compared to studies in *Bos taurus* cows. Similar to previous studies, there was no negative impact on the survival of pregnancy following oxytocin challenge, despite a decrease in P4.

In addition to the decrease in P4, a decrease in circulating E2 was also observed in oxytocin treated cows. This is an interesting finding, as E2 concentrations following oxytocin challenge at day 30 of gestation have not been reported previously. Estradiol levels are low during early pregnancy compared to the follicular phase of the estrous cycle

and final trimester of gestation [45, 46]. Wettemann et al. reported an increase in circulating E2 at day 40 of gestation that returned to previous concentrations by day 50, indicating that the increase throughout gestation might not be linear and that E2 concentrations may fluctuate during pregnancy [47]. At day 15 of the estrous cycle, a $PGF_{2\alpha}$ pulse has been shown to increase LH secretion [48] which supports increased E2 secretion by the ovary; however it is unknown what a rebound in P4 concentration would contribute. Despite these observations, the potential role of E2 and the regulatory mechanisms during the period of embryonic development is unclear.

Significant cow to cow variation existed in concentrations of hormones measured in this study, particularly PGFM. Compared to the estrous cycle, basal concentrations of PGFM are greater during pregnancy [10, 28]. Some cows, however, exhibited significantly greater circulating concentrations of PGFM than other cows (data not shown). Interestingly, peripheral concentrations of P4 and E2 did not differ among groups. Both low and high basal and peak concentrations of PGFM were evenly distributed among PAG classification groups. These results are similar to previously reported studies indicating marked variation in magnitude of PGFM pulses between cows [21, 40]. From a physiological perspective, this variation has not been explained.

One limitation of this study was the singular evaluation of PGFM from the prostaglandin family. There is some debate as to whether circulating PGFM concentrations are representative of $PGF_{2\alpha}$ release by the uterus. Most reports have utilized concentrations of PGFM as a surrogate for $PGF_{2\alpha}$ because it is more stable in circulation and prevents the need to catheterize the uterine vasculature [49, 50]. Despite the frequent

use of this metabolite to monitor $PGF_{2\alpha}$ levels, Cooper et al. reported that PGFM concentrations may not correspond closely with $PGF_{2\alpha}$ concentrations in all physiological conditions [51]. Additionally, it has been suggested that the ratio between $PGF_{2\alpha}$ and PGE_2 may be more important than the concentration of $PGF_{2\alpha}$ alone for pregnancy maintenance. In sheep and cattle, there is evidence that elevated concentrations of PGE_2 promote luteal resistance by stimulating P4 secretion during the maternal recognition of pregnancy [52-55]. During the second month of gestation, the mechanisms which protect the CL from elevated basal concentrations of $PGF_{2\alpha}$ and the return of $PGF_{2\alpha}$ pulses may be, in part, explained by this relationship. In addition to the endometrium, it has been shown that binucleated trophoblast cells (BNC) in bovine placenta during later stages of gestation can convert $PGF_{2\alpha}$ to PGE_2 [56, 57]. Binucleated trophoblast cells appear in the bovine chorionic epithelium around day 17 of gestation and secretory products, including PAG, can be detected in maternal circulation by day 24 of gestation [9, 58]. As previously mentioned, differences in PAG production are observed in cows that undergo pregnancy loss and are directly influenced by BNC function, which may also have the capability to alter prostaglandin profiles during the period of active placentation. Additionally, a recent *in vitro* study reported an increase in relative mRNA abundance of Prostaglandin E synthase from endometrium explants after 24 hours of PAG treatment exposure [59]. Although no difference was observed in hormone response following oxytocin challenge in cows with different circulating PAG concentrations, the BNC population in individual placentas could play a role in prostaglandin synthesis and regulation to allow maintenance

of the CL and pregnancy in cattle. Further research is needed to identify the prostaglandin profile in cows that undergo late embryonic mortality.

In summary, circulating PAG concentrations do not appear to be related to concentrations of PGFM following oxytocin challenge in *Bos indicus*-influenced cows. A significant oxytocin-induced release of $PGF_{2\alpha}$ results in a temporary decrease of P4 indicating an effect on CL function. The return to basal concentrations within 2 hours and absence of pregnancy loss, however, indicate a single significant release of $PGF_{2\alpha}$ does not have long term negative impacts at day 30 of gestation.

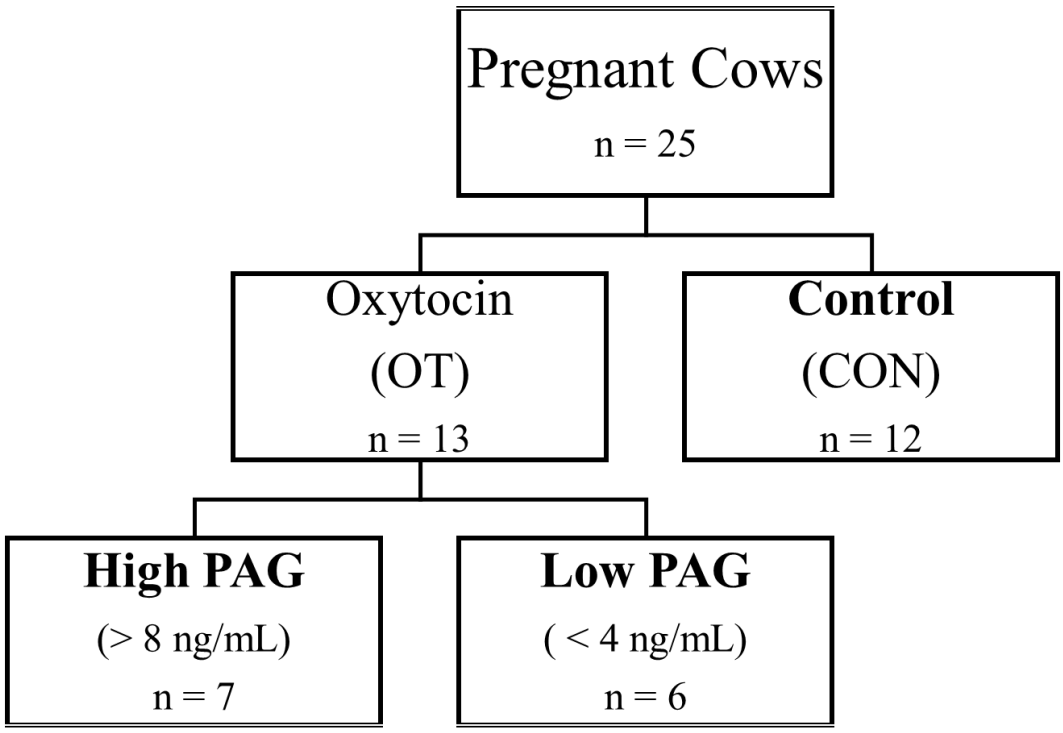

Figure 3-1. Hierarchy of treatment group classifications
Groups in **bold** are those that were used for analysis. Cows treated with oxytocin were subsequently divided for analysis while the control group was analyzed singularly.

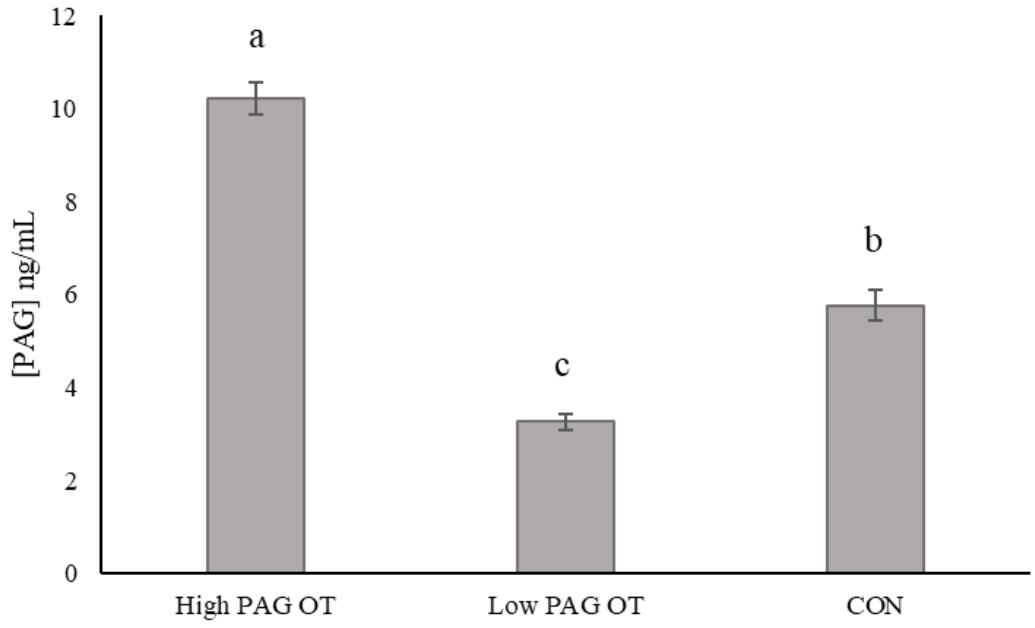

Figure 3-2. PAG Concentrations by treatment group
Mean ± SEM circulating PAG concentrations for each treatment group. Cows in the oxytocin treatment groups were separated into groups based on PAG concentration at the baseline sample period (High PAG OT and Low PAG OT). Letters indicate differences at $P < 0.05$.

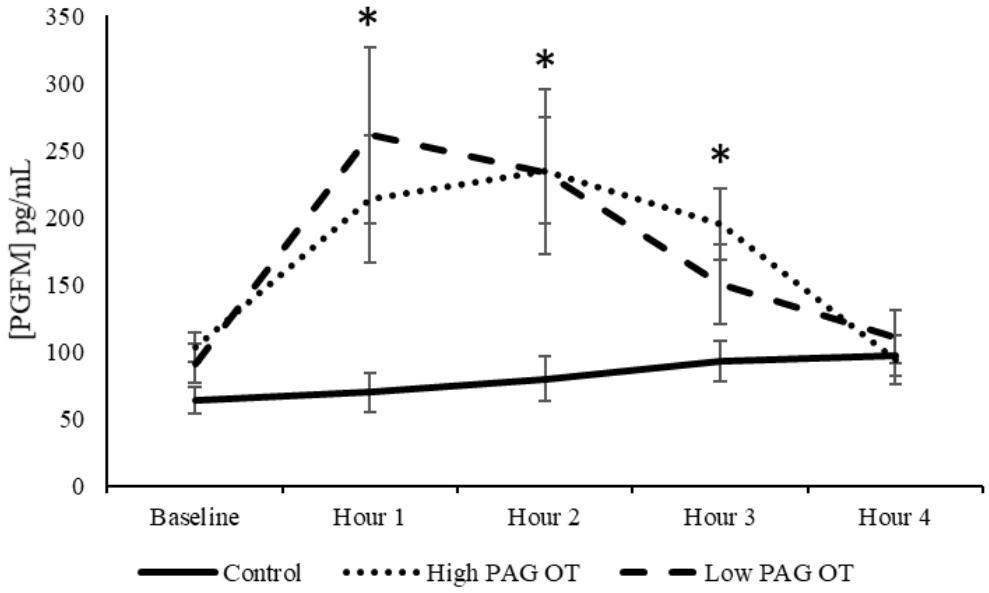

Figure 3-3 PGFM concentrations during oxytocin challenge
Concentrations of PGFM in oxytocin-treated and control cows over the 5 hour treatment period. An asterisk indicates a difference ($P < 0.05$) between control and oxytocin challenge cows. There was no difference between High PAG OT and Low PAG OT concentrations of PGFM at any time point ($P > 0.1$).

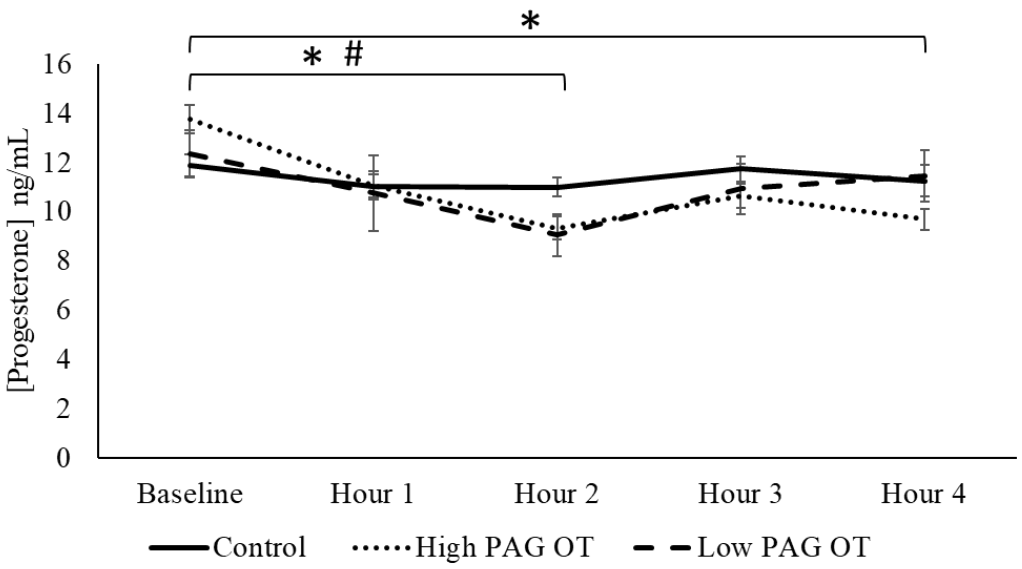

Figure 3-4 Progesterone concentrations during oxytocin challenge
Mean P4 (± SEM) concentrations in control and oxytocin challenged (OT) cows throughout the sampling period. Brackets indicate decreased concentrations ($P < 0.05$) within groups (asterisk indicates High PAG OT; number sign indicates Low PAG OT) compared to baseline levels.

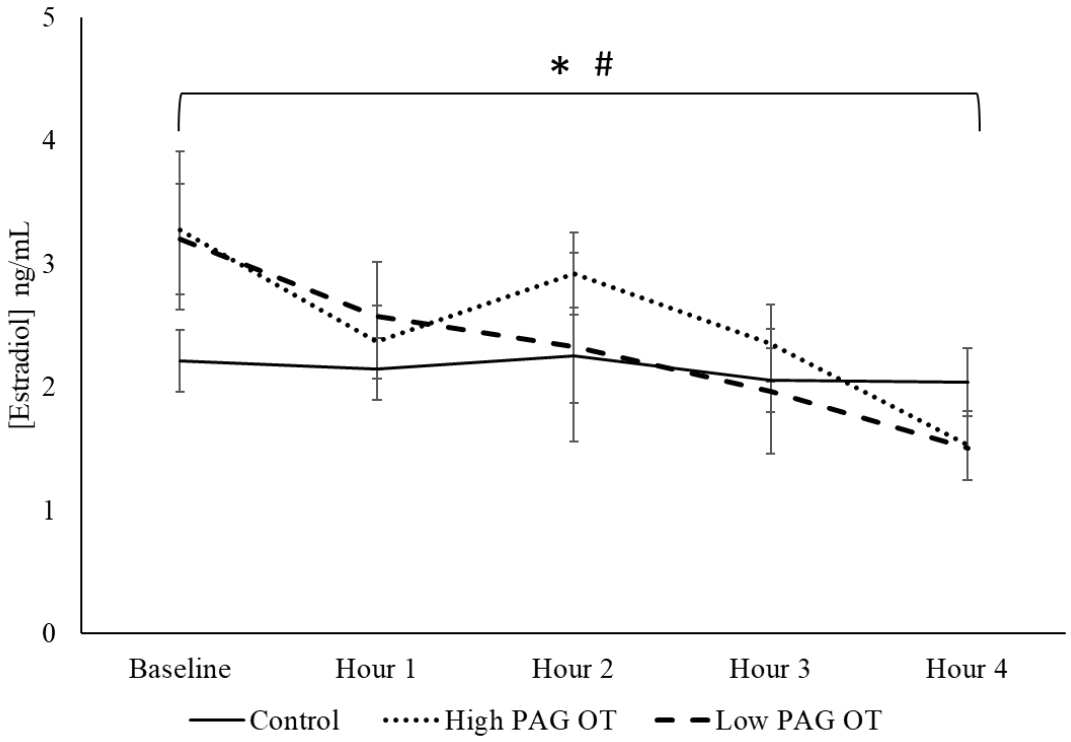

Figure 3-5 Estradiol concentrations during oxytocin challenge
Mean concentrations of E2 (± SEM) in control and oxytocin challenged (OT) cows throughout the sampling period. A time effect ($P < 0.05$) was observed between baseline and hour 4 concentrations in High PAG (asterisk) and Low PAG (number sign) cows but there was no change in concentrations of E2 over the sampling period for control cows.

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4. COCCYGEAL VEIN CATHETERIZATION FOR SAMPLING OF BOVINE FEMALE REPRODUCTIVE TRACT DERVIVED PRODUCTS

4.1. Introduction

Studies involving reproductive endocrinology at the organ or cellular level of cattle have been difficult to conduct due to the challenges associated with collecting blood or lymph samples prior to dilution and metabolism in general circulation. Many uterine vein cannulation procedures, including saphenous vein cannulation, require heavy sedation or anesthesia and immobilization in a recombinant position [1]. Use of flank laparotomy to access the uterine vein can be invasive, requires advanced surgical skill, and presents potential post-surgical risks [2, 3]. Alternatively, coccygeal vein cannulation is a relatively non-invasive, easily performed technique in normal cattle working facilities. Further, upon healing, there is little visible damage to the associated blood vessels. Coccygeal vein cannulation is also easily maintained and accessed for frequent sampling. Cannulation of the coccygeal vein and artery have been used for many years [4-6]; yet, details regarding the procedure have not been well described or updated for many years. The objective of this paper is to provide a detailed procedure for cannulation of the coccygeal vein and model for the ideal distance of cannula placement for blood collection of the uterine drainage.

4.2. Materials and Methods

4.2.1. Animals

All protocols were approved by Texas A&M University Institutional Animal Care and Use committee. Cows and heifers of mixed breeds were used in the validation of the surgical technique for placement of the coccygeal vein cannulas. A subset of these animals (n = 4) were utilized for confirmation of placement at the site of uterine drainage through P4 quantification. Multiparous beef cows were used in this study, ranging in body weight from 453 - 589 kg. Both subspecies, *Bos taurus* (n = 2) and *Bos indicus* (n = 2), were utilized. Cows were pregnant, ranging from 15 to 30 days of gestation, with an active CL.

4.2.2. Catheter placement and maintenance

The procedure described in this study for cannulation of the caudal vena cava is a modification of the procedure first described by Sears et al. (1978) [4] using a small incision rather than blind needle insertion. The previously described procedure is difficult to replicate, as vessel location varies by individual animal and blind needle insertion increases the likelihood of damaging the vessel impeding placement of the catheter. Prior to catheter insertion, cows were restrained in a squeeze chute and given a caudal epidural (Lidocaine hydrochloride, 100 mg). The area was cleaned with povidone and iodine scrub for 2 minutes and air dried. The tail was raised over the back of the animal and a tourniquet was applied immediately to the base of the tail to prevent blood return and aid in isolation of vein. A surgical drape was applied to prevent fecal material contamination due to close proximately of the anus. To begin, a 2-inch incision was made between the 2nd and 3rd coccygeal vertebrae in the ventral vertebral groove. Both the middle tail artery and vein

lie within this central groove; however, pressure caused by the tourniquet and raising the tail cause the vein diameter to increase while the artery diameter decreased (Figure 4-1). There appears to be no pattern to predict the orientation of the vein compared to the artery (posterior, anterior, left or right) and much variation exists between individual animals in the amount and types of tissue surrounding the vein as well as collateral flow. Some vessels run superficially, while others require significant dissection of tissue and muscle fibers to locate. In a research setting, animals with scar tissue or vessel damage from previous venipunctures were more complicated to cannulate. A clear isolation of the vein from surrounding tissues and artery was fundamental to facilitate the proper cannula insertion. Once the vein was isolated from the artery and surrounding tissue, the tourniquet was released. Isolation of both artery and vein is recommended to ensure cannulation of correct vessel, as they are commonly found adjacent to each other. At times, it can be difficult to differentiate the coccygeal vein from the coccygeal artery; however, the artery was clearly identified by bright red, highly pressurized blood flow. Following vein and artery isolation, one or two suture lines were passed behind the vessels in order to isolate it and provide options for tying off the vessel after insertion of the cannula (Figure 4-2). The rostral suture was used to tie off the artery to stop any auxiliary bleeding. A 100 cm polyethylene catheter (BD Intramedic, ID 0.047”, OD 0.067”) was inserted through a nick in the vein using a cardiac vein pick (OSCOR, Palm Harbor, FL). Cardiac guidewires (Amplatz SuperStiff, Boston Scientific, Marlborough, MA) were used to provide structure to the catheters during placement. The catheters were marked with permanent marker to aid in identifying the correct placement distance. A square knot can be used to secure the

vein and catheter prior to suturing the incision. For closure of the incision, a simple interrupted suture pattern allows for movement of the catheter which helps to maintain patency for greater periods of time (Figure 4-3).

The catheters are sealed with a bidirectional valve cap (MILA International, Florence, KY) allowing for blood collection and flushing of the catheter to prevent blood clots and introduction of air. Catheters were trimmed to leave approximately 20 cm exposed and fastened to the tail using self-adhering, flexible bandaging. Bandaging was not removed for sample collection; however, if a clot or kink within the tubing occurred the catheter was repositioned using a guidewire to regain function. Catheters were flushed with 0.9% saline solution and locked with 20 IU/mL heparin-saline solution at least every 8 hours or whenever sample collection occurred to maintain long term patency. Bandages were changed once per day to evaluate incision site and maintain cleanliness.

4.2.3. Uterine drainage identification

To ensure correct placement of the catheter for collection of uterine/ovarian derived products, P4 concentrations were evaluated. Progesterone concentrations peak at the site of uterine/ovarian drainage and decrease as the concentrated uterine/ovarian blood flow is diluted in general circulation. Samples were collected beginning once the catheter was inserted 45 to 55 cm. Additional samples were collected every 5 cm as the catheter was progressed until it was inserted 90 cm or could no longer be moved forward freely. Samples were collected into EDTA vacutainer tubes, inverted 5 times, and immediately placed at 4℃. After centrifugation at 2500 g for 15 minutes, plasma was aliquoted and stored at -20℃. Progesterone concentrations were quantified using a double antibody RIA

(MP Biomedical, Salon, OH) in duplicates following manufacturer recommendations previously validated in our lab [7] with a sensitivity of 0.1 ng/mL. Intra-assay and inter-assay CV's were 5.12% and 6.83%, respectively. Once the correct distance was identified, a guidewire was reintroduced to the catheter and correctly positioned within the coccygeal vein for the remainder of the collection period.

4.3. Results and Discussion

This procedure is a proven method for collection of uterine-ovarian drainage for the assessment of uterine derived products. While inconsistencies in the anatomy of the tail vasculature complicate the placement procedure, our group has achieved a catheterization success rate of up to 80%. Visualization of the blood vessels and isolation with the suture lines adds using this method adds precision that cannot be achieved using the method described by Sears et al [4]. This technique has multiple applications for studies utilizing sampling schedules that may be difficult to accommodate with other techniques. Coccygeal vein catheters are robust enough for frequent samplings (minutes) but also stable enough for collections occurring every 6-8 hours. Catheters were maintained up to 12 days; however, catheter failure occurred in 30% of them prior to the end of the collection period.

In previous studies, catheter placement is generally reported at a set distance of 65-75 cm [5, 8, 9]. We aimed to identify if a standard placement distance could be utilized for uterine ovarian drainage collection. The four cows used for P4 evaluation had small to average sized tracts, score 1 or 2 as evaluated according to the guidelines outlined in

Young et al [10]. Peak concentrations of P4 were observed when the catheter was placed between 58 and 80 cm into the coccygeal vein (Fig 4-4). For the most accurate collections, catheters should be placed with regard for individual P4 measurements. No relationship was detected between body weight, parity, or reproductive tract score ($P > 0.05$); however, a larger sample size may be necessary to detect meaningful correlations. Factors such as frame score, body composition and type, in combination with weight and reproductive tract size, could provide additional variation to the internal distance of uterine ovarian drainage. It may be possible to have a standardized placement for heifers within a contemporary group, as was observed by Kotwica et al [5], but there is likely too much variation in cows. Progesterone sampling is inexpensive, relatively quick and can ensure proper placement for the collection of the desired samples.

Based on increasing concentrations prior to the peak P4 concentration, it appears that accessory vessels or back flow may contribute to elevated levels of P4 prior to the connection of the uterine vein. Blood flow diluted the P4 concentration to same level as the pre- drainage levels at 10 to 15 cm past the distance where the peak concentration was observed. In Figure 4-4c, this was unable to be evaluated because the catheter could not be progressed forward beyond 80 cm due to obstruction or vessel wall issue. This illustrates the issues associated with a single distance which has been prescribed in earlier studies, a placement of 75 cm would likely be unable to detect differences compared to general circulation in a cow with a peak at 58 cm. Most of the original data suggests 70 cm but with a range of 60 to 80 cm this could significantly alter the results when collecting uterine or ovarian derived products prior to general circulation.

Despite the difficulties presented by the inconsistent vasculature between individual cows, this technique does not require significant surgical input or specialized facilities and accommodates a wide variety of sampling schedules. Coccygeal vein catheters can be used in pregnant animals without harm to the pregnancy, have low risk of infection and heal without impairing function of the vasculature or tail.

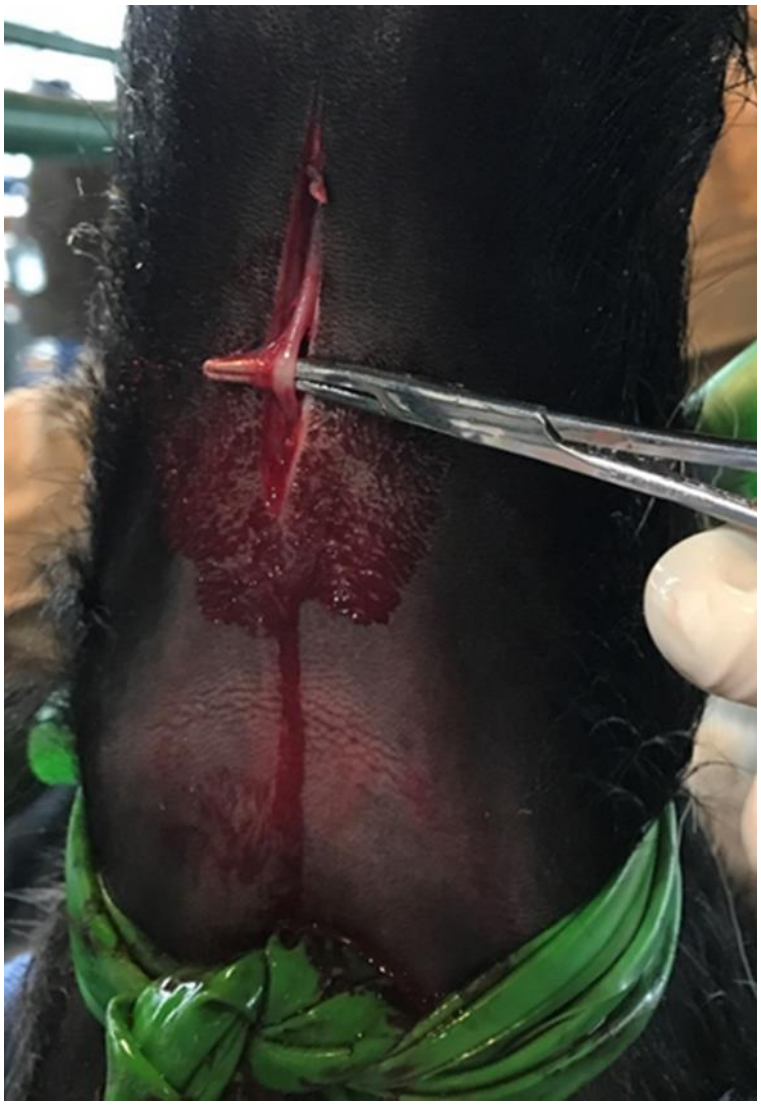

Figure 4-1. Coccygeal vein isolation.
A small incision was made between the 2nd and 3rd coccygeal vertebrae to visualize the coccygeal vein. Hemostats were used to isolate the vein from surrounding tissue. Photo: S. Reese.

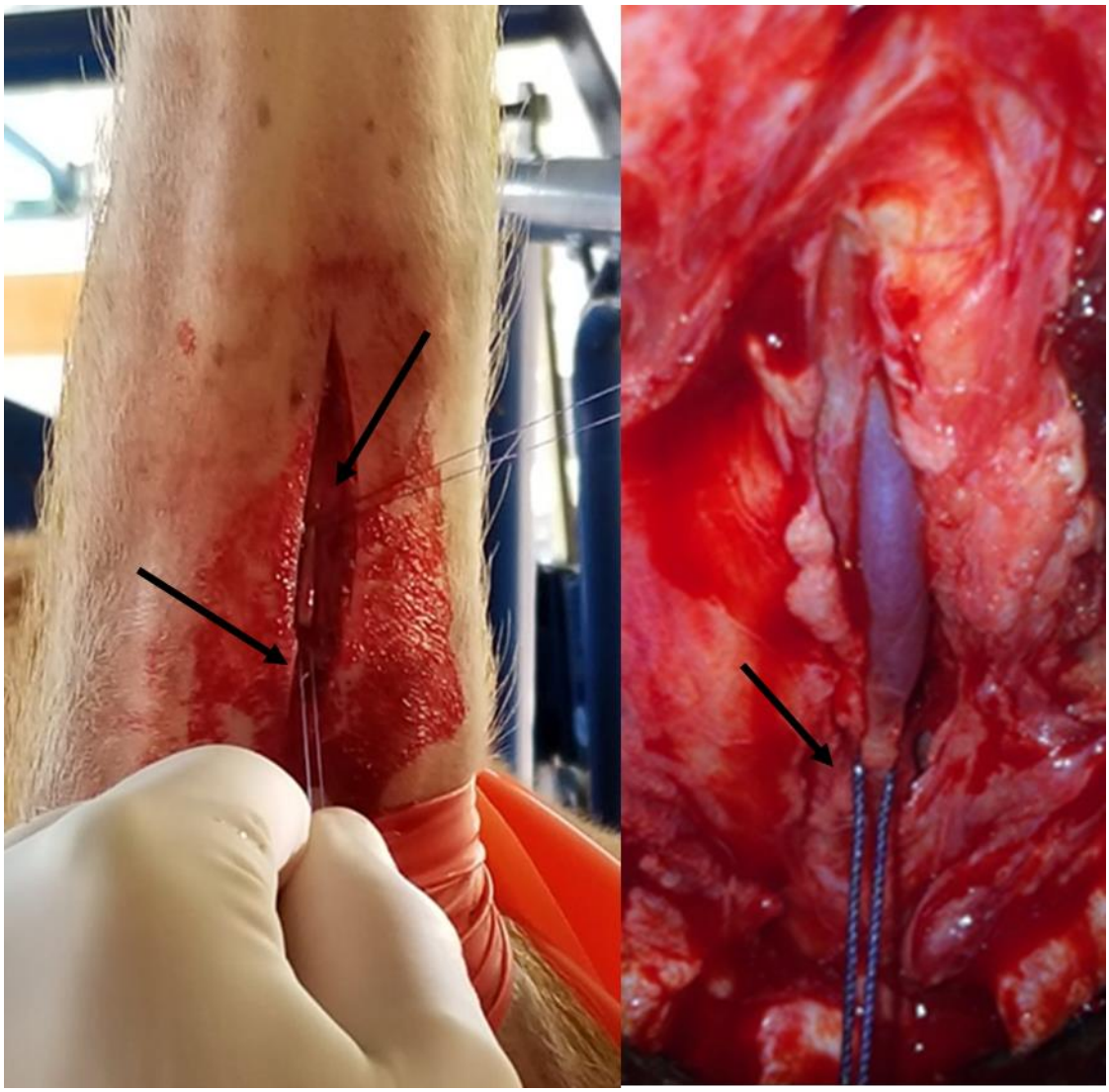

Figure 4-2. Using suture lines to control vein for catheter placement.
Using the hemostats, suture (black arrows) was placed behind the vein to maintain control of blood flow during catheter insertion. Photos: S. Reese.

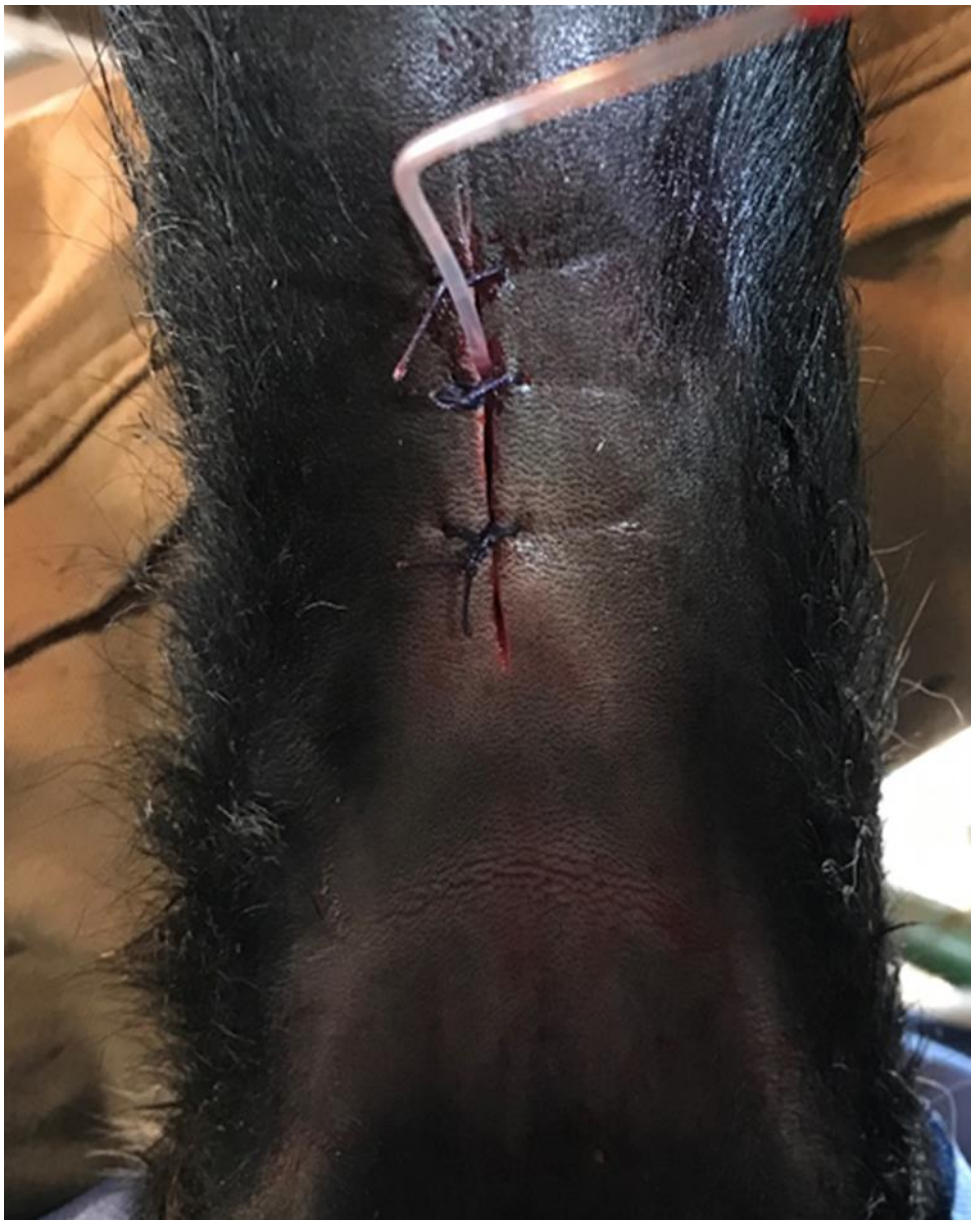

Figure 4-3 Incision site closure.
The incision was closed with a simple interrupted suture pattern to allow for movement of the catheter if required during the sample collection period. Photo: S. Reese.

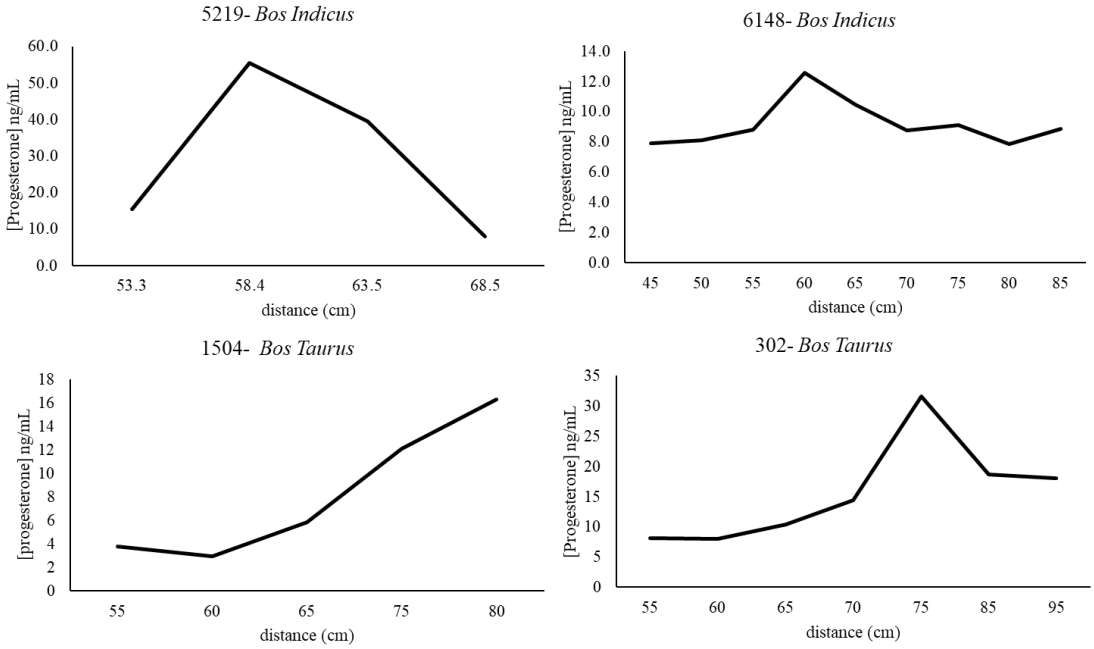

Figure 4-4. Progesterone profiles used to determine correct catheter placement P4 samples of blood samples collected as the catheter was progressed forward into the vein to identify the sight of uterine ovarian drainage for final catheter placement.

4.4. References

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5. VARIATION IN PROSTAGLANDIN PROFILES IN COWS THAT UNDERGO LATE EMBRYONIC MORTALITY

5.1. Introduction

Despite decades of research, genetic evaluations and improved management strategies, reproductive failure remains a significant problem to cattle industries. Late embryonic/early fetal mortality (LEF), or pregnancy loss occurring between day 28 and 60 of gestation, affects 8 to 15% of all pregnancies in cattle [1, 2]. The defining feature of this period of pregnancy is active placentation with placentome development. Around day 28 to 30 of gestation, active placentation begins with the appearance of primal cotyledons and initial development of maternal caruncular villi [3, 4]. Active placentation continues between day 35 and 40 with in increasing number and length of caruncular villi [5]. Simultaneously, trophoblast cells establish close contact with the uterine epithelial cells within the villi crypts anchoring the fetal membranes to maternal tissues [5]. By day 45 of gestation, the embryo transition to the fetal stage is underway and the placenta has 30 or more placentomes [4]. The placenta continues to develop and the number of placentomes grows as gestation progresses, however, basic cotyledon and caruncular structure is established by the end of embryonic development [4, 5]. During this crucial period of development, insufficient placental development may result in pregnancy failure.

Basal levels of prostaglandins (PG), specifically $PGF_{2\alpha}$, increase during the second month of gestation without corpus luteum (CL) regression occurring [6, 7]. Additionally, Bridges et al. [8] reported increased concentrations of $PGF_{2\alpha}$ between day 31 and 35 in cows that maintained pregnancy compared to cows that underwent embryonic mortality.

Increased frequency of $PGF_{2\alpha}$ pulses are responsible for the luteolytic effect seen during the estrous cycle [9]; however, limited information is known about the pulsatility of $PGF_{2\alpha}$ when basal concentrations are elevated during the second month of gestation and its effect on pregnancy maintenance. Additionally, even less is known about the most ubiquitous of all prostaglandins, PGE_2, during this period. Evidence suggests that PGE_2 may contribute to luteoprotective mechanisms and counteract the properties of $PGF_{2\alpha}$ [10] during early gestation; however, the relationship during later stages of gestation are unknown.

The mechanisms driving LEF losses are relatively unknown, therefore it is imperative to profile normal pregnancies and identify deviations in endocrine relationships that may contribute to LEF. The objectives of this study are to 1) profile concentrations of PG and their relationship with concentrations of P4 and pregnancy associated glycoproteins (PAG) in cows that maintain pregnancy or undergo LEF and 2) examine pulse patterns of $PGF_{2\alpha}$ at various time points in the late embryo development period. Our hypothesis is that increased basal concentrations of PG are required for proper placentation development and alterations in prostaglandin release, specifically the pulse patterns, would negatively affect pregnancy success.

5.2. Materials and methods:

5.2.1. Animals

All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of the respective supervising institution. Cows of mixed breeds and parities (n = 150) at 3 different research stations (Middle Tennessee Ag Research and Teaching Center, Spring Hill, TN; Texas A&M Animal Science Beef Production Systems

facility, College Station, TX; Texas A&M AgriLife Research and Extension Center, Overton, TX) were utilized. All cows were synchronized using 7- day Co-synch + CIDR protocol beginning with CIDR insertion and GnRH administration. After 7 days, the CIDR was removed, 25 mg of $PGF_{2\alpha}$ was administered and an estrus detection patch was applied. Cows were observed for estrus 2 to 3 times daily and inseminated 12 hours after detection of estrus. If estrus was not observed 62 to 66 hours post CIDR removal, cows received GnRH and were inseminated. Cows designated to the non-pregnant control group (CON; $n = 7$) were sham inseminated with heat treated semen. Breeding date is identified as day 0. On day 16, CON cows received a CIDR to maintain high P4 levels. The CIDR was replaced on day 27. Pregnancy status was evaluated by ultrasound and catheter placement occurred on day 29. Only cows with visibly normal CL and embryos with apparent heartbeats were selected to undergo the vein cannulation procedure. The sample collection period lasted from the time of catheter insertion until day 40 of gestation (schematic of the experimental design, Figure 5-1) or until the catheter was deemed nonfunctional. Ultrasound was used to monitor the viability of pregnancies every 3 to 4 days. Once the catheter was removed at day 40, another pregnancy diagnosis was performed, and the final pregnancy evaluation was conducted between day 60 and 75 of gestation. Two pregnancy loss periods were identified and analyzed separately: loss between day 30 and 40 (L1) and loss between day 41 and 60 (L2).

5.2.2. Sample collection

Pregnant and CON cows were fitted with coccygeal vein catheters on day 29 of gestation ($n = 47$). Polyethylene catheters (Intramedic, Beckton Dickinson, Sparks, MD) were placed between 60 and 75 cm into the coccygeal vein as described in the previous

chapter to collect blood from uterine ovarian drainage. Catheters were loosely sutured into the vein to allow for movement of the tubing to maintain patency. A bidirectional injection cap (MILA International, Florence, KY) allowed for blood collection via syringe. Blood samples were collected every 6 hours until day 40 of gestation. Blood was immediately put into 6 mL EDTA K2 vacutainer tubes (BD Vacutainer, Franklin Lakes, NJ) with 10 µM/mL indomethacin to prevent *ex vivo* eicosanoid formation and mixed thoroughly. Catheters were flushed with 0.9% saline solution and locked with 20 I.U. heparin solution. In the event blood was unable to be collected from the catheter, a sample was collected via jugular venipuncture and treated with indomethacin. Samples were stored on ice until centrifugation. Plasma was separated by centrifugation for 15 min at 2,500 x g within 1 hour of collection and stored at -20℃.

5.2.3. Pulse characterization

A subset of cows (n = 4) and heifers (n = 4) underwent a more frequent collection schedule to assess the prostaglandin pulse profiles. Blood samples were collected every 15 minutes for 6 hours on d 29, 31, 34,37, and 39 of pregnancy. Blood samples were treated the same as previous collections and all animals were part of the larger trial.

5.2.4. Assays

Prostaglandin $F_{2\alpha}$ metabolite (PGFM): Concentrations of PGFM, specifically 15-keto-13, 14-dihydro-PGF_{2a}, were quantified using an ELISA described by Mezera et al. [11] using a 1: 16,000 dilution of primary antibody (gift from Dr. William Thatcher, University of Florida) and PGFM- HRP conjugate (gift from Dr. Milo Wiltbank, University of Wisconsin). Serum from a cow treated with flunixin meglumine was used as low control

for all prostaglandin assays. The intra-assay and inter- assay CVs were 6.23% and 14.97%, respectively. All samples were assayed for PGFM concentration.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$): A subset of samples were analyzed for $PGF_{2\alpha}$ using a commercial assay (#516011, Cayman Chemical, Ann Arbor, MI) according to manufacturer instructions for serum. The intra-assay and inter- assay CVs were 7.91% and 7.25%, respectively. Samples were assayed for $PGF_{2\alpha}$ concentration if P4 concentrations indicated that the catheter was near the site of uterine ovarian drainage.

Prostaglandin E_2 metabolite (PGEM): Samples were assayed for concentration of PGEM using a commercially available ELISA (#514531, Cayman Chemical, Ann Arbor, MI) previously utilized in bovine serum according to manufacturer instructions [12, 13]. The intra-assay and inter- assay CVs were 9.13% and 11.37%, respectively.

Progesterone (P4): Progesterone concentrations were quantified via RIA using a commercial kit (MP Biomedicals, Santa Ana, CA) previously validated in our lab [14] with high and low P4 controls and standard curves at the beginning and end of each assay. The intra-assay and inter-assay CVs were 6.87% and 7.31%, respectively. All samples were assayed for P4 concentration.

Pregnancy associated glycoproteins (PAG): Concentrations of PAG were quantified using an in-house ELISA validated by Green et al [15] using a polyclonal antibody raised against PAG expressed in early gestation that was validated by Reese et al [16]. Plates were controlled using a standard curve, positive controls from a pool of late gestation pregnant cow serum and negative controls using pooled steer serum. The inter-assay and intra- assay CVs were 7.83% and 8.65%, respectively. Circulating PAG concentrations were assayed every 12 hours.

5.2.5. Analysis and Statistics

Cows were grouped by pregnancy outcome based on pregnancy diagnoses at day 40 and day 60 of gestation. The response variables $PGF_{2\alpha}$, PGFM, PGEM, and PAG were analyzed using PROC MIXED on SAS 9.4 (SAS Institute Inc., Cary, NC) using sample as repeated measures. Fixed effects in the model included day, time, pregnancy status group and their interactions. Random effects included cow within group, breed, parity, and experimental round. If overall model was significant, Tukey's HSD procedure was utilized to identify mean differences between pregnancy status groups. Peaks and basal concentrations of PGFM were analyzed using AutoDeacon [17] using the Pulse2 fit with a half-life adjustment of 60 minutes [18, 19]. No assumptions were made on predicted basal secretion or number of secretion events. When appropriate, data are presented as average ± SEM. Significance was set at $P \leq 0.05$ and a trend was defined between $0.05 < P \leq 0.1$.

5.3. Results:

5.3.1. Pregnancy rates

After the initial pregnancy diagnosis, pregnant ($n = 42$) and CON ($n = 5$) cows were successfully fitted with coccygeal vein catheters. Subsequent pregnancy diagnoses determined cows that underwent pregnancy loss between day 30 and 40 of gestation (L1; $n = 4$), cows that lost pregnancy between day 41 and 60 (L2; $n = 4$), and cows that successfully maintained pregnancy (PS; $n = 34$).

5.3.2. PGF2α

Concentrations of $PGF_{2\alpha}$ from the uterine ovarian drainage were evaluated in pregnant cows ($n = 21$), CON cows ($n = 4$), L1 cows ($n = 3$) and L2 ($n = 1$). Samples were

only considered for $PGF_{2\alpha}$ analysis if the P4 concentrations were increased compared to circulating P4 concentrations collected from jugular venipuncture indicating that sampling from uterine ovarian drainage was successful. Predicted basal concentrations ranged from 14.41 to 47.63 pg/mL. Peak concentrations of $PGF_{2\alpha}$ ranged from 50.41 to 288.76 pg/mL. Concentrations of $PGF_{2\alpha}$ were decreased ($P < 0.05$) compared to PGFM; however, observed patterns and peaks were similar between hormone and metabolite (Figure 5-2). In order to include the greatest possible number of animals, PGFM was used in all further analysis.

PGFM

Concentrations of PGFM were analyzed in all cows. Basal PGFM concentrations did not differ between groups ($P = 0.26$; Figure 5-3). One cow in the L2 group had significantly elevated PGFM concentrations compared to other L2 cows, thus weighed heavily on the average concentrations for that group. Concentrations of PGFM varied significantly by individual animal; basal concentrations of PGFM ranged from 10 to 150 pg/ mL. Over the sampling duration, periods of elevated PGFM concentrations (peaks) were observed. Peak concentrations of PGFM ranged from (99.48 to 755.78 pg/mL), generally proportional with basal concentrations. Additionally, there was no difference ($P = 0.33$) in the ratio of peak PGFM concentration to basal concentration. The number of peaks, however, varied between pregnancy outcome groups (Figure 5- 4). Cows in the L2 group had a significantly greater number of peaks compared to PS and CON cows ($P = 0.04$; L2: 2.8 ± 0.37 peaks vs CON: 1.6 ± 0.40 peaks and PS: 1.66 ± 0.18 peaks). During these periods of increased concentrations, PGFM peak concentrations were numerically increased in PS cows compared to L1 cows (227.12 ± 26.95 vs. 179.27 ± 13.75 pg/ mL)

but not statistically different ($P = 0.12$). In cows with 2 or more peaks, the time between peaks varied. Cows that experienced pregnancy loss tended (L1, $P = 0.09$; L2, $P = 0.07$) to have fewer hours between each peak even if they exhibited the same number of peaks as PS cows. There was no difference between any pregnancy outcome group on day which peaks occurred ($P = 0.36$) .

5.3.3. PGFM pulse characterization

Pulses of PGFM were characterized on days 29, 31, 34, 37 and 39 in a subset of females ($n = 8$; 4 heifers, 4 cows). As with the long-term profiles, significant variation in PGFM concentrations was observed between individuals that maintained pregnancy ($n = 6$). Each animal had peaks during the sampling period (range: 2 to 8). In pregnant cows, there was no difference in peak concentration by day ($P = 0.68$) or by parity ($P = 0.76$). Basal concentrations of PGFM at day 31 were increased in heifers ($P = 0.03$) and tended to be increased in cows ($P = 0.09$) compared to other days of gestation. The number of pulses was greatest at day 31 (2.2 ± 0.57 peaks) and decreased day 34 (0.25 ± 0.25 peaks) through day 39 (0.33 ± 0.13 peaks). Progesterone concentrations did not differ ($P > 0.05$) by day, parity, or pregnancy outcome, nor were significant fluctuations observed during the 6 hour collection periods.

Of the 8 animals profiled for pulse characterization, 2 (1 cow, 1 heifer) underwent LEF between day 41 and 60. The LEF females had 2 different PGFM phenotypes. The heifer's PGFM profile did not differ in basal concentration, peak concentration, number of pulses or any observable factor compared to PS animals that maintained pregnancy. The cow, however, had significant deviations in PGFM profile compared to PS cows. This cow had increased ($P < 0.01$) PGFM concentrations at day 31, 34 and 37. Additionally, the

number of peaks observed during the collection period was increased compared to PS cows (8 peaks vs average 2.4 peaks; $P < 0.05$).

5.3.4. PGEM

Concentrations of PGEM were analyzed in pregnant cows ($n = 32$), CON cows ($n = 5$), L1 cows ($n = 4$) and L2 cows ($n = 4$). Two separate PGEM profiles were observed between the 4 pregnancy outcome groups. Concentrations of PGEM peaked in L1 and CON cows between days 31 and 35 (range: 15.9 – 22.3 pg/mL). However, L2 cows had decreased PGEM concentrations similar to PS cows that maintained pregnancy (range: 6.7 – 13.1 pg/mL), indicating different profiles based on pregnancy success and timing of pregnancy loss (Figure 5- 5). There was a group*day interaction ($P = 0.01$) and concentrations differed ($P < 0.05$) independently between PS and CON and L1, as well as between L2 and CON and L1. There was no difference ($P > 0.1$) of PGEM concentrations between CON and L1 cows on any day evaluated, nor was there a difference between PS and L2 cows. Concentrations of PGEM did not fluctuate significantly within individual animals during the trial period ($P > 0.05$) and no significant pulses were observed in individual animal profiles. Prior to day 35 of gestation, L1 cows had a greater PGE_2:$PGF_{2\alpha}$ ratios compared to PS cows and L2 cows that would undergo pregnancy loss later in gestation ($P < 0.05$).

5.3.5. Progesterone (P4)

Progesterone concentrations were measured in all cows. Concentrations were significantly lower ($P < 0.01$) in CON cows with a CIDR compared to pregnancy cows regardless of pregnancy outcome. All cows in the PS and L2 groups maintained their CL function with circulating P4 concentrations greater than 6 ng/ mL and CON cows with

CIDRs maintained circulating concentrations between 2 and 3 ng/mL. Only one L1 cow (pregnancy loss around d 33) regressed the CL by day 40 of gestation, all other L1 cows (pregnancy loss between day 37 and 40) maintained CL and circulating P4 concentrations until day 40. Due to the use of P4 to track catheter location and regular movement of the catheter to maintain patency, P4 concentrations were not taken from a consistent location within cows; therefore, could not be used for comparisons between groups or within cow.

5.3.6. PAG

Concentrations of PAG were measured in all cows. Concentrations were not different in cows that underwent LEM (L1 and L2) compared to PS cows (Figure 5- 6). Except 1 CON cow with residual PAG from a previous pregnancy, CON cows did not have detectable circulating PAG. There was no change in PAG concentration from day 30 to 38 in any group ($P > 0.05$).

5.4. Discussion:

Innumerable factors may contribute to the incidence of late embryonic mortality, a majority of which are unknown. This study provides foundational knowledge about prostaglandin profiles during this stage of development. Beyond recordings of embryonic and placental size and development [4], little research has focused on the interactions of maternal environment and conceptus growth during the corresponding period of active placentation. Basal concentrations of PGFM in the current study were increased compared to concentrations of PGFM that have previously been reported at earlier stages of gestation, including MRP [20, 21]. Early studies indicated that $PGF_{2\alpha}$ concentrations are increased around day 30 of gestation [6, 8]. Drum et al. [22] reported that basal PGFM concentrations

and $PGF_{2\alpha}$ concentrations in response to oxytocin administration increased as pregnancy progressed into the second month of gestation in dairy cattle. Additionally, prostaglandins significantly increased in response to oxytocin challenge but did not differ between cows expected to maintain pregnancy (high PAG) and those with an increased likelihood to experience LEF (low PAG; Reese et al. 2020b). Despite elevated $PGF_{2\alpha}$ concentrations around day 30 of gestation in this study and others, CL regression was not observed, suggesting that there is some mechanism of luteolytic resistance and prostaglandins may have a necessary physiological role for the stage of pregnancy development. This study evaluates the endocrine profile in normal and LEF pregnancies throughout late embryonic mortality.

Our tail cannulation model allowed for direct sampling from uterine ovarian drainage for analysis of prostaglandins prior to metabolism in general circulation. Progesterone concentrations allowed for identification of targeted sample location and confidence to accurately quantify $PGF_{2\alpha}$ concentrations. To utilize the greatest number of animals, however, PGFM concentrations were used for most analyses. A prostaglandin $F_{2\alpha}$ metabolite, specifically 15-keto-13, 14-dihydro-PGF_{2a}, has been used in multiple species as a proxy for $PGF_{2\alpha}$ concentrations [18, 19, 24]. Similarly, as shown in Figure 5- 2, PGFM and $PGF_{2\alpha}$ profiles were comparable in cows that had properly placed catheters. Concentrations of PGFM and $PGF_{2\alpha}$ were not similar in cows that the catheter was placed in the caudal vena cava either in front of or behind the uterine vein drainage, indicating dilution with general circulation or prostaglandin metabolism prevents comparison between these two groups of cows with varying catheter placements.

The functional mechanism of $PGF_{2\alpha}$ is the frequency of pulses, as demonstrated by the requirement of sequential pulses to induce luteolysis [9]. During this period of active placentation, $PGF_{2\alpha}$ is released via the inducible COX-2 pathway as demonstrated by oxytocin challenges during this period [22, 25] but CL function remains. In this trial, cows in the L2 group had a significantly greater number of periods of elevated PGF_{2a} from day 30 to 38 compared to PS cows that maintained pregnancy. A frequent sampling period (every 15 min) of 6 hours was used to evaluate the occurrence of true pulses in a subset of animals. Two L2 animals (1 heifer, 1 cow) were evaluated on days 29, 31, 34, 37 and 39 along with 6 PS animals. While the heifer's PGFM pulse pattern did not differ from the PS animals, the cow had obvious deviations from the normal pattern. This observation supports the principle that pregnancy loss during late embryonic and early fetal development may be caused or driven by multiple factors. Over the course of the intensive sampling days, the L2 cow had 2.6 times more pulses compared to PS cows. On day 31, the L2 cow had 4 identifiable pulses compared to an average of 2 ± 0.33 pulses. Additionally, basal concentrations of PGFM were significantly increased compared to the PS cows. Despite a functioning CL and a viable pregnancy at day 40 of gestation, alterations in $PGF_{2\alpha}$ pulsatility at the early stages of placentation may indicate or contribute to pregnancy failure during early fetal development. Prostaglandin induced gene expression is well defined in many reproductive tissues and physiological processes [13, 26, 27]. In the cow, Atli et al. [28] reported that luteal gene expression in the CL was significantly altered after the second pulse of $PGF_{2\alpha}$ of luteolysis. Based on data from the pulse challenge, the average number of pulses is greater on day 29 and 31 compared to day 37 and 39. Despite these peaks early in the evaluation period, most L1 cows lost pregnancy

after day 35 of gestation. Early pulses of $PGF_{2\alpha}$ around day 31 did not significantly impair CL function by negatively influencing P4 concentrations. It may have, however, negatively influenced the coordinated processes required for successful placentation. From day 30 to 38, basal concentrations of PGFM did not differ between pregnancy outcome groups; however, basal concentrations between individual animals varied significantly. Compared to previous studies where basal PGFM concentrations did not vary [22, 23], this study used a larger sample size and a mixed population of breeds and parities. The physiological mechanisms dependent on $PGF_{2\alpha}$ during active placentation may be reliant on frequency of pulses rather than basal concentrations as seen in the mechanisms of luteolysis.

Physiologically, PGE_2 has opposite functions and properties compared to $PGF_{2\alpha}$. Within the reproductive tract, PGE_2 is proposed to have a luteoprotective role and mediates endometrial receptivity and myometrial quiescence [29, 30]. Additionally, PGE_2 has angiogenic properties and stimulates vasodilation [31] which should seemingly be beneficial for placentation. During early pregnancy and secretion of INFT, a decreased ratio of $PGF_{2\alpha}$ / PGE_2 is observed [32]. Little information, however, has been identified regarding PGE_2 during the late embryonic/early fetal period of development. Surprisingly, PGEM concentrations were decreased in PS cows compared to non-pregnant CON cows. Cows that lost pregnancy prior to day 40 (L1 group) also had increased PGEM concentrations similar to CON cows. Increased concentrations of PGE_2 are also observed in cases of inflammatory viral infections in calves [33]. Cheng et al. [34] demonstrated that BVDV infection stimulated PGE_2 production and decreased $PGF_{2\alpha}$ production through an endocrine switch of the production pathways in uterine endometrial cells. While the cows in the present study were free from infection, the immunosuppressive properties of PGE_2

changing local innate immune responses may provide evidence of potential causes of LEF [34, 35]. Innate immune cell response in pregnancy is a carefully coordinated event and the recruitment of natural killer cells and T cells is crucial to placentation in ruminants [36, 37]. Prostaglandin E_2 has a well-defined regulatory role of cytokines, including IL-10, IL-12, TNFα, and changes in specific PGE receptors can alter immune defenses [38]. Non-infectious causes of changes in PGE_2 production may alter immune function causing deficiencies in placental development and contribute to LEF.

Contrary to previously published reports, there was no difference in circulating PAG concentrations between PS cows and those that experienced embryonic mortality in either pregnancy loss period [14, 16, 39-41]. This could be attributed to different causes of late embryonic mortality in each case. The small sample size in the current study may prevent differences from being observed; however, it also emphasizes the complexity of understanding late embryonic mortality.

5.5. Conclusion

The causes of LEF are complex and varied; however, PG may play a regulatory role in pregnancy loss during placentation. Basal $PGF_{2\alpha}$ is increased during the second month of gestation and sporadic pulses do not negatively influence CL function and pregnancy maintenance; however, in some cases increased pulse frequency may contribute to LEF. Prostaglandin E_2 is increased in cows undergoing pregnancy loss between day 30 and 40 but not day 41 and 60 indicating different causes of pregnancy loss during the late embryonic and early fetal development. These findings are in general agreement with the regulatory roles of PG in pregnancy and open an area for future investigation of the causes of LEF.

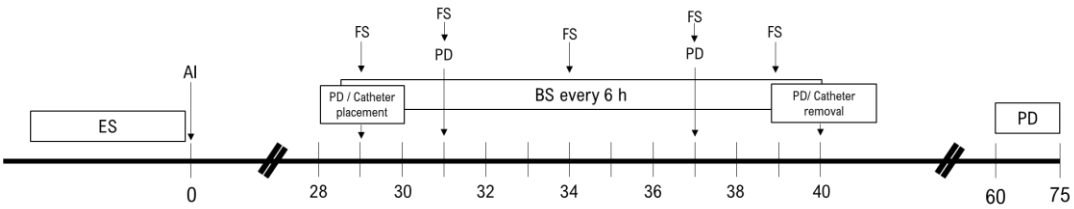

Figure 5-1. A timeline of experimental procedures
ES = estrous synchronization; AI = artificial insemination; PD = pregnancy diagnosis by ultrasound; BS = blood sample collection; FS = Frequent sampling every 15 minutes for 6 hours

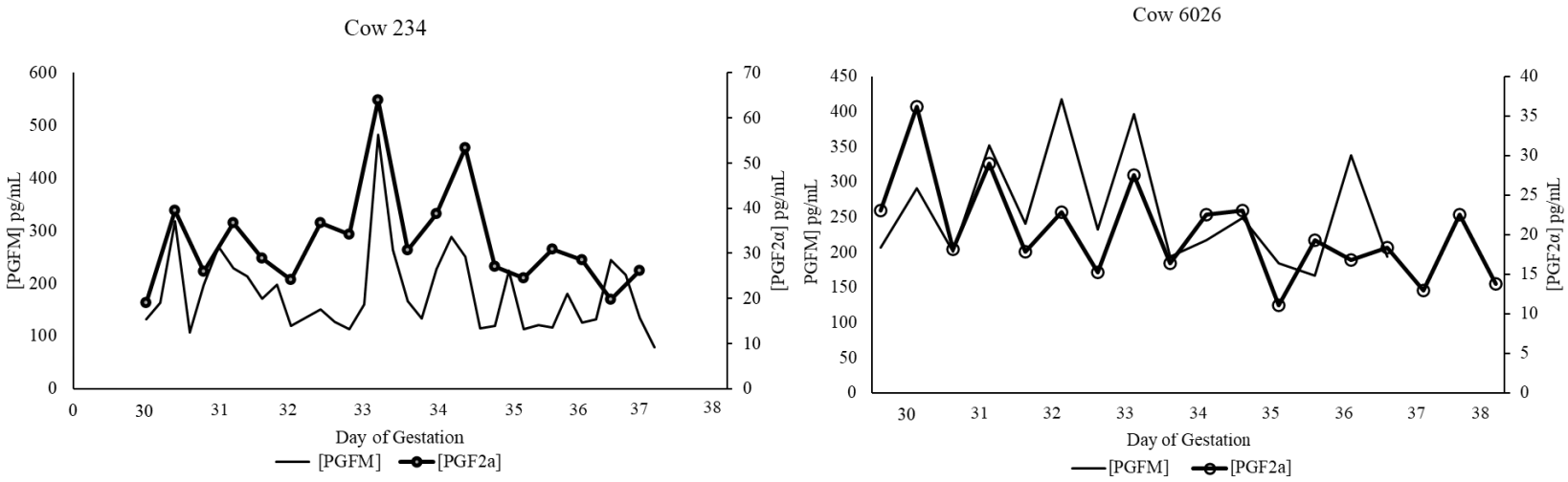

Figure 5-2. A comparison between PGF2α and PGFM profiles in 2 cows.
Profiles of PGFM were reflective of concentrations of $PGF_{2\alpha}$ although concentrations of PGFM were higher due to slower metabolism.

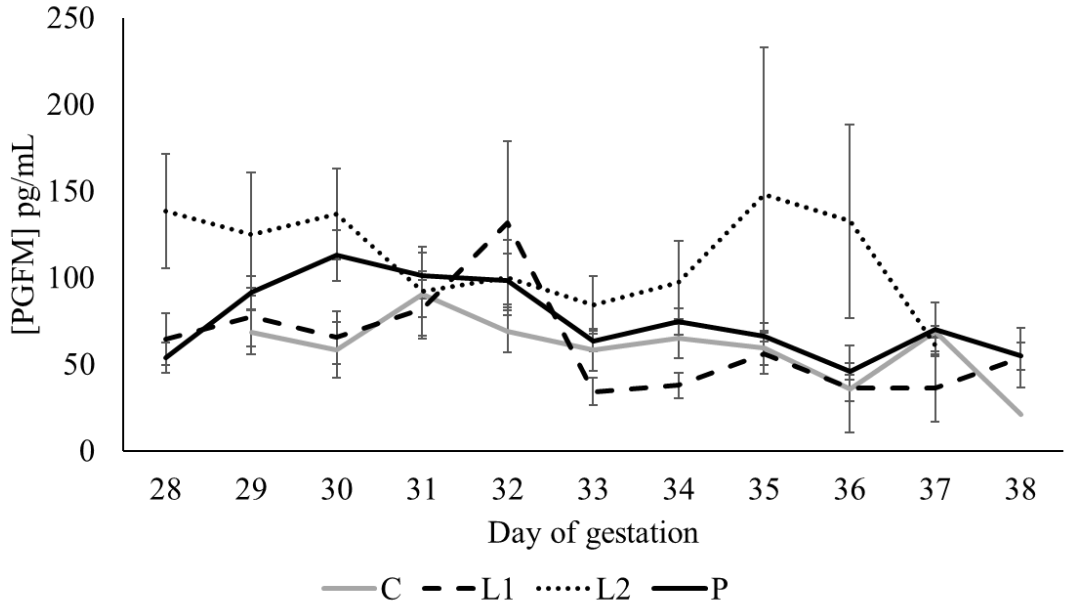

Figure 5-3. Profile of PGFM concentrations from day 28 to 38 of gestation.
PGFM concentrations were not different between pregnancy groups.

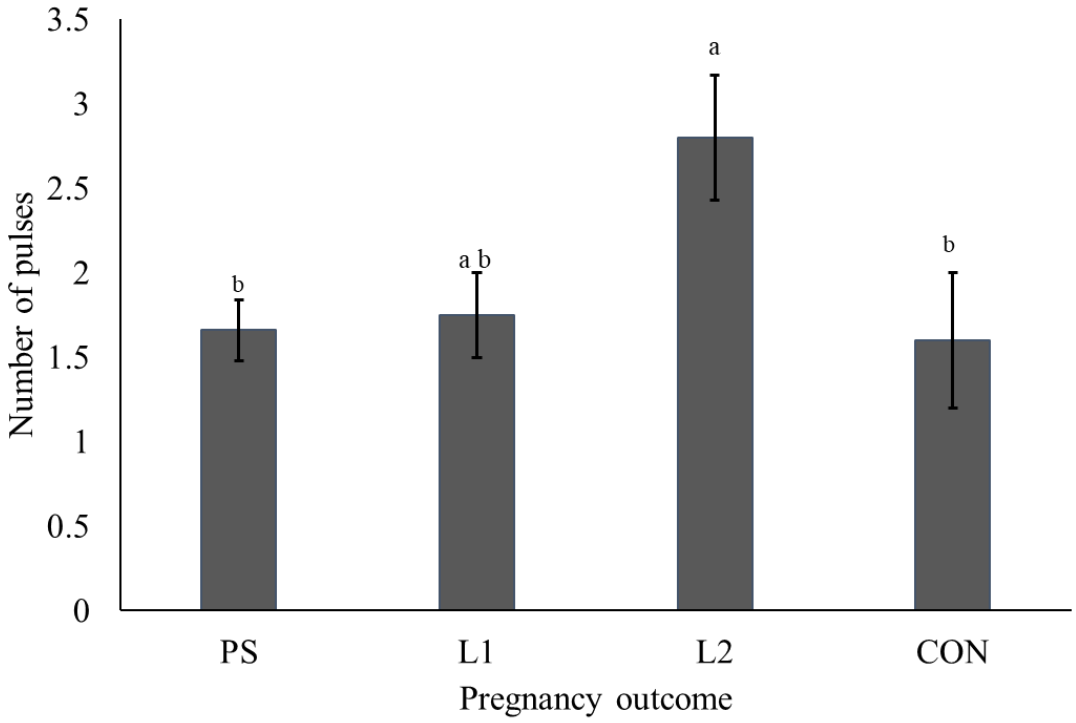

Figure 5-4. Number of peaks from day 30 to 38 of gestation by pregnancy outcome. Cows that lost pregnancy between day 40 and 60 had a significantly greater number of peaks compared to control and pregnancy success cows. Connecting letters indicate no significant difference between groups.

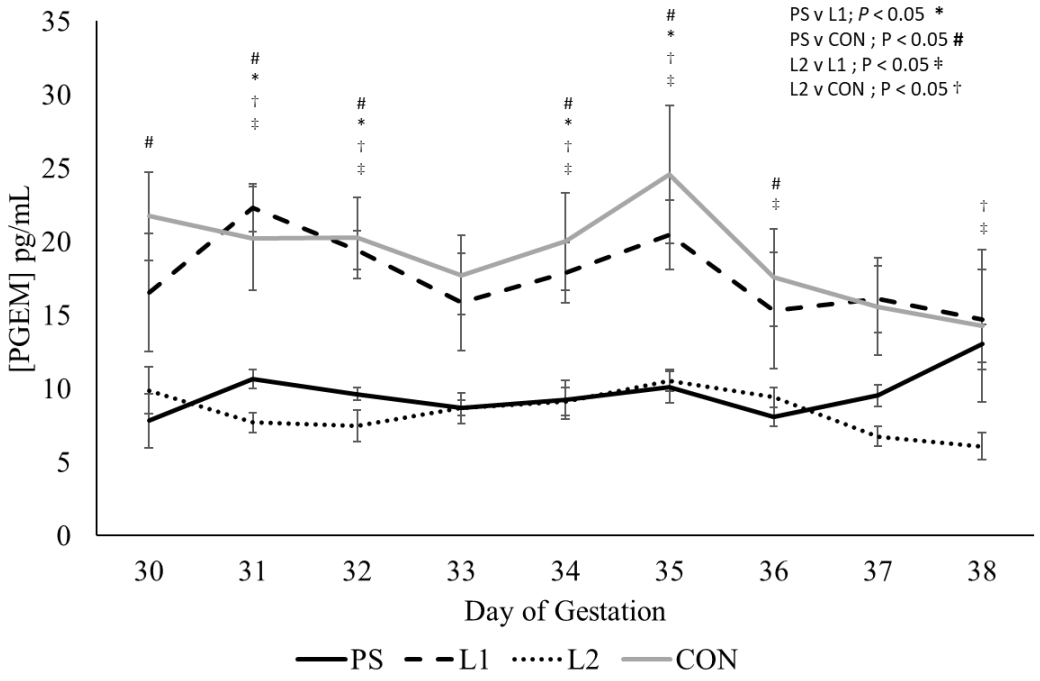

Figure 5-5. Profile of PGEM concentrations from day 28 to 38 of gestation.
Two profiles of PGEM were observed. Cows in the PS and L2 groups had significantly decreased PGEM compared to nonpregnant CON cows and L1 cows.

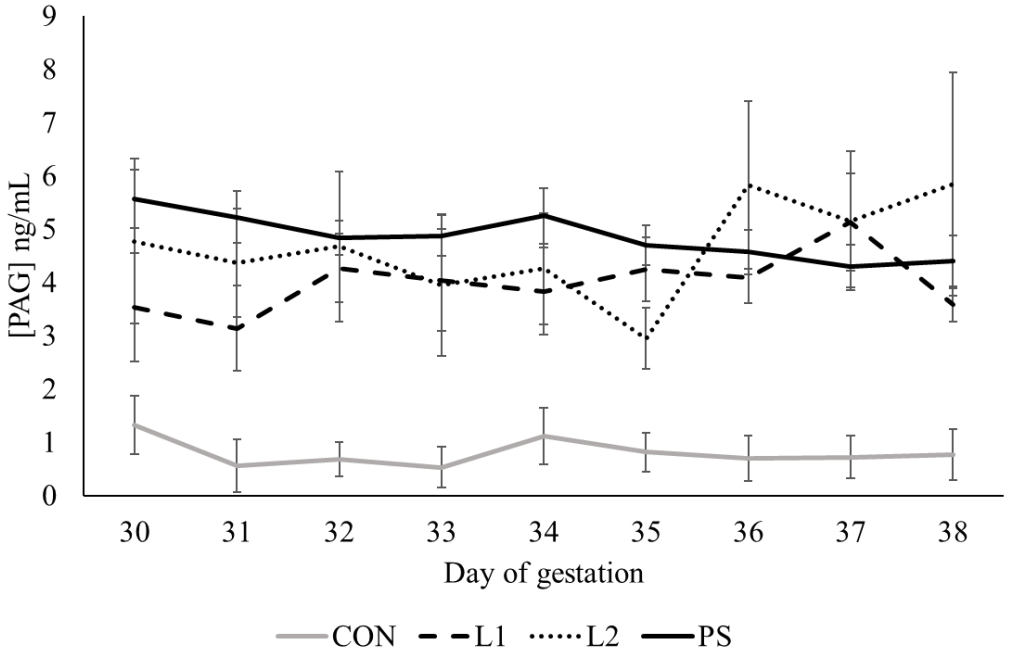

Figure 5-6. Concentrations of PAG by experimental group.
PAG concentrations did not differ between pregnant cows, regardless of pregnancy outcome on any day. Nonpregnant cows had decreased ($P > 0.05$) PAG concentrations through the sampling period.

5.6. References

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CONCLUSIONS

Pregnancy loss in cattle will remain a significant issue to the livestock industry until the mechanisms that cause pregnancy loss are understood and can be regulated. Our meta-analysis demonstrated that reproductive failure materializes differently for beef cattle compared to dairy cattle with fewer incidences of late embryonic loss. Extensive management practices within the beef industry, however, contribute to the significant economic impact of such losses. A complete understanding of a normal, successful pregnancy is crucial to recognizing deviations that contribute to reproductive failure. Using pregnancy associated glycoproteins are markers of pregnancy viability, there was no difference in uterine production of $PGF_{2\alpha}$ when stimulated by oxytocin. Collection of blood directly from the uterine ovarian drainage indicated that prostaglandins are altered in basal concentrations and secretion patterns between cows that maintain pregnancy and those that undergo late embryonic/ early fetal mortality. These findings suggest that future studies should determine if prostaglandin alterations are a mechanism of pregnancy loss or a result of causative processes.