INVESTIGATING THE SIRE CONTRIBUTION TO EMBRYONIC MORTALITY IN CATTLE

A Dissertation

by

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ABSTRACT

First, we characterized the association between sire fertility and pregnancy loss in cattle. Bos Taurus beef cows had a large variance in pregnancy loss between days 24 and 31 of gestation (1.8 to 11.7%) and between days 31 and 60 of gestation (2.3 to 12.6%) among service sires used for timed artificial insemination (TAI). Similarly, same variance was observed among sires used for TAI and timed embryo transfer (TET) in dairy cattle. Pregnancy loss during the second month of gestation ranged from 5 to 35-40% among sires used in both TAI and TET, and no correlation was observed with their respective sire conception rate (SCR) index. These sire phenotype characterization studies suggest that current methods to evaluate sire fertility may be limited in assessing overall reproductive success and incidence of late gestation pregnancy loss should be considered when evaluating sire fertility, as it can significantly affect final pregnancy rate. To investigate the physiological mechanism of paternal contribution to conceptus formation, parthenogenetic embryos (PA) were compared to control embryos (CON) during blastocyst stage and post elongation stage. Pregnancy development was monitored by ultrasonography and blood based placental secretions. In all 19 cows that established a pregnancy with PA embryos, circulating concentration of placental products (PAG and ISG) throughout gestation were lesser compared to cows carrying CON embryos. Even though these embryos survived up to day 40-45 of gestation, no active site of implantation and attachment to endometrium was observed, suggesting that trophoblast tissues are not properly formed in the absence of paternal genes. These findings strongly suggest that

paternal genetics contribute significantly to placenta formation in cattle, which could explain most of the sire variance observed in pregnancy loss during the period of active placentation. The development of markers to identify sires of high or low pregnancy loss would improve sire fertility evaluations and increase beef and dairy reproductive efficiency.

DEDICATION

A minha familia, que sempre foi meu porto seguro!

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1. INTRODUCTION [1]

1.1. Sire Fertility Evaluation

In most livestock species, the male is responsible for multiple pregnancies per year and up to hundreds of thousands of pregnancies if used for artificial insemination or in vitro embryo production. Use of sub fertile or infertile sires can have devastating impacts regarding the reproductive efficiency of a beef herd. Yet, our ability to predict the fertility potential of a male's semen sample seldom explain half of the variation among males [1, 2].

With the advances in molecular, genomic and computer techniques our ability to evaluate pregnancy development expanded rapidly [3], but one of the major challenges in evaluating sire fertility remains defining the fertility phenotype. Fertility is a broad term used to define the ability to produce a viable offspring, but it is meaningless if the outcome measure is not stipulated [4]. Between mating and birth, there are several time points that are used to evaluate fertility success in livestock, including fertilization rate, non-return to estrus rate, conception rate, pregnancy rate and calving rate which are correlated with different aspects of the reproductive cycle [4]

Fertilization rate can be evaluated *in vitro* by presence of first cell division after 48h of insemination or *in vivo* by flushing the uterus 7 days after insemination to recover embryos or unfertilized oocytes [5], but the technique of flushing does not guarantee that all embryos will be recovered, limiting the use of this index as a true estimate of fertilization. Conception rate represents the percentage of pregnant animals over number of animals inseminated after detection

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of estrus, similar to fertilization rate. However, due to limitations of early pregnancy diagnosis, conception rate is usually reported as pregnant animals at some point after breeding (e.g. 30 to 45 days) and therefore, account for not only fertilization rate but also early pregnancy loss. Pregnancy rate is the most common reported measurement of herd fertility. It accounts for the number of animals that conceived in a defined time period over the number of animals eligible for breeding during the defined time period. This time period can be either the 21-day estrus interval, a single day of timed AI, or breeding season interval (e.g. 90 days, 120 days). [1]. Identification of pregnant animals are often obtained using rectal palpation, ultrasonography, or blood-based pregnancy tests.

From a productive and economic point of view, the most appropriate measurement of fertility is calving rate. Defined as the number of calves born from total number of cows inseminated or exposed to a bull, this index accounts for both fertilization rate and all subsequent pregnancy losses. This measurement, however, provides little advantage to a producer because it can only be obtained 9 months after the breeding season. In cattle, most male fertility traits have only been studied in relation to fertilization and early embryonic development, represented by conception or pregnancy rate. Historically, pregnancy loss after this time is usually associated with female infertility only. However, recent studies have shown paternal genetics provide a significant contribution to embryonic/fetal mortality in cattle [6-8] that can drastically affect calving rate and should be accounted for when measuring male effect on herd fertility. An ideal male fertility test must be economically practical, provide consistent results and can measure multiple variables as spermatozoa must meet many requirements for successful fertilization. A perfect test should be able to evaluate not only the ability of the spermatozoa to reach the site of fertilization, but also the ability to fertilize the oocyte, establish and maintain a successful pregnancy [3].

1.2. Embryonic mortality in cattle

In beef and dairy cattle, reproductive inefficiency costs producers over $1 billion dollars annually [9, 10]. Incidence and timing of embryonic and fetal mortality in cattle vary greatly based on animal genetic composition, production status, environment, and management conditions. [11, 12]. Pregnancy losses during the first month of gestation are commonly classified as early embryonic mortality (before day 28 of gestation) and include fertilization failure, maternal recognition of pregnancy failure as well as early embryonic losses related to genetic defects and uterine asynchrony. In cattle, 28-35% of pregnancies fail during the first 7 days of embryonic development, and another 15-20% is lost between days 16 and 32 which sums over 45% of pregnancies lost during the first month of gestation. Historically, maternal recognition of pregnancy failure was believed to account for a significant percentage of pregnancy loss [5], but recent research suggests that there is large incidence of pregnancy loss happening between days 24 and 31 of gestation, post maternal recognition of pregnancy [13, 14]

Late embryonic and early fetal mortality make up a small percentage of overall pregnancy losses; however, due to management practices, many go undetected and result in greater economic deficits [15]. It is estimated that late embryonic mortality affects 5 to 8% of beef cattle pregnancies while upwards of 15% of dairy pregnancies may fail during this period [11, 12]. Causes of late embryonic mortality are less understood as research has focused primarily on examining the contributing factors to early embryonic loss. During late embryonic development, hallmark placentome formation and exponential placental expansion occurs [16]. Deficiencies in placental growth or function can have severe consequences and may be an overlooked factor of pregnancy loss. Many reproductive biologists have focused on the female contribution in reproductive processes, and much less attention has been given to male derived factors associated with fertility

or causes of embryonic mortality originating from the sire post fertilization and initial embryonic development. Taking into consideration the paternal genetic influence on placenta formation there is an increased interest in understanding the sire contribution to embryonic mortality in cattle.

1.3. Placentation in cattle

After blastocyst hatching, there is rapid development of the extraembryonic membranes. For most domestic species, including cattle, the formation of extraembryonic tissues occurs during the pre-attachment period and involves extensive folding of germ layers to generate the amnion, chorion, allantois, and allanto chorion. In the cow, the conceptus undergoes extensive elongation from 3mm at day 13 to about 250mm by day 17, forming a filamentous threadlike blastocyst that covers the length of the uterine horn [17]. Around day 15, apposition and adhesion of the trophoblastic cells to the uterine epithelium begins, in which the trophoblast develops finger-like villi that penetrate the opening of uterine glands [17, 18], providing a temporary anchor for the conceptus. After day 19, the elongating conceptus adheres to the luminal epithelium and is characterized by the appearance of giant multinucleated cells of the trophoblast. At this point, placentation begins and the trophectoderm and endometrial luminal epithelium are extensively interdigitated in both caruncular and intercaruncular areas.

Proper placentation is required to correctly exchange nutrients, gas and waste products at the fetal-maternal interface. The bovine placenta has been characterized morphologically and histologically as cotyledonary synepitheliochorial and considered to be one of the least invasive placental types [19-22]. Despite visual similarities, sheep have a more invasive placental type than originally believed where the uterine luminal epithelial cells are almost completely engulfed and eliminated which does not occur in the bovine [23].

Early morphological changes associated with bovine placentation begin during the 3rd week of gestation. Major events in early placentation include the following: 1) the allantois becomes visible on day 23; 2) the chorion reaches the tip of the nongravid horn on day 24; 3) the adhesion phase is complete in both uterine horns by day 27; 4) first chorionic indentation into the endometrium is visible on day 32; 5) a few regular projections from the endometrium are evident on the caruncular surface on day 28 to 33; and 6) approximately 35 cotyledons are formed by day 38 [16, 24]. Initial formation of cotyledons occurs near the embryo proper between day 30 and 37, with 17 to 25 cotyledons present on the chorion adjacent to the embryo on day 35 [16, 24].

During placentome formation and interdigitation, fetal chorionic villi migrate towards maternal caruncular tissue and subepithelial capillaries [22]. On a cellular level, interdigitation allows for migration of giant binucleate trophoblast cells to the maternal epithelium [21, 25]. Giant trophoblast cells make up 15- 20% of placentome cell population [21] and contain fetal products including hormones [26], pregnancy associated glycoproteins (PAGs) [27, 28], and placental lactogen [29]. Since their discovery in 1980's, PAGs have been a target for pregnancy diagnosis with the first detectable increase in general circulation between days 22 to 24 of gestation. Concentrations of PAG continue to increase through day 36, followed by subsequent decrease in concentration until day 60 of pregnancy followed by a steady increase through the second and third trimesters of pregnancy [28, 30-32]. Several studies have shown a strong correlation between pregnancy success and concentration of PAGs during early gestation, suggesting that PAG could be a good marker for placental competence and pregnancy viability [32-36].

1.4. Paternal vs Maternal Genome Tug of War (Conceptus Genome Establishment)

Both paternal and maternal genomes contribute quantitatively equal amount for conceptus formation, but the functionality varies due to epigenetic modifications. Genomic imprinting is a process that selectively silences or promotes the expression of one of parental inherited allele of same gene [37, 38]. The term "parental tug of war" was first used by Moore and Haig (1991) [39] to describe the conflicting expression of paternal vs maternal genes in relation to transfer of nutrients from mother to offspring. Briefly, authors described the hypothesis that states that increasing the availability of maternal nutrients to the embryo will allow for growth and greater survival rates; however, to great of a nutrient allocation to the embryo may negatively stress the maternal system and potentially impact lactation and offspring survival. Therefore, preferentially paternally expressed genes encourage nutrient exchange to the fetus while maternally expressed genes reduce those demands [39]. Investigating the parental contribution to conceptus formation can help elucidate the mechanism that leads to placenta insufficiency and consequent pregnancy failure. Comparison of development of uniparental embryos (parthenogenetic vs androgenetic) with normal biparental embryos is an interesting tool to distinguish the mechanism that control fetus and placental development as well as develop possible markers to predict pregnancy success.

1.5. Parthenogenetic embryo production

Uniparental embryos allow examination of the contributions that are made by each individual parental genome (paternal vs maternal) to conceptus development and have been extensively used for this purpose in the mouse [37, 38, 40-46]. Parthenogenetic embryos (maternal genome only) can be efficiently generated by artificial oocyte activation and can develop readily to the blastocyst stage when oocyte maturation, method of activation, and culture conditions are

optimized [47-49]. Parthenogenetic embryos have also been used as a possible source of embryonic stem cells for cell therapy in humans [50-52] .

The genetic composition and ploidy of mammalian parthenotes vary depending on the method of oocyte activation [53]. A majority of mammalian oocytes are arrested in metaphase II until activation by spermatozoa fertilization or artificial stimulus (parthenogenetic activation). During oocyte fertilization, a series of periodic oscillations of intracellular calcium [54] cause a cortical reaction, meiosis resumption, pronuclear development and mitotic cleavage [48, 55, 56].

Artificial oocyte activation aims to mimic these physiological changes happening on oocyte during fertilization. Increased cytoplasmic levels of calcium can be induced by incubation with ionomycin [49], ethanol [49], calcium ionophore A23187 [57] and strontium [58]. Oocytes can also be activated through electrical stimulation [59]. Moreover, the addition of protein synthesis inhibitors (example CHX) [60] or histone kinase inhibitors (example: 6-dimethylaminopurine (6-DAMP)) [61, 62] have been used to improve parthenogenetic embryonic development rates [61, 63-65]. The most commonly used method for bovine oocyte activation includes incubation with ionomycin followed by 6-DMAP, which yields a blastocyst development rate of over 42% [48, 66]. When oocyte is activated at the second metaphase resulting in the extrusion of the second polar body leads to formation of a haploid parthenote. Overall, haploid parthenotes have decreased developmental competence compared to normal embryos and to diploid parthenotes [67]. Table 1 summarizes bovine parthenogenetic and androgenetic embryonic development success rates using different techniques.

1.6. Androgenetic embryo production

Production of androgenetic embryos (paternal genome only) is more complex and requires micromanipulation of zygote pronuclei. In mice, this technique has been extensively applied [38, 40, 42, 45, 46] due to the ease of pronuclei visualization during embryonic development. Despite the ability to visualize the pronuclei in murine embryos, androgenic embryo production is hindered by the challenges associated with identifying the male pronucleus and contamination by newly synthetized products from maternal and paternal genome present in egg cytoplasm.[68, 69] Androgenetic embryos can also be produced by in vitro fertilization of enucleated oocytes [70, 71]. Mice androgenetic embryos have lower developmental ability compared to parthenogenetic, but there are reports of development up to 25-somite stage and embryos had a phenotype of extensive trophoblast proliferation but rudimentary embryo proper formation [38, 71].

For ruminant species, androgenetic embryo production is more difficult and less efficient process. Bovine zygotes contain pigment granular material that conceals the translucent pronuclei making it more difficult to visualize and isolate them [72]. Moreover, it is extremely difficult to differentiate the female and male pronuclei. Hagemann and other developed a protocol for pronuclei replacement in sheep and suggested that the female pronuclei is often, but not always, the smaller of the two [72], similar to mice. Fertilization of enucleated oocytes may be the more efficient process to produce androgenetic ruminants embryo [73]. In sheep and bovine, androgenetic embryos have high cleavage rates but low morula compaction and blastocyst formation rates [72, 73].

1.7. Gene expression in uniparental embryos

Approximately 150 imprinted genes have been identified in humans and mice, while 50 have been identified in ruminants (See full list at https://www.geneimprint.com/site/genes-by-

species). Most of the known imprinted genes are highly expressed in the placenta and are crucial for placental and fetal development.[74]. Evaluation of gene expression in uniparental embryos during different periods of embryonic development is used to understand genomic imprinting. Genes that encode insulin-like growth factor type 2 (Igf2) and its receptor (Igf2R), for example, are known to be imprinted in fetal and adult mice, as well as in androgenetic, gynogenetic, and parthenogenetic preimplantation mouse embryos. This suggests that inactivation of these genes occur post implantation [75]. Genes linked to placental development, such as *Slc38a4, HIF2α*, *Gab1* and *Plac9*, are known to be reduced in parthenotes. Alternatively, expression of genes reported to down regulate placental growth, such as *H19, Tssc3/Phlda2, Grb10/Meg1* and *Cdkn1c* are increased in parthenogenetic blastocysts[76, 77]

Viable pups that developed to adult mice were produced from parthenogenic embryos in which imprinted genes *Igf2* and *H19* were manipulated, supporting the hypothesis that gene imprinting is a barrier to parthenogenetic development in mice [78, 79]. Using mutant mice with a 13-kilobase deletion in the *H19* gene, a parthenogenetic embryo with increased *Igf2* activity combined with monoallelic expression of *H19* gene was produced. The predominantly paternal expressed gene, *Igf2* gene encodes a growth-promoting factor (IGF-II) and is a crucial regulator of fetal growth [80-82], while *H19* is expressed from the maternal allele and encodes a transcript which downregulates cellular proliferation [83]. Controlled manipulation of these two very important genes caused modification of a wide range of genes and allowed normal development of parthenote embryos [78, 79]. Moreover, in rodents, deletion of the paternally expressed *Igf2, Peg1/Mest, Peg3* or *Ins1/Ins2* genes results in intrauterine growth restriction [84-86], whereas deletion of the maternally expressed *Igf2r* or *H19* genes, or overexpression of the *Igf2* gene, results in fetal overgrowth [80, 82]. In cattle, parthenogenetic embryos had

decreased *Mash2* mRNA, suggesting paternal regulation of this gene expression. *Mash2* is greatly expressed during trophoblast proliferation but silenced after implantation and stimulates mononucleate trophoblast cell proliferation while inhibiting giant multinucleated cell formation [87]. Imprinted genes in the placenta are major regulators of nutrient exchange efficiency and disruption of imprinted genes dynamics during different periods of embryonic development can lead to poor embryonic and placental development and subsequent pregnancy failure [74, 76].

1.8. Conclusion

Understanding embryonic mortality remains a significant problem, yet there is limited data currently available during this period of active placentation in cattle. Uniparental embryos are a great tool to investigate the mechanism of genomic imprinting and parental contribution to embryonic and placental development.

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Table 1-1 Summary of bovine uniparental published studies

Author	Embryo Type	Production Method	Blastocyst Rate	# cells on day 7	Pregnancy Rate
Gomez et al 2009	Parthenogenetic (mostly diploid)	Ionomycin + 6-DMAP	42%	123	NA
Wang et al 2008	Parthenogenetic (Over 50% Diploid)	Ion + DAMP	28%	92	NA
		Eth + CHX + CB	26%	89	NA
Lagutina et al 2004	Parthenogenetic diploid	Ionomycin + 6-DMAP + culture	43%	100	NA
	Parthenogenetic haploid	Ionomycin + 6-DMAP	14%	90	
	Androgenetic haploid	Fertilize enucleated oocytes	2.70%	38-71	1 pregnancy of androgenetic blastocyst that lost between days 28 and 35
	Androgenetic diploid	Fertilize enucleated oocytes	22.30%	182	
	Androgenetic diploid	Pronuclear transfer	43%	123	
De la Fuente 1998	Parthenogenetic (mostly diploid)	Ionomycin + 6-DMAP	27%	67	NA
Boediono and Suzuki 1994	Pathenogenetic diploid	Ethanol + aggregation of four 8-cell stage embryos	17-29%	NA	3 of 5 recipients pregnant at day 42 Estrus was prolonged up to day 67
Fukui et al 1992	Parthenogenetic	Ethanol + transfer 2 embryos/cow on day 6 or 7	2-10%	NA	1 out of 4 cows pregnant at day 35
Susko-Parrish et al 1994	Parthenogenetic	Ionomycim + 6-DMAP + transfer 2 blastocyst/cow on day 6 or 7	21%	70-88	7 out of 22 cows had prolonged estrus and uterine vesible. 1 animal had a fetal mass

2. SIRE CONTRIBUTION TO PREGNANCY LOSS IN DIFFERENT PERIODS OF EMBRYONIC AND FETAL DEVELOPMENT OF BEEF COWS[2]

2.1. Introduction

Pregnancy loss is recognized as a major cause of reproductive failure in cattle, resulting in significant economic consequences for both beef and dairy industries. Early embryonic mortality (EEM) can be defined as pregnancy losses between insemination (day 0) or embryo transfer (day 7) and day 28-30 of gestation and late embryonic/early fetal mortality (LEM) as pregnancy losses during the second month of gestation (between days 30 and 60 of gestation). The first period includes fertilization failure, which occurs in about 28% of single inseminations in beef cattle, as well as failures of embryo elongation and maternal recognition of pregnancy [1]. In beef cows, EEM range from 34% [2] to 62% [3] with an average of 48% [1], while LEM has been reported to range from 2-10% [3, 4]. Some studies suggest that the majority of EEM occurs before day 14 or 18 of gestation [5, 6], but advancements of more precise early pregnancy markers suggest that a considerable amount of pregnancy loss (~16%) happens between days 20 and 30 of gestation, during embryo implantation period [7].

It is known that paternal genetics contribute significantly to pregnancy establishment and maintenance, specifically placenta formation, and contributes to the incidence of pregnancy loss. Using an uniparental embryo model in mice, the maternal genome has shown to greatly contribute to the formation of the embryo itself, while the paternal genome greatly contributes to the

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trophoblast and, therefore, placenta [8, 9]. In cattle, morphological changes of the placenta associated with placentome formation and interdigitation begin during the third week of gestation [10]. Adequate placentation is required for proper exchange of nutrients at the fetal-maternal interface and disruption of these physiological processes could lead to pregnancy failure. Previous research from our lab demonstrated that PAG concentrations were influenced by sire and reflected the probability of pregnancy loss [11]; however, the loss was not stage specific (EEM vs. LEM). Therefore, the primary objective of this study was to evaluate the effects of service sire on pregnancy loss during different periods of embryonic development in beef cattle. We hypothesized that individual sires would have different phenotypes in regard to the amount of pregnancy loss in different periods of gestation.

2.2. Materials and Methods

The experiment was conducted at UTIA Research and Education Center system in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Multiparous (n = 485) and primiparous (n = 173) Angus beef cows at least 30 days postpartum were subjected to 7-day CO-SYNCH + CIDR timed artificial insemination (TAI) protocol. On day -9 of the experiment, all cows received a controlled internal drug release insert (Eazi-breed CIDR; Zoetis Inc., Parsippany-Troy Hills, NJ) and a GnRH dose (100 µg; Factrel, Zoetis Inc.), followed by the administration of $PGF_{2\alpha}$ (25 mg; Lutalyse, Zoetis Inc.) and CIDR removal 7 days later (day -2). A second dose of GnRH was administered at TAI, 60h post CIDR removal. Estrus breeding indicators (Estrotect Breeding Indicator, Rockway Inc., Spring Valley, WI) were placed at CIDR removal and scored at the time of TAI on a scale of zero to four as described by Pohler et al. [12]. Briefly, cows with patch scores one and two were defined to not be in estrus or low estrus intensity,

whereas patch scores of three and four signified estrus had occurred with higher intensity. Cows without a patch at TAI were removed from the analysis that evaluated estrus expression.

2.2.1. Sire Distribution

Cows were randomly inseminated with semen from one of eight Angus sires to assess the effects of individual sire on incidence of pregnancy loss. To ensure randomization, sires were alternated every 5 cows. Mean number of inseminations per sire was 83 (range 61 - 92). Frozen semen was purchased from commercial bull studs that follow all health, ethical, and animal welfare guidelines from Certified Semen Services (CSS) and National Association of Animal Breeders (NAAB) and semen from each sire was from the same collection batch. All semen passed pre- and post- freezing quality tests with a minimum of 30% progressive motility and 70% normal morphology.

2.2.2. Pregnancy Associated Glycoprotein and Progesterone Quantification

Blood samples were collected from all cows at TAI (day 0, baseline) and days 24 and 31 post TAI by venipuncture using a 10-mL vacutainer tube (BD Vacutainer, Becton, Dickinson and Company, New Jersey) and allowed to clot at room temperature for 1 h before being placed in a 4 °C refrigerator for approximately 24 hours. Samples were centrifuged at 1,500 x g for 15 minutes and serum stored at -20 °C for subsequent pregnancy associated glycoprotein (PAG) and progesterone analysis. Serum concentration of PAGs were quantified using a monoclonal-based PAG ELISA described by Green et. al., [13] using a polyclonal antibody (Ab 63) described by Reese et al., (2017) with a sensitivity of 0.28 ng/mL. Each assay was run with duplicates of each sample, a standard curve, a sample from a pregnant cow approximately 60 days into gestation and a pooled sample from non-pregnant cows as controls. Serum concentration of progesterone were

quantified using a double-antibody radioimmunoassay (RIA) as previously described [14]. Intra assay and inter assay coefficients of variance were less than 10% for both assays.

2.2.3. Pregnancy status determination

Pregnancy status for this experiment was classified by the following: 1) pregnant at day 24 was determined by increased circulating PAG concentration on day 24 compared to day 0 and circulating progesterone concentration greater than 0.7 ng/ml [14] on day 24. Animals that did not have increased PAG at day 24 but were diagnosed pregnant at day 31 were also considered pregnant; 2) on days 31 and 60, pregnancy diagnosis was performed by transrectal ultrasonography using an Aloka 500V ultrasound (Aloka, Wallingford, CT) with a 7.5 MHz transrectal linear probe. After the last pregnancy diagnosis, cows were divided into four groups: 1) pregnant (pregnancy established and maintained to day 60), 2) pregnancy loss between days 24 and 31 of gestation (EEM; cows that were pregnant at day 24 but no embryo present at day 31), 3) pregnancy loss between days 31 and 60 of gestation (LEM; cows with a viable embryo with a heartbeat at day 31 but diagnosed non pregnant at day 60) or 4) failure to establish pregnancy (cows without elevated PAG at day 24 and no embryo at day 31).

2.2.4. Data Analysis

Sire classification was retrospectively determined according to the amount of pregnancy loss during different periods of embryonic and fetal development, following an approach previously described by [11]. Briefly, for the first interval of pregnancy loss (between days 24 and 31 of gestation) sires were classified as either high or low early embryonic mortality (EEM), while for the second interval (between days 31 and 60 of gestation) sires were classified as either high or low late embryonic/early fetal mortality (LEM). One-way ANOVA (GLIMMIX procedure,

SAS 9.4, Institute Inc., Cary, NC) was used to test differences in the dependent variables pregnancy rate, pregnancy loss and PAG concentration. Fixed effects included sire classification (high or low EEM and LEM), estrus expression (high or low), parity (multiparous or primiparous) and their interactions. Cow BCS, days postpartum and farm location were included as random variable in all models. All data were analyzed using cow as the experimental unit and means were separated using LSMEANS and adjusted according to the Tukey-Kramer test. Frequency of pregnancy rate and pregnancy loss was compared between variables using odds ratio (FREQ procedure, SAS 9.4, Institute Inc., Cary, NC). For all analyses, significance was set at $P \leq 0.05$ and tendencies were determined when $0.05 < P \leq 0.15$, results are presented as mean ± SEM.

2.3. Results

To facilitate the understanding of the effect of sire on pregnancy loss the results were divided into two embryonic/fetal developmental periods: 1) between days 24 and 31 of gestation (EEM) and 2) between days 31 and 60 of gestation (LEM).

2.3.1. Pregnancy results in the first interval: between days 24 and 31 of gestation

Pregnancy at day 24 was determined by an increased circulating PAG concentration compared to the baseline sample and progesterone concentration greater than 0.7 ng/ml on day 24. Animals that were pregnant at day 31 but had a negative diagnosis at day 24 (false negatives, n=113) were also classified as pregnant. Overall pregnancy rate at day 24 was 54.86% (361/658) and pregnancy loss, as defined by animals with pregnant at day 24 but no embryo present at day 31, was estimated at 5.54% (20/361; Table 1). Primiparous and multiparous animals had similar pregnancy rate ($P = 0.48$) and pregnancy loss ($P = 0.86$) between days 24 and 31 and, therefore, parity was included as a random variable in all subsequent analysis.

3.2 Pregnancy results in the second interval: between days 31 and 60 of gestation

Overall, pregnancy rate at day 31 was 51.8% (341/658) and pregnancy loss between days 31 and 60 was 6.74% (23/341; Table 1). Primiparous and multiparous animals had similar pregnancy rate ($P = 0.39$) and pregnancy loss between days 31 and 60 ($P = 0.37$) and was included as a random variable on the subsequent analysis.

2.3.2. Classification of sire by pregnancy loss for interval 1

Pregnancy rate per sire at day 24 ranged from 47% to 63% and no differences between sires were observed ($P = 0.31$). Subsequently, sires were retrospectively classified according to the percentage of pregnancy loss happening between days 24 and 31 of gestation (Table 2). Four sires accounted for 75% of the total EEM and were classified as high EEM (average = 8.93%) while the other four sires accounted for the remaining 25% and were classified as low EEM (average = 2.59%; Fig. 1A). Cows inseminated with high EEM sires had 3.7 greater odds of undergoing pregnancy loss between days 24 and 31 compared with cows inseminated with low EEM sires ($P = 0.01$). No difference ($P = 0.56$) was observed in circulating PAG concentration on day 24 from pregnancies sired by high EEM sires compared with pregnancies sired by low EEM sires (1.32 ± 0.37 vs 1.44 ± 0.37 ng/ml; Fig. 1B).

2.3.3. Classification of sire by pregnancy loss for interval 2

Pregnancy rate at day 31 did not differ ($P = 0.31$) by individual sire, with values ranging from 44% to 61% (Table 2). Three sires accounted for 74% of the embryonic mortality occurring between days 31 and 60 of gestation and were classified as high LEM (average = 11.04 %) while the other five sires accounted for the remaining 26% of the total pregnancy loss and were classified as low LEM (average = 3.21%, Fig. 2A). Cows bred with high LEM sires had 3.7 greater odds of experiencing pregnancy loss between days 31 and 60 compared to cows bred with low LEM sires ($P = 0.004$). No difference was observed in circulating PAG concentration at day 31 from

successful pregnancies by sires classified as either high or low LEM (6.55 ± 1.81 vs 6.97 ± 1.80 ng/mL; $P = 0.49$) for this period of gestation (Fig. 2B). Moreover, initial pregnancy rate at day 31 was greater ($P = 0.03$) in cows bred with high LEM sires (56.8% vs 47.6%), but final pregnancy rate at day 60 was similar (50.7% vs 46.6%, $P = 0.30$) to cows inseminated with low LEM sires (Fig. 3).

2.3.4. Estrus expression and pregnancy loss on intervals 1 and 2

Estrus expression prior to TAI influenced pregnancy status. Overall, cows that expressed estrus had higher ($P = 0.0002$) pregnancy rates at day 24 (63 vs 48%), but similar ($P = 0.33$) incidence of pregnancy loss during the first interval compared with cows that did not express estrus (4.3 vs 6.8%). Moreover, the effect of estrus expression on pregnancy rate was similar ($P = 0.71$) within sire groups. Cows that were bred with low EEM sires had a 17% increase in pregnancy rate when animals expressed estrus or not, while cows bred with high EEM sires had a 14% increase (Fig. 4A).

During the second interval, cows that express estrus prior to TAI had greater ($P < 0.001$) pregnancy rate at day 31 (61% vs 45%) but similar ($P = 0.47$) incidence of pregnancy loss between days 31 and 60 of gestation compared with cows that did not express estrus (8.27% vs 6.17%). Effect of estrus expression on pregnancy rate differed between sire groups. Cows that were bred with low LEM sires had a greater ($P = 0.05$) increase (23% increase) in pregnancy rate at day 31 when estrus was expressed compared with cows that expressed estrus bred to high LEM sires (7% increase; Fig. 4B).

2.4. Discussion

Embryonic mortality, if strictly classified according to physiological events during gestation, should refer to losses during the embryonic period which is from conception to the end

of the differentiation stage, around day 42 of gestation in cattle [15]. As in this study, it is common to include both fertilization failure as well as losses happening right after fertilization as embryonic mortality, since there is no available diagnostic method that accurately identifies fertilization success without terminating pregnancy. Moreover, beef herd management strategies often utilize pregnancy diagnosis around day 30 and later at day 60 or 90 of gestation, requiring the evaluation of late embryonic and early fetal mortality together. This study uniquely characterizes pregnancy loss in beef cattle, utilizing an early blood test pregnancy diagnosis with traditional transrectal ultrasonography allowing characterization of embryonic mortality in different periods of development that had not been done previously in beef cows. Conceptus derived products, such as PAGs, are secreted exclusively during pregnancy and can be used accurately to detect pregnancy [12, 16-18]. Commercial PAG pregnancy diagnosis assay is currently recommended to be performed around day 28 of gestation, but recent studies in dairy [19] and beef cows [20] suggest that measurements of PAG as early as day 24 post insemination may accurately (80-85%) differentiate pregnant and non-pregnant animals.

In this study, we observed similar accuracy (80%) using day 24 sample, with a higher probability of detecting true non pregnant animals (93%). Cows that were pregnant but had no detectable PAG on day 24 (false negatives, 17%) were still included in the analysis in order to obtain a better representation of incidence of pregnancy loss during this period. In this study, animals pregnant at day 24 but no embryo present at day 31, were classified as pregnancy loss between days 24 and 31 (EEM; 5.5%). A recent meta-analysis described an average of 15% of pregnancy loss between days 16 and 32 in beef cows [1]. Unfortunately, the current study was unable to capture this early period of pregnancy loss (days 16 to 24) and this reflects our findings of reduced incidence of EEM. From days 16 to 31 of gestation, there are critical milestones in

embryo growth and extraembryonic membrane development that could provide numerous opportunities for pregnancy failure. Trophoblast expansion, accompanied by rapid differentiation and migration of multinucleated giant cells with concurrent changes in embryonic shape, happens from days 16 to 20 of embryonic development. During this same period, the allantois expand and forms villous-like projections that invade the chorionic cavity and subsequently attaches to the lining of the uterus giving rise to the formation of placentomes [10, 21]. Even though past reports suggest that most early embryonic mortality happens before day 16 of gestation in cattle, prior to maternal recognition of pregnancy [3, 6, 22, 23], this complex embryo-uterine relationship and the role that the rapidly growing trophoblast plays in embryo survival is suggestive that failure in pregnancy maintenance during this time is noteworthy. In sub-fertile heifers, increased embryonic mortality was observed during the post embryo elongation period, between days 17 and 28 post embryo transfer, likely due to a dysregulation of conceptus-endometrial interaction that compromises normal implantation and placentation [7, 24]. Late embryonic/early fetal mortality of 6.7% in the current study was similar to previous studies that reported to range from 2-10% [3, 4]. The exact mechanisms that lead to embryonic mortality during this time are poorly understood and could be related to embryo development itself or inadequate formation of extra embryonic membranes as it coincides with time of embryo attachment and initiation of placentation [12, 25].

In regard to factors that contribute to pregnancy success post fertilization, most research has focused on maternal and environmental factors [26-29] and only a few studies report the effect of sire on embryonic mortality [30, 31]. Paternal genetics play an important role in placenta formation and seems to be critical during later stages of embryonic development. In post-implantation chimaeric murine fetuses, parthenogenetic cells (with maternal genome only) were confined to the embryo proper, while androgenetic cells (with paternal genome only) formed the

trophoblast [32, 33]. Similar results were observed in cattle. Parthenogenetic embryos develop up to day 35 of gestation after embryo transfer with a clear embryonic vesicle present, but with very rudimentary or even absent embryo proper mass [34, 35]. In this study, we observed large variance on the percentage of pregnancy loss among sires in both periods of embryonic development analyzed. Moreover, the sires that were classified as high EEM did not always equate to the same phenotype profile for the LEM period and vice versa. Sires classified as high LEM (between days 31 and 60) had increased pregnancy rate at day 31 and would probably be classified as high fertility by current methods of sire fertility evaluation. After accounting for the percentage of LEM, however, final pregnancy rates were similar to those classified as low LEM. Similar results were reported using *Bos indicus* beef cattle [11] and dairy cows [36], in which the service sire used for breeding affects the incidence of pregnancy loss during the second month of gestation. Starbuck et al. [31] reported that pregnancy rate at day 35 was similar among sires used for AI but pregnancy loss varied, including one sire with substantial higher pregnancy loss during the second month of gestation. Similarly, Lopez-Gatius et al., [30] reported that lactating dairy cows bred to an individual sire had 3.4 times greater chance of pregnancy loss during second month of gestation compared to cows bred with other service sires used. Sires used in this experiment passed standard commercial semen evaluations and met all minimum requirements for use in the field; therefore, no difference in fertility could have been predicted at the beginning of the experiment. In spite of this, large variance on the incidence of pregnancy loss by sires during both periods evaluated were observed in this study, with shifts in the sire fertility ranking depending on the period analyzed, reinforcing the importance of evaluating different milestones of embryonic development when classifying sire fertility.

Circulating PAGs have been identified as an indicator of LEM [4, 37, 38] and were previously correlated with sire fertility in regards to pregnancy loss [11]. The presence of placental development-associated transcripts, such as *PAG5*, *PAG7* and *PAG10* in spermatozoa suggest that sperm-borne transcripts might contribute to pregnancy beyond early embryonic development [39]. These transcripts may have significant roles during implantation and placentation, which corresponds with the timing of the pregnancy losses analyzed here. In the current study, we did not observe differences in circulating concentrations of PAG in cows inseminated with either high or low LEM sires, potentially due to smaller sample sizes and higher number of sires utilized, but future studies to further evaluate the relationship of sire fertility and PAG secretion could establish a potential marker for sire fertility in regards to embryonic mortality.

A secondary objective of this study was to evaluate the relationship between sire classification and estrus expression. Estrus expression, caused by elevated concentrations of estradiol from the dominant follicle, is an indicator of female fertility and is well-established to be positively correlated with pregnancy establishment [40, 41]. Cows that express estrus had an average increase of 15% in pregnancy establishment, which is similar to other studies using TAI with a GnRH-based estrus synchronization protocol [40, 42], but this effect of estrus on pregnancy rate was highly variable among sires. Elevated estradiol concentrations at estrus causes several changes within the reproductive tract, including changes in the composition and amount of cervical mucus, enhanced uterine contractions and decreased uterine pH, leading to decreased sperm motility and subsequent increase in sperm lifespan within the female tract [43]. Differences in semen composition may allow certain sire sperm to have a longer viable life-span in the uterus, independent of estradiol induced changes, which would explain the higher pregnancy rate in the non-estrus scenario for those specific sires. Estrus expression alters endometrial gene expression

during the implantation period, mostly genes involved with adhesion and immune system, that could favor embryonic implantation and proper placentation [44]. An inadequate estradiol concentration, however, could impair uterine environment regulation that could lead to subsequent failure in pregnancy maintenance. Herein, cows that were bred with sires classified as high LEM had higher pregnancy rates at day 31 in a non-estrus scenario, which could partially explain the increase in pregnancy loss in these group of animals. Similarly, in a previous experiment from our group, cows inseminated with specific sires had higher pregnancy rates in the absence of estrus expression, consequently, that led to higher LEM. Further studies are needed to understand the differences in sperm morphology that could explain the variation observed.

2.5. Conclusion

Even when bulls are selected for artificial insemination and pass all standard semen evaluations, there is still considerable variance in pregnancy rate and pregnancy loss. These differences in the ability to establish and maintain pregnancy cannot be explained by variation in visual semen analysis. Moreover, the sire phenotype in regard to pregnancy loss differed depending on the interval of embryonic and fetal development. Thus, making sire fertility decision solely based on a single pregnancy check an inaccurate assessment. The development of markers that can identify sires of high or low pregnancy loss would be very beneficial to improve sire fertility evaluation and increase beef and dairy reproductive efficiency.

2.6. References

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Table 2-1 Description of the number of animals in each period of pregnancy diagnosis.

	TAI[1]	**Pregnant at day 24**[2]	**Pregnant at day 31**[2]	**Pregnant at day 60**[2]	**EEM**[3] **(day 24 - 31)**	**LEM**[3] **(day 31 - 60)**
Primiparous	173	96	91	83	5.20%	8.79%
Multiparous	485	265	250	235	5.66%	6.00%
Total	**658**	**361**	**341**	**318**	**5.55%**	**6.74%**

[1] TAI – Fixed time artificial insemination
[2] Pregnancy diagnosis at day 24 were done based on serum PAG concentration and at day 31 and day 60 by visualization of fetal heartbeat by transrectal ultrasonography.
[3] EEM – early embryonic mortality
[4] LEM – late embryonic/early fetal mortality

Table 2-2 Sire classification based on pregnancy loss during different periods of embryonic and fetal development.
For the first interval of pregnancy loss (between days 24 and 31 of gestation) sires were classified as either high (◄) or low early embryonic mortality (EEM), while for the second interval (between days 31 and 60 of gestation) sires were classified as either high (♦) or low late embryonic/early fetal mortality (LEM). Results are represented as [least squares means (n/n)].

Sire	Pregnancy rate at day 24 (%)	EEM (%)	EEM Classification	Pregnancy rate at day 31 (%)	LEM (%)	LEM Classification
1	47.13% (41/87)	2.43% (1/41)	Low EEM	45.9% (40/87)	5.1% (2/40)	Low LEM
2	50.75% (34/67)	11.76% (4/34)	High EEM◄	44.8% (30/67)	3.4% (1/30)	Low LEM
3	63.64% (56/88)	8.92% (5/56)	High EEM◄	57.9% (51/88)	9.9% (5/51)	High LEM♦
4	50.00% (45/90)	8.88% (4/45)	High EEM◄	45.5% (41/90)	2.5% (1/41)	Low LEM
5	55.93% (33/59)	6.06% (2/33)	High EEM◄	52.5% (31/59)	3.3% (1/31)	Low LEM
6	54.35% (50/92)	4.00% (2/50)	Low EEM	52.0% (48/92)	12.6% (6/48)	High LEM♦
7	54.12% (46/85)	2.17% (1/46)	Low EEM	52.9% (45/85)	2.3% (1/45)	Low LEM
8	62.22% (56/90)	1.78% (1/56)	Low EEM	61.1% (55/90)	11.0% (6/55)	High LEM♦

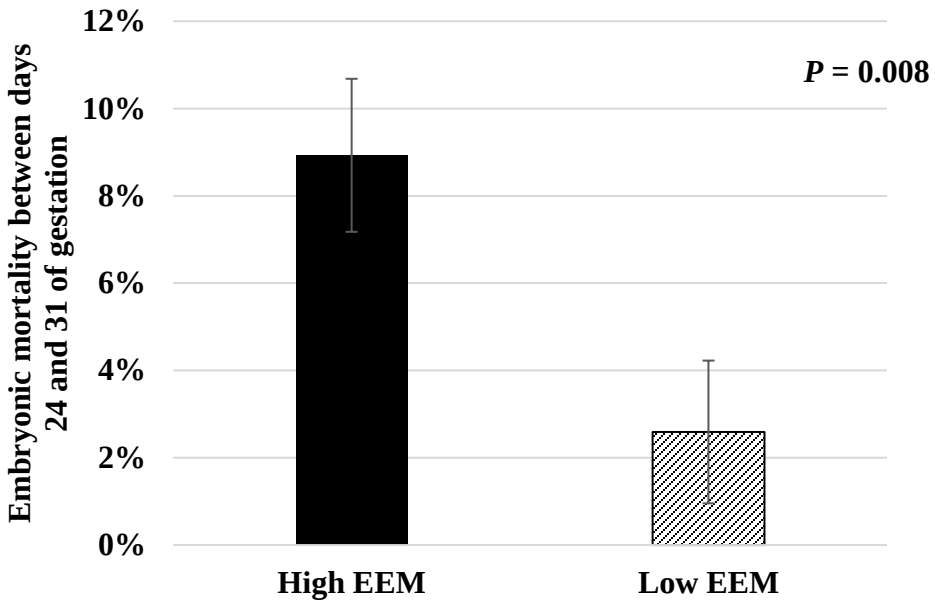

Figure 2-1 Percentage of early embryonic mortality (EEM) between days 24 and 31 of gestation by first interval sire classification.
Cows inseminated with high EEM sires had greater percentage of pregnancy loss compared to cows inseminated with low EEM sires (8.93 vs 2.59 %; *P* = 0.008).

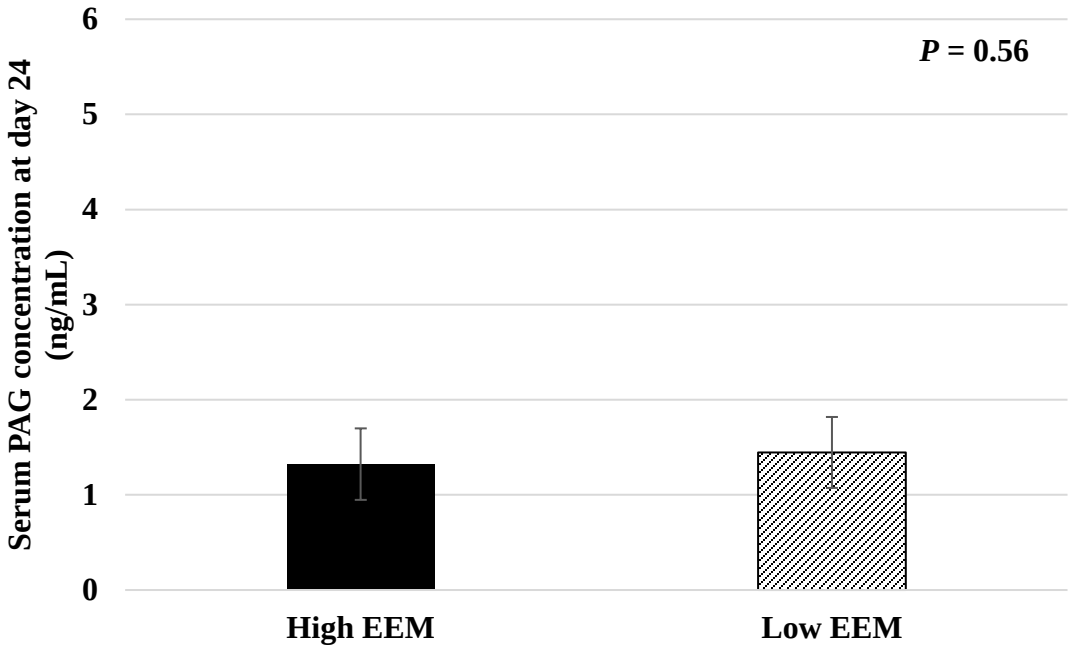

Figure 2-2 Circulating pregnancy associated glycoprotein (PAG) concentration by sire classification at day 24 of gestation.
Serum concentrations of PAG in successful pregnancies of cows bred with sires classified as high early embryonic mortality (EEM) were similar (P = 0.56) at day 24 of gestation compared with cows bred with sires classified as low EEM.

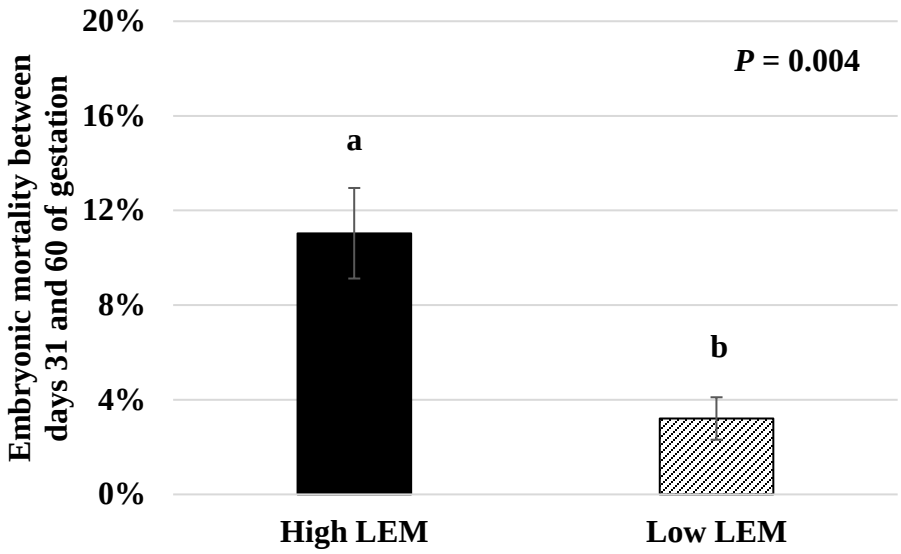

Figure 2-3 Percentage of late embryonic/early fetal mortality (LEM) between days 31 and 60 of gestation by second interval sire classification.
Cows inseminated with high LEM sires had greater percentage of pregnancy loss compared to cows inseminated with low LEM sires (11.04 vs 3.21 %; *P* = 0.004).

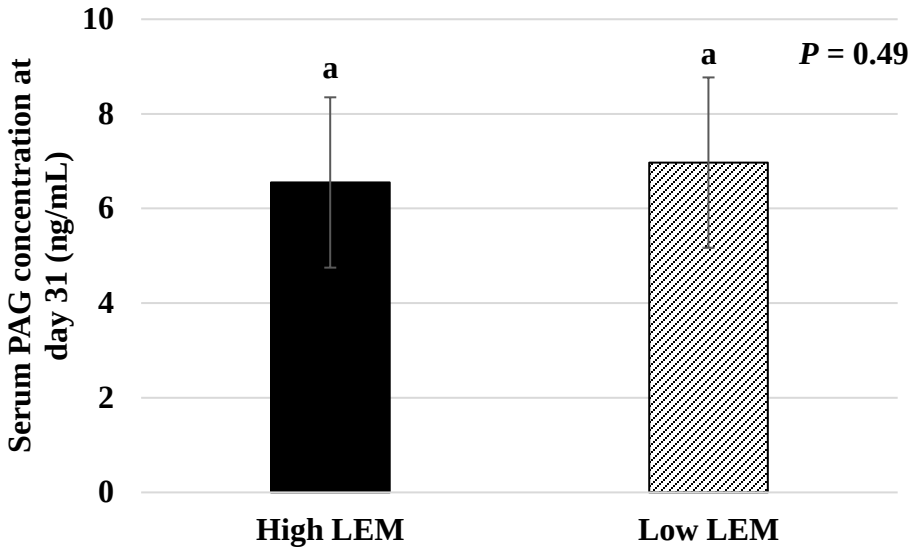

Figure 2-4 Circulating pregnancy associated glycoprotein (PAG) concentration by sire classification at day 31 of gestation.
Serum concentrations of PAG in successful pregnancies of cows bred with sires classified as high late embryonic/early fetal mortality (LEM) were similar ($P = 0.94$) at day 31 of gestation compared with cows bred with sires classified as low LEM.

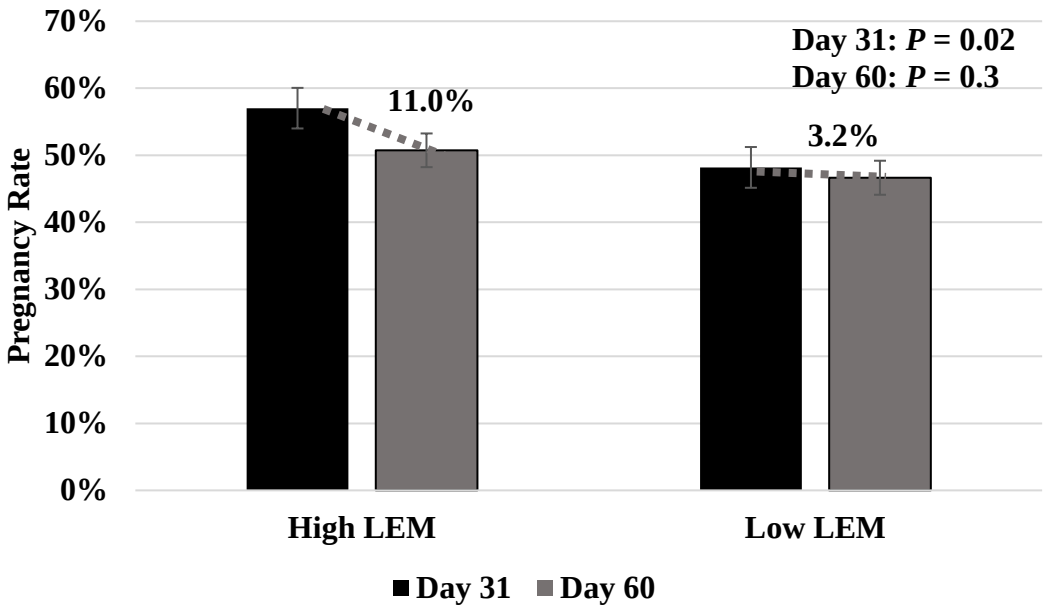

Figure 2-5 Pregnancy rate and late embryonic/early fetal mortality (LEM) by second interval sire classification.

Sires were retrospectively classified according to amount of pregnancy loss between days 31 and 60 of gestation. Cows inseminated with high LEM sires had greater (P = 0.01) initial pregnancy rate at day 31 but similar (P = 0.30) final pregnancy rate at day 60 compared with cows inseminated with low LEM sires. The amount of LEM was 11.0% for the high LEM group against 3.2% for the low LEM group (P = 0. 04)

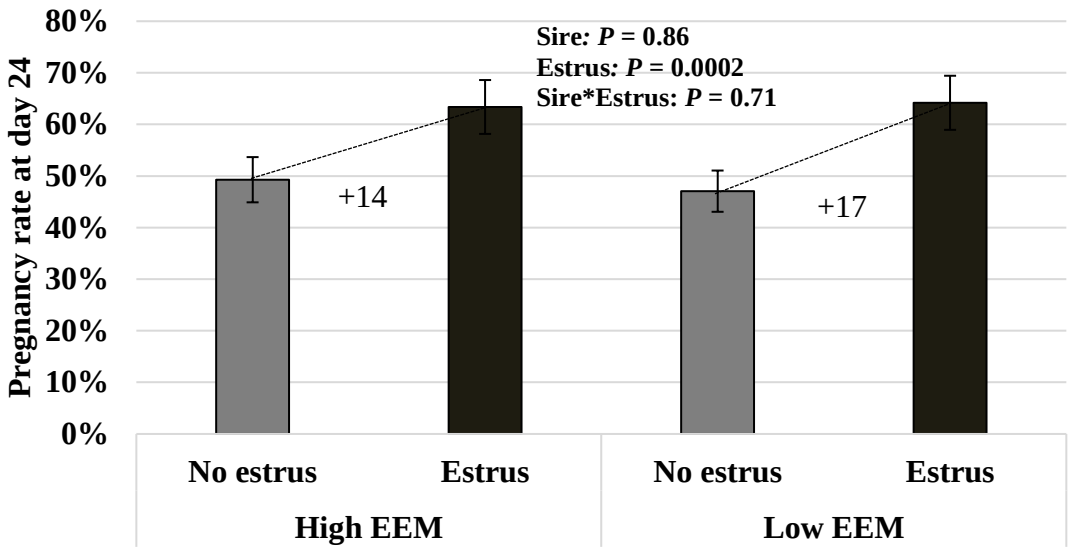

Figure 2-6 Differences in pregnancy rate at day 24 by estrus expression prior to TAI. Cows that were bred with low early embryonic mortality (EEM) sires had no difference in pregnancy rates at day 24 when estrus was expressed prior to AI compared to cows bred with high EEM.

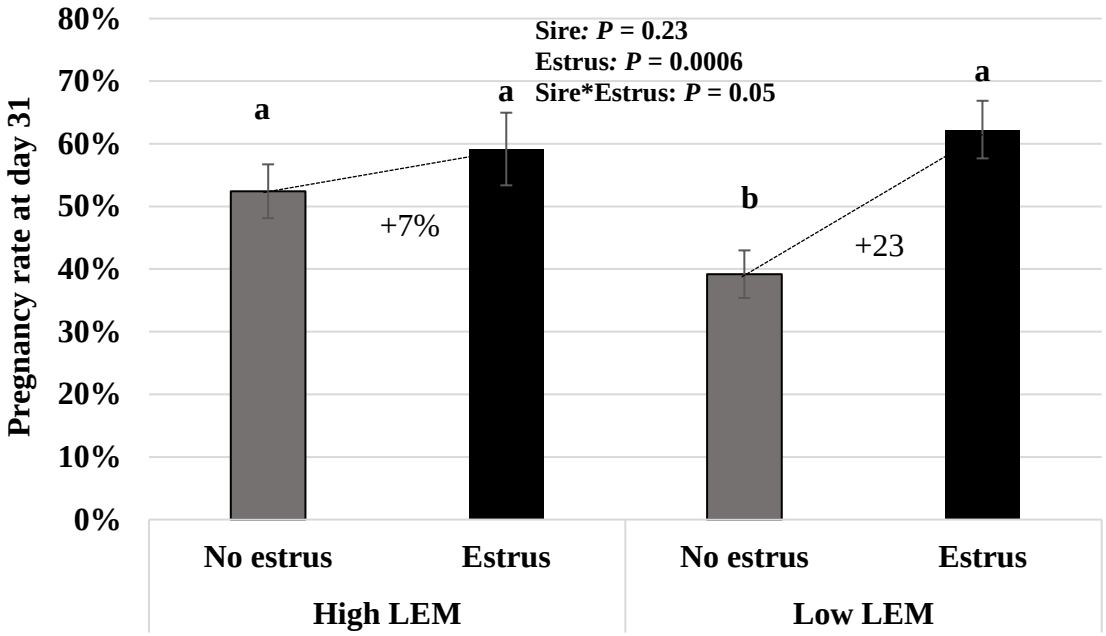

Figure 2-7 Differences in pregnancy rate at day 31 by estrus expression prior to TAI. Cows that were bred with low late embryonic/early fetal mortality (LEM) sires had a greater difference in pregnancy rates at day 31 when estrus was expressed prior to AI compared to cows bred with high LEM.

3. SIRE EFFECT ON PREGNANCY RATE AND PREGNANCY LOSS IN DAIRY COWS: DOES FIELD FERTILITY ASSOCIATE WITH SIRE CONCEPTION RATE (SCR)?

3.1. Introduction

Improving sire fertility estimations has been one of the major goals of the dairy industry to hasten the improvement on reproductive performance in recent decades. According to current estimation methods, the term "sire fertility" could be related to any measurements throughout gestation, including fertilization rate, embryonic formation, conceptus product formation or, ultimately, calf birth [1]. The lack of a standardized time point measurement decreases accuracy and causes limitations of population-based approaches to estimate fertility of a dairy sire. Pregnancy failure post-conception is not commonly attributed to the sire but considering the role of male genetics in embryo development, implantation and placenta formation, this dogma should be revisited. Low fertility sires produce lower quality preimplantation embryos and have higher pregnancy loss during the implantation period [2]. In rodents and humans, paternal genetics drive trophoblast cell proliferation and placenta formation [3-5]. Similarly, in cattle, genes that regulate trophoblast cell proliferation (e.g. Mash2) are regulated by the paternal genome [6]. Adequate placentation is required for proper exchange of nutrients at the fetal-maternal interface and disruption of these physiological processes could lead to pregnancy failure [7].

Previous research from our lab has demonstrated that the sire contributes significantly to incidence of pregnancy loss in beef cows throughout embryonic/fetal development, which drastically affects final pregnancy rate. In *Bos indicus* beef cows, no difference in pregnancy rate at day 30 was observed among sires used for TAI, but pregnancy loss during the second month of gestation was highly variable (1.4 to 11.1%) among sires [8]. Similarly, *Bos taurus* beef cows had significant variation in pregnancy loss between days 24 and 31 of gestation (1.8 to 11.7%) and between days 31 and 60 of gestation (2.3 to 12.6%) among sires used for TAI [9]. In dairy cows, it has also been observed that pregnancies from a subset of sires were more likely to undergo embryonic mortality [10, 11], where cows inseminated using semen from one of the six bulls tested had 3.4 times higher incidence of late embryonic/early fetal mortality [10]. These studies suggest that the incidence of late embryonic/early fetal mortality should be considered when evaluating sire fertility, as it can significantly affect final pregnancy rate.

Evaluating sire fertility and identifying genetic markers to accurately predict fertility traits is a complex process, partly due to poorly defined phenotypes. Reproductive processes that can be affected by sire, aside from fertilization, are mostly unknown. In 2008, USDA's Animal Improvement Programs Laboratory (AIPL) unified the existing sire summaries to create a single indicator of semen fertility for major dairy breeds. Sire conception rate (SCR) is a national indicator of semen fertility of bulls used for AI. It includes data from all US Dairy Record Processing Centers and is calculated and distributed by the Council on Dairy Cattle Breeding (CDCB). SCR is updated tri-annually

to include additional breeding and is expressed as a relative conception rate and represents the deviation of the breed average. For example, a Holstein bull with SCR of +2.0 is expected to have a 2.0% higher conception rate than the average for the Holstein breed, and 4.0% higher conception rate than a Holstein bull with score -2.0 [12]. In 2015, more AI centers were included in SCR publications and the model was re-examined to confirm correlation with previous model and accuracy in predicting fertility [13]. Even though this index has standardized dairy bull fertility evaluation nationwide and become a valuable tool for sire selection, challenges exist in preventing optimization of the model. The current model does not use a standardized day for pregnancy diagnoses; therefore, pregnancy loss may or may not be properly quantified. Our objective was to characterize pregnancy rate and pregnancy loss of individual sires across large herds diagnosed on uniform data sets and correlate to the published SCR. The major hypothesis is that variance in fertility parameters cannot be explained completely by variance in the SCR and pregnancy loss can drastically change the accuracy.

3.2. Materials and Methods

3.2.1. Overall Data Collection

An 8-year (2011-2018) retrospective analysis was performed using data from 55 commercial dairy herds (average herd size = 800) in the southeast region of Brazil where fixed-time artificial insemination (TAI) or fixed-time embryo transfer (TET) was used. These data are combined from several previous experiments with some of the results published in previous scientific manuscripts [14-17]. More than 45,000 breeding records

were initially obtained. Initial screening excluded data from cows with more than 50% of Bos Indicus genetics and data from sires with less than 50 services. After final screening, 31,857 breeding records were included for TAI (6.570 cows) and TET (16,847 cows and 8,440 heifers). Throughout the experimental period, cows were housed in free stall barns and were milked 3 times daily. Nutrition was balanced to meet or exceed the nutritional requirements of lactating dairy cows. Females averaged 123 ± 87 DIM, yielding 20.3 ± 7.6 kg of milk/d, lactation number of 1.4 ± 1.5, and had been bred 3.9 ± 2.8 times before positive pregnancy diagnosis.

3.2.2. Reproductive Technologies Procedures

Animals were synchronized using an industry standard protocol using progesterone implant and estradiol [17] where on day 0, cows received TAI or on day 7, cows received a single fresh *in vitro*-produced grade 1 embryo (TET). All cows were evaluated for pregnancy using transrectal ultrasound and embryo fetal viability confirmed by heartbeat visualization around day 30 (P30) and a second diagnosis occurred around day 60 (P60) of gestation. Pregnancy rates were calculated using the number of pregnant cows at P30 or P60 divided by the total number of cows serviced. Pregnancy loss (PL) was calculated by dividing the number of cows that had embryonic mortality between P30 and P60 over the number of pregnant cows at P30.

3.2.3. Sire Classification

A total of 39 sires were used for TAI with an average of 168 (range 50 to 420) services per sire and a total of 81 sires were used for TET with an average of 310 (range

50 to 1040) services per sire. Published SCR from the period prior to individual breeding service were obtained for each sire used for artificial insemination or embryo production. Scores were used to classify sires into quartiles, with quartile 1 representing the highest SCR (i.e. expected higher fertility) **(Table 1 and 3)**. Sires were also classified according to percentage of PL between P30 and P60 into high (greater than 20%), average (between 10 and 20%) or low PL (less than 10%).

3.2.4. Statistical Analysis

Records from date of service, cow genetic composition, milk production, parity, service sire, sire SCR were obtained for each service. Many levels of crossbreeding (*Bos indicus* Gir vs *Bos Taurus* Holstein) were reported and animals with more than 50% Gir genetics were removed. Animals bred or receiving embryos from May through September were classified as winter breeding, while animals bred or receiving embryos from October to April were classified as summer breeding. The MIXED procedure (SAS 9.4, Institute Inc., Cary, NC) was used to test differences in the dependent variables pregnancy rate at P30 and P60, pregnancy loss (PL). Fixed effects included SCR classification (Quartiles 1 – 4), PL classification (high, average or low PL) with season, parity, cow genetic composition and milk production as independent effects. Animals with multiple services within the same year were accounted for in repeated measures. Year and location were included as random variables in all models. All data were analyzed using cow as the experimental unit and means were separated using PDIFF when the *P*-value for the main effect was ≤ 0.05. Data is presented as mean ± SEM. Frequency of pregnancy rate and PL

was compared between variables using odds ratio (FREQ procedure, SAS 9.4, Institute Inc., Cary, NC). Pearson correlation with PROC CORR (SAS 9.4, Institute Inc., Cary, NC) was used to test the correlation between SCR and fertility parameters. For all analyses, significance was set at $P \leq 0.05$.

3.3. RESULTS

3.3.1. Artificial Insemination Data

Overall pregnancy rate at P30 was 33.1% (2,177/6,570), and 27.6 % for P60 (1815/6,570) and PL was 16.6% (362/2,177). Cows bred during the winter season (n = 3,864) had greater (P < 0.0001) pregnancy rate at both P30 (40.9 vs 25.0%) and P60 (33.8 vs 21.1%) compared with summer season (n = 2,706), but no difference (P = 0.23) was observed in percentage of PL (17.3 vs 15.2%). Pregnancy rate (P30 and P60) and PL incidence was highly variable among sires and no clear correlation was observed between these variables **(Figure 1A**). Next, sire effect was evaluated based on SCR and incidence of pregnancy loss classification.

Sire Fertility Correlation with SCR. SCR from sires used for TAI range from -3.2 to 3.7. Individual sire's SCR had poor correlation with P30 (P = 0.07; r = 0.2) and P60 (P = 0.07; r = 0.2) **(Figure 2A)**. When classifying into quartiles, sires from quartile 1 had greater (P < 0.05) pregnancy rate at P30 (39% vs 31%, 30% and 32%) and P60 (33% vs 27%, 25% and 27%) compared with sires from quartile 2, 3 and 4 respectively, but no difference (P > 0.05) in pregnancy results among quartiles 2, 3 and 4 . Pregnancy loss between P30 and P60 was similar (P > 0.05) among all sire quartiles (**Figure 3A**).

Sire Correlation with Pregnancy Loss. Pregnancy loss between P30 and P60 ranged from 0 to 38% among sires used. Cows bred with high PL sires had 1.8 (95% CI:1.4 – 2.3) greater odds of undergoing pregnancy loss compared with cows bred to average sires. On the other hand, cows bred to low PL sires were 60% less likely (OR 0.4, 95% CI: 0.2 – 0.6) to undergo PL compared with average. Sires initial pregnancy rate at P30 was greater ($P < 0.001$) for low PL sires but similar ($P = 0.07$) between average and high PL. Final pregnancy rate at P60 was significantly lower in sires classified as high PL compared with average PL ($P = 0.004$) and low PL ($P < 0.001$) (**Figure 4A**).

3.3.2. Embryo Transfer Results

Overall pregnancy rate at P30 was 47.8% (12,082/25,287) and 40.5% for P60 (10,246/25,287) with an overall PL of 15.2% (1,836/12,082). Cows bred during winter season (n = 13,825) had greater (P < 0.0001) pregnancy rate at both P30 (50.8 vs 46.9%) and P60 (42.8 vs 39.6%), but no difference ($P = 0.78$) was observed in percentage of PL (15.2 vs 15.0%) compared with cows bred during summer (n = 11,462). Heifers (n = 8440) had greater ($P = 0.0032$) pregnancy rate at P30 (56.2 vs 44.9%) and P60 (48.9 vs 37.2%, $P = 0.0009$) and lower PL (12.8 vs 16.7, $P < 0.0001$) compared with cows (n = 16,847). Pregnancy rate and pregnancy loss were highly variable among services sires used and no correlation was observed between these variables **(Figure 1B**). Next, sire effect was evaluated based on SCR and incidence of pregnancy loss classification.

Sire Fertility Correlation with SCR. Sire conception rate from sires used for TET ranged from -6.3 to 4.5 and they were classified into quartiles as presented in **Table 2**.

Individuals' sire SCR had no correlation with P30 (P = 0.8; r = -0.03) and P60 (P = 0.7; r = 0.03) **(Figure 2B).** No difference (P > 0.05) in pregnancy rate at P30 (48.2%, 49.7%, 49.5% and 48.1%), P60 (40.9%, 41.9%, 41.0% and 40.7%) and PL (14.5%, 15.1%, 16.5% and 14.7%) from sires from quartiles 1, 2, 3 and 4, respectively **(Figure 3B)**.

Sire Correlation with Pregnancy Loss. Pregnancy loss between P30 and P60 ranged from 5 to 36% among sires. Pregnancies from high PL sires had 1.3 (95% CI:1.2 – 1.5) greater chance of undergoing PL compared with pregnancies by average PL sires. On the other hand, pregnancies by sires classified as low PL were 30% less likely (OR 0.7, 95% CI: 0.6 – 0.8) of undergoing PL compared with sires classified as average. Initial pregnancy rates at P30 were similar among groups, but final pregnancy rate at P60 was significantly lower (P = 0.0016) from sires classified as high PL (**Figure 4B**).

3.4. DISCUSSION

Adoption of genomic selection in the last decade has drastically improved genetic gain for lowly heritable traits, including cow fertility [18]. Despite these advances, reproductive performance of dairy cows worldwide remains optimal to suboptimal. Final conception rate for US Holsteins average 34% resulting in an average calving interval of almost 400 days [19]. Pregnancy loss during different periods of embryonic development is one of the major causes of reproductive inefficiency. It is estimated that almost 40% of pregnancies are lost between days 19 and 62 of gestation [20]. Pregnancy success and embryonic mortality are affected by a combination of paternal, maternal, and/or

embryonic factors [21, 22]. Although both parental genomes affect reproductive success, much of the research in dairy cattle has been directed toward female fertility and little is known about paternal effect on pregnancy development post fertilization and early embryonic development.

Evaluation of dairy sire fertility is heavily dependent on SCR, an indicator of bull fertility published by USDA expressed as relative conception rate compared with breed average [12]. To date, SCR remains a phenotypic trait and no genetic variants beyond fertilization [23] have been associated with SCR as for other fertility traits in females [18, 24, 25]. This implies that there is either no genetic component contributing to SCR which is unlikely given the consistency of the bulls producing fewer pregnancies in different scenarios; or that given the number of biological processes from fertilization to pregnancy establishment, SCR at this moment is not able to capture the right phenotypes to make genetic associations for this trait.

In this study, there was considerable variance in P30, P60 and PL among sires when AI or ET was used but there was none or poor correlation with published SCR. The lack of correlation in either reproductive technology suggests that variance in sire fertility is not merely a fertilization issue, since that variable is removed in the embryo transfer process. Similar results were reported by Abdalla [26] where 81 sires classified by SCR had similar pregnancy rates at day 30 and day 70. In another study, high fertility sires (greater SCR) produced higher quality blastocysts *in vitro* and *in vivo*, but there was no difference in conceptus recovery or length on day 16 and pregnancy rate at day 32

compared with low fertility sires [2]. Older studies, when sire fertility was classified based on estimated relative conception rate (ERCR) by Dairy Records Management Systems (DRMS; Raleigh, NC) also showed no difference in pregnancy rate by sires with expected higher fertility [27]. SCR is updated tri-annually to include more data and subsequently increase prediction reliability. For this set of data, we investigated the correlation with the five latest available SCR scores to verify if the index changed over time. Each SCR was correlated with each other, but not correlated with the reproductive parameters. Sires used in this study had an average of 90.3% of reliability on published SCR and considering the large number of breeding records analyzed we expected to find a stronger correlation between pregnancy rates and SCR. Together these results indicate the need to further dissect how the male affects pregnancy establishment and maintenance, and to enhance our ability to predict these effects and selecting sires to improve herd conception rate.

Another objective of this study was to characterize sire contribution to late embryonic mortality in dairy cows. Previous studies from our lab have shown that sire has a major contribution to pregnancy loss in *Bos indicus* [8] and *Bos taurus* [9] beef cows. In this study, there was large variance in the percentage of pregnancy loss that drastically affected final pregnancy rate at P60. Interestingly, pregnancy loss phenotype does not always correlate with initial pregnancy rate phenotype. In other words, sires that have a high pregnancy rate at P30 do not necessarily have low pregnancy loss or high pregnancy rate at P60. Sires that were classified as high pregnancy loss had similar P30 pregnancy

rate but lower P60 pregnancy rate for both AI and ET. Moreover, three sires were used for both technologies and had similar pregnancy loss phenotype classification (Sire A: 11.5 and 12.6%; Sire B: 12.5 and 15.9%; Sire C: 11.1 and 13.5%, for TAI and TET respectively). Similarly, Abdalla [26] also described larger variance of pregnancy rate at day 70 than at day 30, a result of increased variance on pregnancy loss during this interval. Starbuck [28] and López-Gatius et al [10] reported that dairy cow pregnancy rate at day 35-38 was similar among sires used for AI but pregnancy loss varied, including one sire with substantially higher pregnancy loss during the second month of gestation. Similar results were observed with *Bos taurus* beef cows that had a significant variation in pregnancy loss between days 24 and 31 of gestation (1.8 to 11.7%) and between days 31 and 60 of gestation (2.3 to 12.6%) among service sires used for AI [9].

The reduced ability of low fertility sires to establish a successful pregnancy is multifactorial, including fertilization capacity, preimplantation embryonic development and placenta and embryo development after conceptus elongation [2]. Research into paternal genetic contributions to pregnancy maintenance has been focused on early stages of pregnancy and little is known of its effect on placentation and pregnancy development after the first month of gestation. In many species, paternal genetics may contribute significantly to placenta formation and subsequent pregnancy maintenance throughout embryonic development. Using a uniparental embryo model in mice, the formation of the embryo is primarily reliant on the maternal genome, while the paternal genome greatly contributes to trophoblast development and, therefore, the placenta [4, 5, 29-31]. Recent

studies have suggested that sperm carries a repertoire of coding and non-coding RNAs that could be translated into protein in oocyte after fertilization [32] and regulate gene expression during embryonic development [33]. In cattle, fewer studies investigate this relationship, but there is evidence of paternal imprinted genes associated with trophoblast proliferation [6]. Moreover, the presence of intact transcripts of pregnancy-associated glycoproteins (PAGs) in the spermatozoa suggest a possible influence of sperm transcripts beyond early embryonic development [34]. Previous studies from our group described a sire effect on maternal serum PAGs in *Bos indicus* beef cows [8, 35], but no clear correlation with sire fertility was defined. Adequate placentation is required for proper exchange of nutrients at the fetal-maternal interface and disruption of these physiological processes may lead to pregnancy failure. Investigating paternal contributions to placenta formation may increase our understanding into late embryonic mortality mechanisms and improve sire fertility predictions. There is clear evidence of the need to evaluate different gestation periods when classifying sire fertility.

3.5. Conclusion

In conclusion, there is a significant variation in pregnancy rate observed in sires whose semen passes the quality control checks in AI centers. Field fertility phenotype does not correlate with SCR in these groups of animals evaluated and may be partially explained by variance in pregnancy loss between P30 and P60 observed among sires, that is not accounted for in SCR model. Current methods use to estimate sire fertility lack accuracy and dependability due to an unreliable fertility phenotype. Future studies are

needed to identify biomarkers that are different between bulls of varying fertility (e.g. high or low pregnancy loss). High-throughput sequencing approaches evaluating bulls across a wide range of the fertility spectrum might explain some of the fertility variation observed.

3.6. References

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Table 3-1 Relationship of SCR quartiles and pregnancy loss (PL) incidence of sires (n = 39) used for timed artificial insemination (TAI).

	SCR[1]	Average PL (%)[2]
Quartile 1 (n = 10)	2.9 ± 0.01 (3.7 – 1.9)	14.3[a]
Quartile 2 (n = 10)	1.6 ± 0.006 (1.9 – 1.1)	13.4[a]
Quartile 3 (n = 10)	0.54 ± 0.01 (1 – -0.2)	18.8[a]
Quartile 4 (n = 9)	-1.8 ± 0.01 (-1.2 – -3.2)	18.3[a]

[1] Published SCR from the period prior to individual breeding service were obtained for each sire from the Council on Dairy Cattle Breeding Website (https://uscdcb.com). Values represent average ± SEM (range).

[2] Rows with different superscripts differ $P < 0.05$

Table 3-2 Relationship of SCR quartiles and pregnancy loss (PL) incidence of sires (n = 81) used for timed embryo transfer (TET).

	SCR[1]	Average PL (%)[2]
Quartile 1 (n = 21)	2.6 ± 0.007 (4.5 – 1.9)	14.6[a]
Quartile 2 (n = 20)	1.4 ± 0.003 (1.8 – 0.8)	15.2[a]
Quartile 3 (n = 20)	-0.02 ± 0.008 (0.7 – -1.3)	16.6[a]
Quartile 4 (n = 20)	-2.7 ± 0.006 (-1.4 – -6.3)	14.8[a]

[1] Published SCR from the period prior to individual breeding service were obtained for each sire from the Council on Dairy Cattle Breeding Website (https://uscdcb.com). Values represent average ± SEM (range).

[2] Rows with different superscripts differ $P < 0.05$

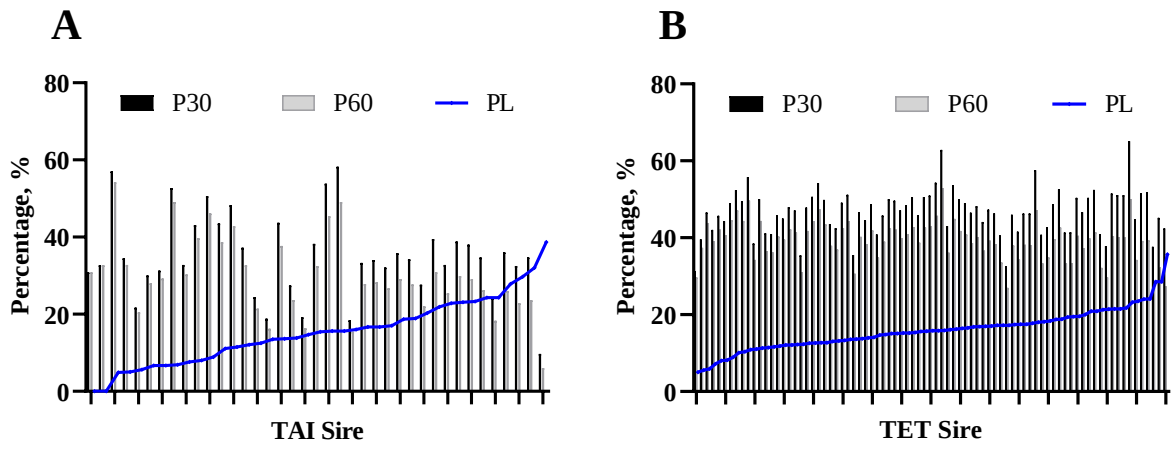

Figure 3-1 Reproductive results by service sire.
Pregnancy rate at day 30 (P30), day 60 (P60) and incidence of pregnancy loss (PL) in Holstein or Holstein crossbred dairy cows/heifers distributed by service sire. Panel A – Timed artificial insemination (TAI) sires (n = 39) and **B** - timed embryo transfer (TET) sires (n = 81). Sires are represented on X axis based on incidence on PL from lowest (left) to highest (right).

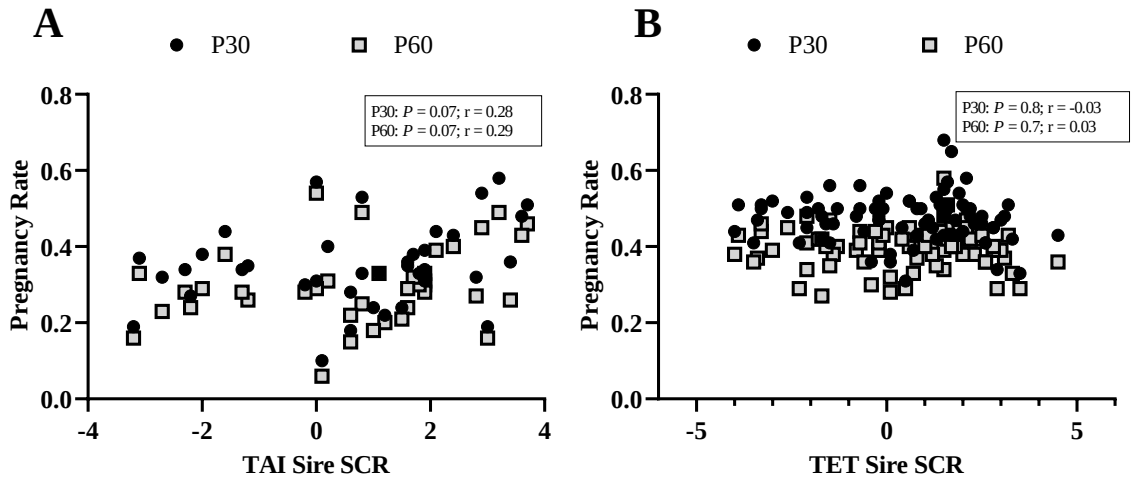

Figure 3-2 SCR correlation with pregnancy results.
Pearson correlation of Holstein or Holstein crossbred dairy cows/heifers pregnancy rate at day 30 (P30, black circle) and day 60 (P60, gray square) and SCR score of individual mating sire used for: **A** - timed artificial insemination (TAI); **B** - timed embryo transfer (TET). r = correlation coefficient.

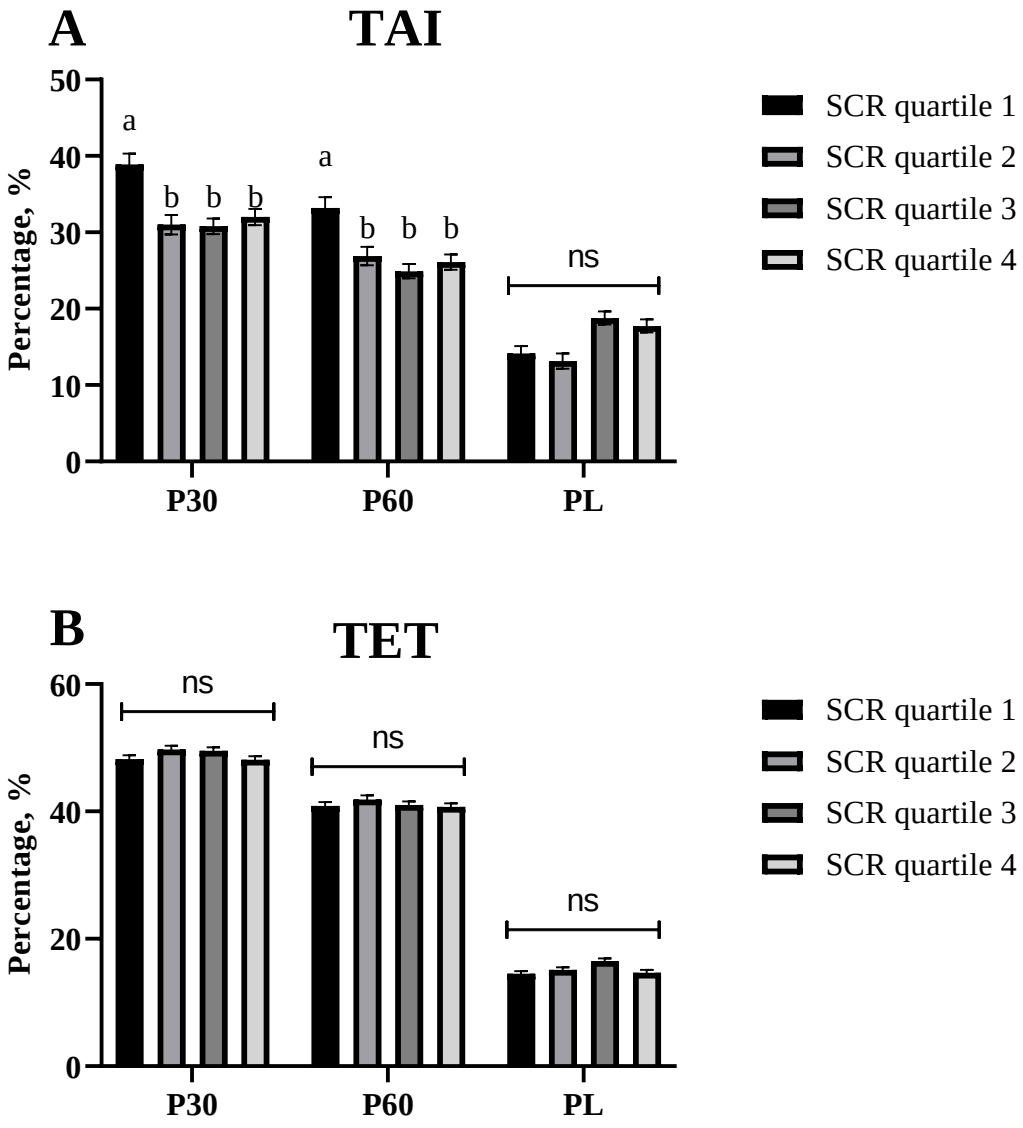

Figure 3-3 Reproductive results by sire SCR quartiles.

Pregnancy rate at day 30 (P30) and day 60 (P60) and incidence of pregnancy loss (PL) in Holstein or Holstein crossbred dairy cows/heifers mated with sires ranked into quartiles based on published SCR (where quartile 1 represents highest SCR and quartile 4 the lowest). **A** - timed artificial insemination (TAI); **B** - timed embryo transfer (TET). Data are presented as mean ± SEM. Bars with different superscripts differ ($P < 0.05$). NS = non-significant.

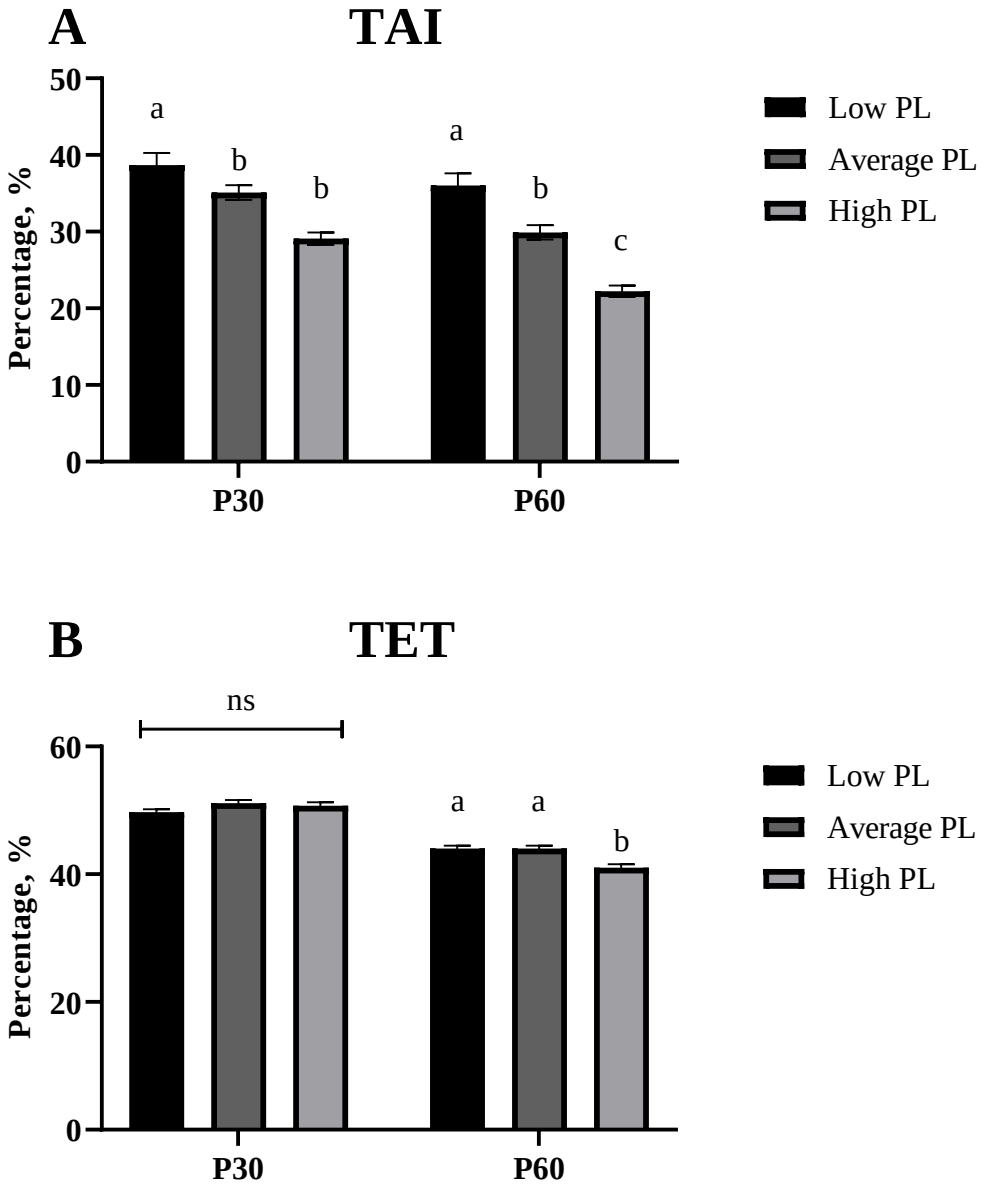

Figure 3-4 Pregnancy results by sire pregnancy loss classification.
Holstein or Holstein crossbred dairy cows/heifers pregnancy rate at day 30 (P30) and day 60 (P60) mated with sires classified based on incidence of pregnancy loss (PL) between P30 and P60 into high PL (greater than 20%), average PL (between 10 and 20%) or low PL (less than 10%). **A** - timed artificial insemination (TAI); **B** - timed embryo transfer (TET). Data are presented as mean ± SEM. Bars with different superscripts differ ($P < 0.05$). NS = non-significant.

4. EMBRYONIC DEVELOPMENT OF PARTHENOGENETIC VS BIPARENTAL BOVINE EMBRYOS: FROM BLASTOCYST TO POST-ELONGATION STAGES

4.1. Introduction

The use of uniparental embryos, parthenogenetic and androgenetic, to investigate parental genomic imprinting and its role in conceptus development have been used by several research groups. A series of classical studies in 1980s using mouse models have shown that, even though the presence of both male and female pronucleus is necessary for proper embryonic development to term, each parental genome plays a different role in conceptus formation. The formation of the embryo proper is primarily reliant on the maternal genome, while the paternal genome greatly contributes to trophoblast development and, therefore, the placenta [1-5]. Parthenogenesis, specifically, is the development of an embryo without paternal genome contribution [6]. It is a common phenomenon in the animal kingdom and the typical reproductive strategy of many insects such as flies, ants, honeybees as well as small vertebrates such as lizards, snakes, fish and amphibians where a female gives birth to offspring without a paternal contribution [7, 8]. The Komodo dragons (*Varanus komodoensis*) switch between asexual and sexual reproduction, depending on the availability of a mate [9]. In mammals parthenogenesis is not a natural reproduction form, but can be replicated through chemical or electric oocyte activation [10]. Using these activation protocols, parthenotes can develop to the blastocyst stage at reasonable rates in mice [1-5, 11], pigs [12], sheep [13], rabbit [14] and monkeys [15], however limited information exist on in vivo development post implantation stage mainly because parthenogenetic embryos are inherently limited in their developmental capacity [16].

In bovine, previous studies have shown the ability of parthenogenic embryos to establish and maintain pregnancy up to 43 days after transfer [17], but further molecular characterization of conceptus and/or implantation mechanisms were not described [17-19]. Bovine reproductive biology has often focused on the female's role in reproductive processes, and much less attention has been given to male derived factors associated with fertility or causes of embryonic mortality originating from the sire post fertilization and initial embryonic development [20, 21]. The increased use of assisted reproductive technologies and therapy, in both humans and livestock have boosted the interest in understanding the paternal effect on embryo development. These techniques increase the possibility of developing an embryo from sperm that carries undesirable genetic defects, which would normally not be possible under normal or natural breeding conditions and methods [22].

There is no doubt that paternal genome is required for proper embryonic development in mammals, but the advancement of genomic and epigenomic techniques are expanding our traditional view of sperm biology beyond oocyte fertilization in the oviduct [23]. Using mules as a mammal hybrid models, Wang and collaborators identified 15 imprinted genes in trophoblast tissue, in which 10 were paternally expressed and demonstrated the plasticity of imprinted genes in the placenta. Moreover, a recent study demonstrated parental bias expression of genes in equine placenta with over 200 potentially imprinted genes [24]. Maternally expressed genes were correlated with decreased gestation length, while paternally expressed genes were linked to increased gestation length. This reciprocal interaction between maternal and paternal gene expression directs the "tug of war" dispute for resource allocation between fetus and dam [25]. In

the human placenta, imprinted genes expression is critical for proper embryonic development through modulation of placental function [26-28].

Previous research from our lab have shown a significant paternal contribution to pregnancy loss during active the period of active placentation in cattle [29, 30], suggesting that male genetics might play a significant role in trophoblast/placenta formation and pregnancy maintenance. Investigation of paternal genome effect on conceptus development and placentation is essential to understand the mechanism that leads to pregnancy failure in cattle. Our objective was to characterize bovine embryonic development and placentation in the absence of paternal genome. Our major hypothesis is that the absence of paternal genes will impair trophoblast development and placentation in cattle and be a useful model to understand embryonic mortality during this period.

4.2. Materials and Methods

4.2.1. In vitro embryo production

Parthenogenetic embryos were produced in vitro by chemical activation using a protocol previously described by Timlin [31]. Briefly, oocytes collected in abattoir were matured for 24 h and denuded with hyaluronidase. Oocytes with a visible polar body and dark cytoplasm were selected and incubated with 5 µM Ionomycin for 5 min followed by 3-hour incubation with 6-DMAP. Activated oocytes were incubated in culture medium until reaching blastocyst stage. Three blastocyst stage parthenogenetic embryos were packed into each straw and shipped overnight for embryo transfer. Control embryos were produced in vitro following industry standard protocols

where IVM oocytes were fertilized to a bull with known fertility. A single frozen-thawed blastocyst stage embryo was transferred into recipients to serve as control.

4.2.2. Blastocyst-stage embryo analysis

A subset of parthenogenetic and IVP control embryos were produced to investigate gene expression profiles at the blastocyst stage. Briefly, day 8 blastocyst embryos were either flash frozen for real-time PCR analysis or fixed with 4% PAF for immunolocalization. Pools of 5 embryos or parthenotes (3 replicates) were flash frozen and stored until RNA isolation with PicoPure™ RNA Isolation Kit (Thermo Fisher Scientific, MA, USA). Relative gene expression of OCT4, NANOG, GATA6, SOX2, IFNT2, KRT8, GATA2, TEAD4, FGF4, DNMT3A was performed using real-time PCR using GADPH as a housekeeping gene (Supplemental Table 1). Paraffin-embedded blastocyst embryos (17 PA and 15 CON) were permeabilized, blocked and incubated with primary antibody overnight followed by 1-hour incubation with secondary antibody and, ultimately, nuclear stained for immunolocalization of the proteins GATA6, NANOG, CDX2.

4.2.3. Post-elongation stage embryo analysis

All animal procedures were approved and conducted in accordance with Texas A&M University Institutional Animal Care and Use Committee guidelines. Multiparous beef cows were submitted to a double Prostaglandin $F_{2\alpha}$ (Lutalyse High Con, Zoetis) estrus synchronization protocol and estrus expression was evaluated using Estrotect breeding indicator patches on day 0. Cows with fully activated patch on day 0 and active corpus luteum on day 7 were randomly assigned to received either parthenogenetic embryos (PA) or a single control embryo (CON) of good or excellent quality (according to IETS guidelines). Based on preliminary pilot studies, we determined that transfer of 3 parthenogenetic blastocyst per recipient yield best pregnancy

outcomes (data not published). Coccygeal vein blood samples were collected on day 7 and daily from days 15 to 36. Serum and buffy coat were collected and stored at -80º until further analysis. Pregnancy status was evaluated daily (every other day) by transrectal ultrasonography starting on day 26. Reproductive tracts from PA pregnant cows (n = 4) were harvested on day 31 of pregnancy and several 1 to1.5 square cm sections of uterine wall from the middle of each horn were fixed in 4% paraformaldehyde and paraffin-embedded for immunohistochemistry (IHC) analysis.

4.2.4. Progesterone and PAG Quantification

Concentrations of PAG were quantified using an in-house ELISA established by Green et al [32] using antibodies produced against early secreted PAGs as validated by Reese et al. [33]. Each assay was run with a standard curve, positive controls from a pool of second-trimester pregnant cow serum, and negative pooled steer serum controls. The interassay and intraassay CVs were less than 10%. Progesterone concentrations were quantified using a commercial RIA kit (MP Biomedicals) previously validated in our laboratory in a single assay with high and low progesterone controls and standard curves at the beginning and end. The intraassay CV was less than 10%.

4.2.5. Circulating blood leukocyte gene expression

Leukocyte RNA was extracted using Trizol (Thermo Fisher Scientific, MA, USA) associated with the DirectZol-RNA kit (Zymo Research, CA, USA). Total leukocyte RNA was transcribed into cDNA by using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, CA, USA). Absolute quantification of ISGs (*ISG15, Mx2, OAS1 and IF16)* was performed using QX100™ Droplet Digital™ PCR System (Bio-rad Laboratories, IL, USA)

according to manufacturer's recommendations using EvaGreen ddPCR Supermix (Bio-rad Laboratories, IL, USA) and previously validated primers.

4.2.6. Immunohistochemistry (IHC)

Immunoreactive PAG and E-cadherin proteins were co-localized in paraffin-embedded samples from day 31 pregnant cow uterine tissue using dual immunofluorescence staining microscopy. Antigen retrieval was performed using either boiling citrate or protease. Sections were then blocked in 10% normal goat serum for 1 h at room temperature. These sections were incubated overnight at 4°C with rabbit anti-PAG polyclonal antibody (kindly provided by Jonathan A. Green, University of Missouri-Columbia, Columbia, MO; 1:100) and mouse anti-E-cadherin monoclonal antibody (BD Biosciences; San Jose, CA, USA; 610182; 1:200) simultaneously. Each antibody was used at a validated optimal dilution. Immunoreactive proteins were detected using the appropriate Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies (Life Technologies, Grand Island, NY, USA) for 1 h at room temperature at a dilution of 1:250. Tissue sections were then washed three times for 5 min/wash in PBS. Slides were counterstained with Prolong Gold Antifade reagent containing DAPI (Life Technologies, Carlsbad, CA, USA) and coverslipped. Images were taken using an Axioplan 2 microscope (Carl Zeiss, Thornwood, NY, USA) interfaced with an Axioplan HR digital camera.

4.2.7. Statistical analysis

Data that were not normally distributed according to the Shapiro-Wilk test were log transformed. Concentrations of PAG, P4, and ISGs were analyzed using ANOVA using effects of group, day, and their interaction. The SAS PROC MIXED procedure (SAS version 9.2) was used with a REPEATED statement to account for the autocorrelation between sequential measurements

and with cow-within-group as a random variable. The least significant difference test was used when comparisons between days were made within a group. Results are presented as mean ± 95% CI. Significance was set at $P < 0.05$.

4.3. Results

4.3.1. Blastocyst-stage embryo results

A total of 320 CON and 317 PA embryos were produced in vitro. Overall, chemical activation of oocytes had similar developmental efficiency as in vitro fertilized control embryos. There was no difference ($P > 0.05$) in cleavage rate (i.e. embryos with at least one cellular division), blastocyst rate or blastocyst/cleaved ratio (**Table 1)** between CON and PA embryos. Control embryos had significantly ($P < 0.05$) greater number of trophectoderm cells and lesser ($P < 0.05$) ICM cells compared to PA embryos. Subsequently, TE:ICM ratio was greater ($P < 0.05$) for CON embryos compared to PA embryos.

Blastocyst PA embryos tended ($P = 0.07$) to have less CDX2 gene expression compared to CON embryos **(Figure 1)**, but no differences were observed for the remaining genes KRT8 ($P = 0.21$), GATA2 ($P = 0.21$), TEAD4 ($P = 0.23$), FGF4 ($P = 0.36$), NANOG ($P = 0.36$), OCT4 ($P = 0.51$), SOX2 ($P = 0.95$), GATA6 ($P = 0.32$), DNMT3A ($P = 0.62$) or IFNT2 ($P = 0.52$). Because it was the only gene with differential expression, embryos were immune stained with CDX2 protein. PA embryos had lesser CDX2 staining compared to CON embryos (**Figure 2**).

4.3.2. Post-elongation stage embryo results

4.3.2.1. Pregnancy status

Out of the 36 cows that received parthenogenetic embryos, 12 had a visible embryo-like structure on day 26 that were maintained to between day 40 and 50 of gestation (**Figure 3**). Pregnancy rate in cows receiving control embryos was 55.55% (5 of 9). No embryonic heartbeat was ever detected and the size of the pregnant uterus, as well as amount of fluid, appeared to be smaller than in a normal pregnancy of same developmental day. The corpus luteum blood flow remained active while signs of pregnancy were visible on ultrasound. Moreover, no difference ($P > 0.05$) in circulating progesterone was observed in cows pregnant with PA or CON embryos on any day measured (**Figure 4**).

4.3.2.2. Conceptus tissue collection

Reproductive tracts of pregnant PA cows harvested on day 31 of gestation had similar appearance and tone to a normal pregnant uterus of same developmental period. A corpus luteum was present on same side of pregnancy in all cows. Upon dissection of uteri, large amounts of trophectoderm **(Tr, Figure 5)** were observed extending into both uterine horns. All three embryos were recovered in each animal **(E, Figure 5)**. Embryo size was variable, but all were visually smaller compared to a control embryo of same day. Conceptus tissue was "free floating" in uterine lumen, and no site of implantation and/or embryo attachment was observed.

4.3.2.3. Immunohistochemistry

Because the embryo was free floating within the uterus, interaction of trophoblast cells with the uterine luminal epithelial was not obtained. Upon staining of conceptus, both mononuclear (E-cad; red) and multinucleate (PAG; green) trophoblast cells were present (**Figure 6**). PAG positive cells were present throughout the entire trophectoderm tissue.

4.3.2.4. Circulating conceptus-secreted products

Expression profile of interferon-stimulated genes OAS1, Mx2, ISG15 and IF16 in peripheral blood leukocytes in cows pregnant with PA embryos (Pregnant PA) were similar to non-pregnant cows (Open CON) and decreased when compared to pregnant control animals (Pregnant CON) on day 20 of gestation (**Figure 7**). Cows pregnant with PA embryos had decreased ($P < 0.001$) serum concentration of pregnancy-associated glycoprotein (PAG) on days 26 to 36 of gestation compared to CON pregnant cows (**Figure 8**).

4.4. Discussion

Despite the significant impact of reproductive failure on cattle production, our understanding of embryonic development in domestic species remain suboptimal, especially regarding placental development and function. Embryonic mortality during the second month of gestation affects 6% of beef cows [34] and 12% of dairy cows [35], resulting in significant economic consequences for the industry. This loss is even higher in embryo transfer pregnancies (10%) compared to artificial insemination (5%) pregnancies [34]. The use of assisted reproductive technologies in all species have increased the chance of successfully producing an embryo using semen that would not normally fertilize under natural conditions, which can increase the transmission of undesirable genetic defects and nonviable pregnancies [22]. In the last decade, there has been an increased interest in understanding the male effect in infertility, especially in rodents and humans. With the advancement of genomic and epigenomic technologies the study of sperm biology has expanded beyond the classical roles of fertilization and early embryonic development. Paternal-specific gene expression in the placenta have indicated significant contribution of male genetics to placenta function and proper embryonic development [23].

To our knowledge, this is the first study to characterize bovine parthenogenetic embryo development post-elongation including conceptus product secretion. The extensive amount of trophoblast tissue upon pregnant uteri dissection was surprising. Our blastocyst-stage parthenogenetic embryos had decreased number of TE cells and increased number of ICM cells compared to normal IVF embryos, as well as decrease CDX2 expression. These findings agree with previous studies in which parthenogenetic blastocyst-stage embryos had decreased number of TE cells [36, 37]. These results could suggest impairment of trophoblast development in parthenogenetic blastocyst embryos, and a mechanism to encourage TE overcompensation in post-elongation stage parthenotes.

Pioneer studies using uniparental embryo were critical to determining parental contribution to the formation of various tissues, especially in mice. A series of studies was done in 1980s using both parthenogenetic (maternal genome only) and androgenetic (paternal genome only) murine embryos. The results indicated that the maternal genome drives embryo proper formation while the paternal genome contributes mostly to extra embryonic membrane development.[1-5, 11, 38-40]. From an evolutionary perspective, genomic imprinting has evolved to resolve conflict of interest between paternal and maternal genes within the offspring. While paternal genes are programmed to obtain as much nourishment as possible for the offspring, the maternal genome regulates nutrient exchange to guarantee its own survival [25]. The placenta, particularly invasive trophoblast lineages, is an important focus for potential conflict between the maternal and paternal genes. It is directly responsible for both nutrient exchange and resource allocation between maternal and fetal blood.

In bovine, one of the first studies using parthenogenetic bovine embryos described successful development of blastocyst stage embryos and pregnancy maintenance up to days 35-48 following embryo transfer [18]. Pregnancy status in this study, however, was not visualized rather inferred by lack of estrus cyclicity and no histological characterization of the conceptus was presented. Additionally, Susko-Parrish and others demonstrated that parthenogenetic bovine embryos could initiate limited early pregnancy responses when placed in uteri, characterized by extended estrus cycle [19]. In 1999, birth of reconstructed chimera calves using parthenogenetic and in vitro fertilized embryos of different breeds were reported in both Japan and USA [17, 41]. The calves' chimeric phenotypic was determined by coat color pattern, with black and white from the Holstein breed IVF derived embryo and red pattern spots from parthenogenetic Red Angus embryo. Moreover, it was reported the presence of XX and XY chromosome plates in the same embryo sample, while some samples had only XX or only XY, but no further analysis of distribution of these different cell types were performed in these studies [17, 41].

Lagutina and others (2004) compared developmental capacity of both parthenogenetic and androgenetic bovine embryos using different activation methods [42]. Almost 40% of haploid parthenogenetic progressed through morula compaction and 15% reached blastocyst stage. Diploid parthenotes had a highly efficient development rate, with blastocyst rates similar to control biparental IVF embryos (84-94%). Due to this increased efficiency, our activation protocol aimed to develop diploid embryos with oocyte activation after polar body extrusion. In this study, parthenogenetic embryos had similar embryonic development rates compared to IVF embryos, agreeing with previous studies in mice and bovine, where diploid parthenogenetic embryos develop to blastocysts very efficiently [42, 43].

Upon tissue dissection, we did not observe any site of embryo attachment to the endometrium as would be expected in a normal day 31 pregnancy. Therefore, it may be possible that a potential mechanism contributing to proliferation of TE may be induced by the lack of attachment to increase nutrient exchange rate for the developing embryo. Previous studies have shown down regulation of genes related to placenta development (*IFNT2, ALOX15* and *PLAC8) in buffalo parthenogenetic blastocyst embryos compared to IVP [44] and in mice (Slc38a4, HIF2α, Gab1 and Plac9) [45]*.

Despite the presence of PAG positive cells in day 31 multinucleated trophoblast tissue, we did not detect a significant amount of PAGs in maternal circulation on any day of the study. Since their discovery in 1980's, PAGs have been a target for pregnancy diagnosis with the first detectable increase in general circulation between days 22 to 24 of gestation and 99% accuracy to detect pregnancy by day 31 of gestation [32, 46-48]. Several studies have shown a strong correlation between pregnancy success and concentration of PAGs during early gestation, in which females with low circulating PAG concentration have increase chance of undergoing embryonic mortality [48-52]. The absence of circulating PAGs in cows pregnant with parthenogenetic embryos could be explained by either a lack of embryo attachment to the endometrium preventing the trophoblast cell secretion to reach maternal circulation or lack of altered glycosylation of these proteins preventing the immunoassay to detect these glycoproteins in the maternal serum.

The maintenance of corpus luteum activity despite the lack of ISG expression in peripheral blood leukocytes provides additional questions to previously held dogma of pregnancy establishment. Even though deeper investigation for this finding is beyond the scope of this paper, we could speculate that either the conceptus is secreting enough interferon that can induce a local

response to prevent luteolysis or a secondary mechanism for maternal recognition of pregnancy and luteal protection is taking place in these pregnancies. Previous studies have shown that secretion of IFN-τ in parthenogenetic blastocyst and blastocyst outgrowth embryos were similar in quantity and isoform variability to IVF-derived blastocysts [53], but, to our knowledge, no studies have characterized IFNT-τ secretion in elongated parthenogenetic embryos transferred in vivo.

4.5. Conclusion

In summary, lack of paternal genetics reduced TE cell formation at blastocyst stage and prevented post-elongated embryo attachment to endometrium. Further molecular characterization of these tissues will be necessary to better understand how paternal genetics contribute to embryonic attachment to endometrium and placenta development. Investigating genes that are linked to these events can lead to development of male fertility markers. Moreover, use of uniparental embryos can be a useful model to understand placentation and pregnancy development in cattle.

4.6. References

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Table 4-1. Embryo development and cell counts

					Cell Number			
Group[1]	**n**	**Cleavage (%)[2]**	**Blastocyst (%)**	**Blastocyst / Cleaved (%)**	**TE[3]**	**ICM[4]**	**Total**	**Ratio**
IVP	320	86.9 ± 5.7	34.9 ± 6.7	40.4 ± 5.9	54.8 ± 3.5[a]	31.3 ± 2.5[a]	86.2 ± 5.8	1.75 ± 0.05[a]
Parthenotes	317	79.2 ± 5.7	35.5 ± 6.7	44.4 ± 5.9	44.9 ± 3.7[b]	42.2 ± 2.6[b]	87.1 ± 6.1	1.09 ± 0.05[b]

[1]Values are presented as least square means ± standard error

[2]Embryos that underwent at least one cellular division

[3]Trophectoderm cells

[4]Inner cell mass (equal to total nuclei minus trophectoderm)

[a, b]Numbers with different superscripts differ ($P < 0.05$)

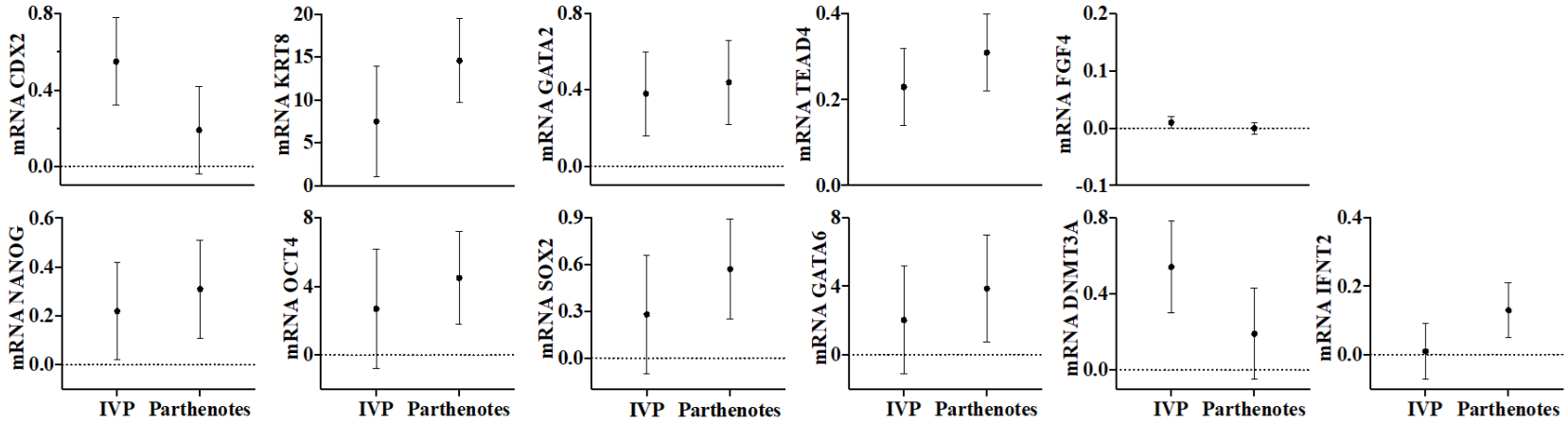

Figure 4-1Relative expression of genes involved in embryonic development
Markers for pluripotency (OCT4, NANOG), hypoblast (GATA6), epiblast (SOX2), trophectoderm (CDX2), and maternal-embryo communication (IFNT2).

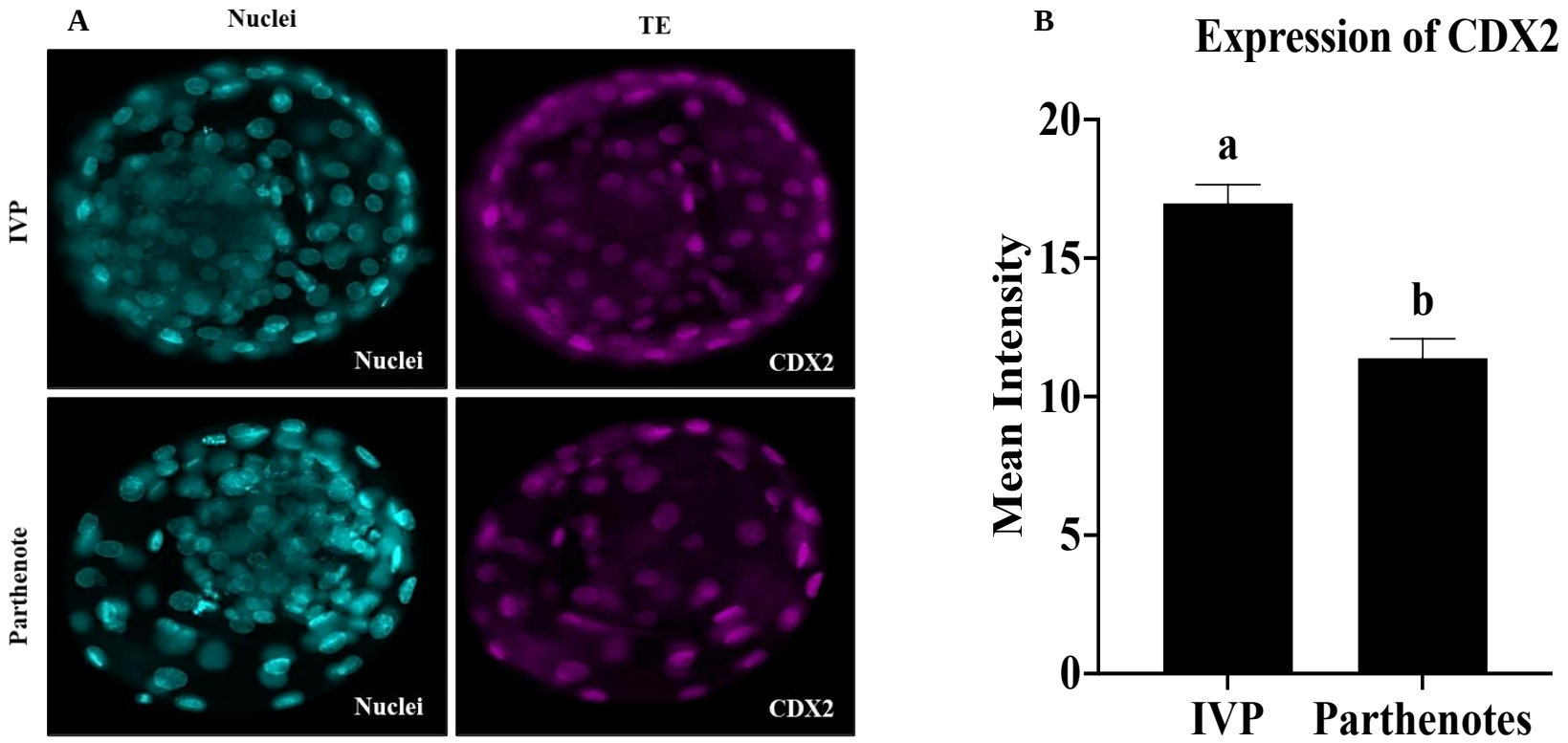

Figure 4-2 - Immunolocalization of CDX2 in parthenotes and control embryos.

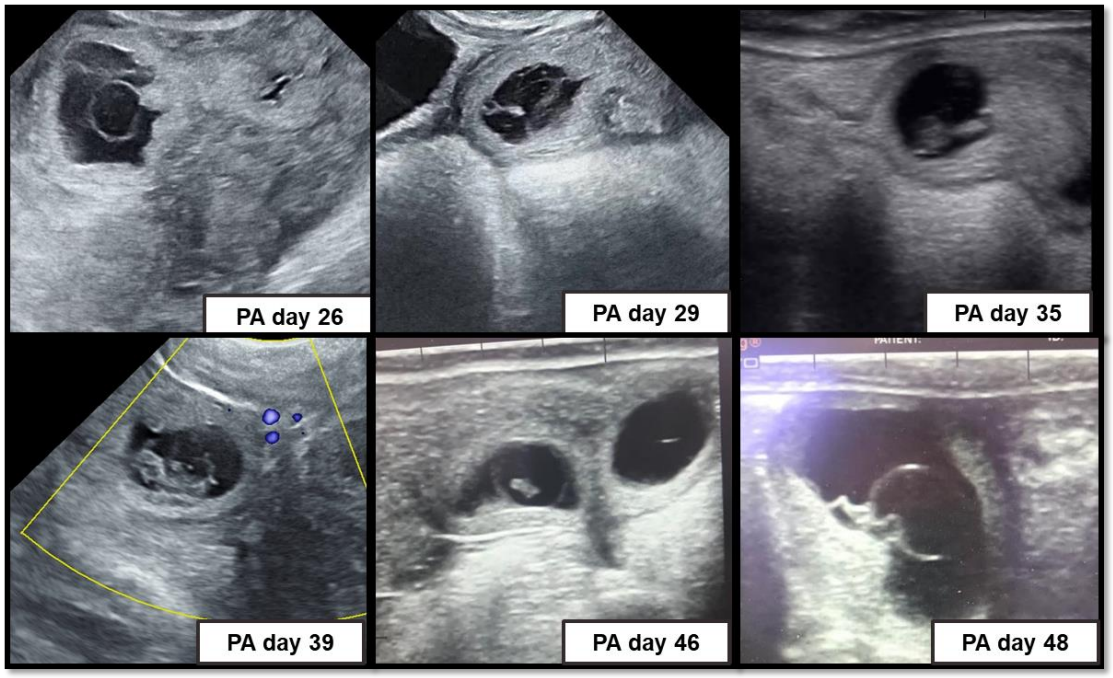

Figure 4-3 - Progressive transrectal ultrasonography images of recipient cows carrying a parthenogenetic embryo.

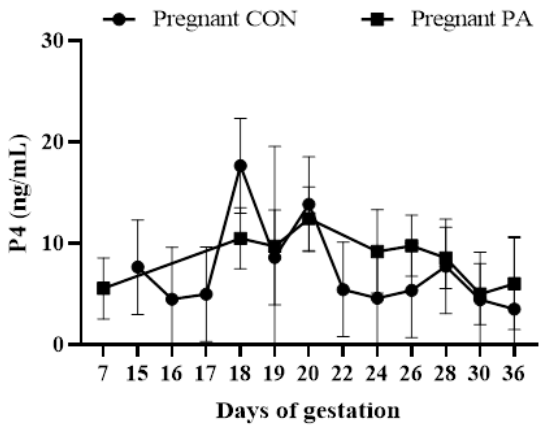

Figure 4-4 Serum concentration of progesterone (P4)
Cows pregnant with parthenogenetic embryos (Pregnant PA) compared to cows pregnant with control embryos (Pregnant CON). Results are presented as mean ± 95% CI.

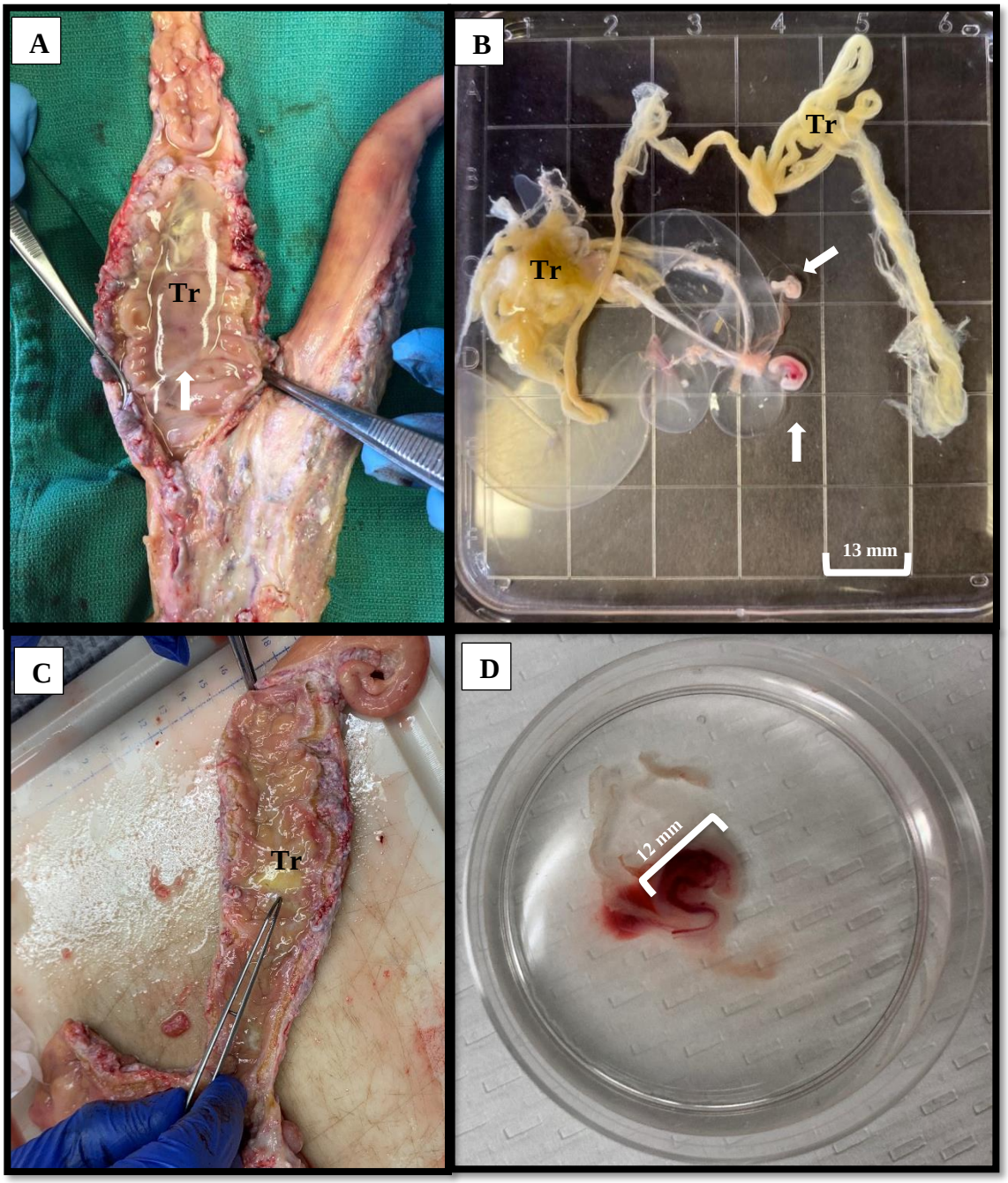

Figure 4-5 Morphological comparison of day 31 parthenogenetic and control pregnancy.

A - Pregnant uterus carrying a parthenogenetic embryo; B – isolated parthenogenetic embryo; C: pregnant uterus carrying a control embryo; D – isolated control embryo. Tr = Trophectoderm, White arrow identify the embryo.

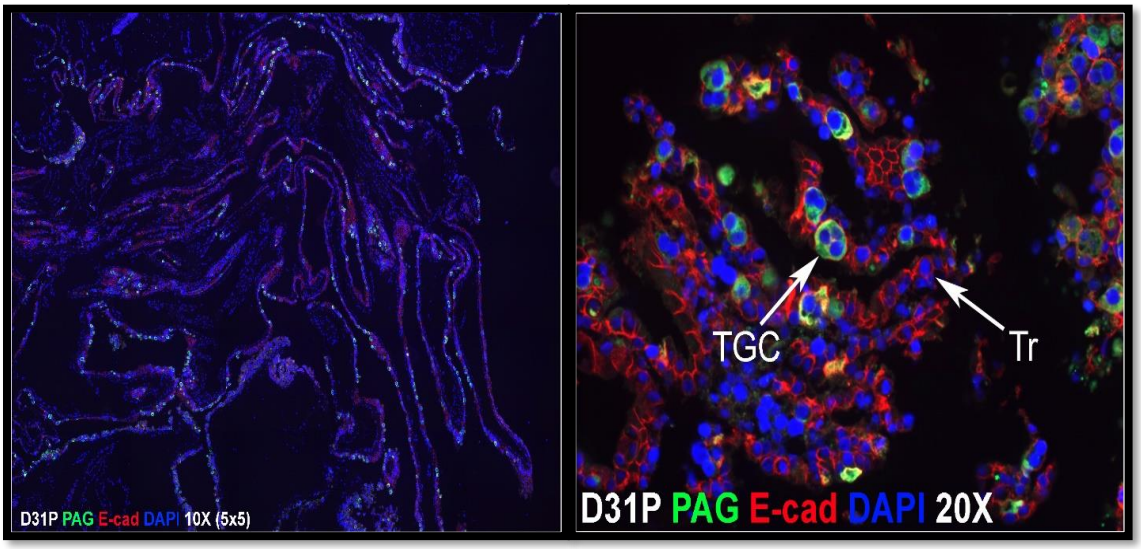

Figure 4-6. Immunohistochemistry of PA trophoblast tissue.
PAG positive cells (green, stains binucleate trophoblast cells) and E-cadherin (E-cad; red, stains mononuclear trophoblast and LE)

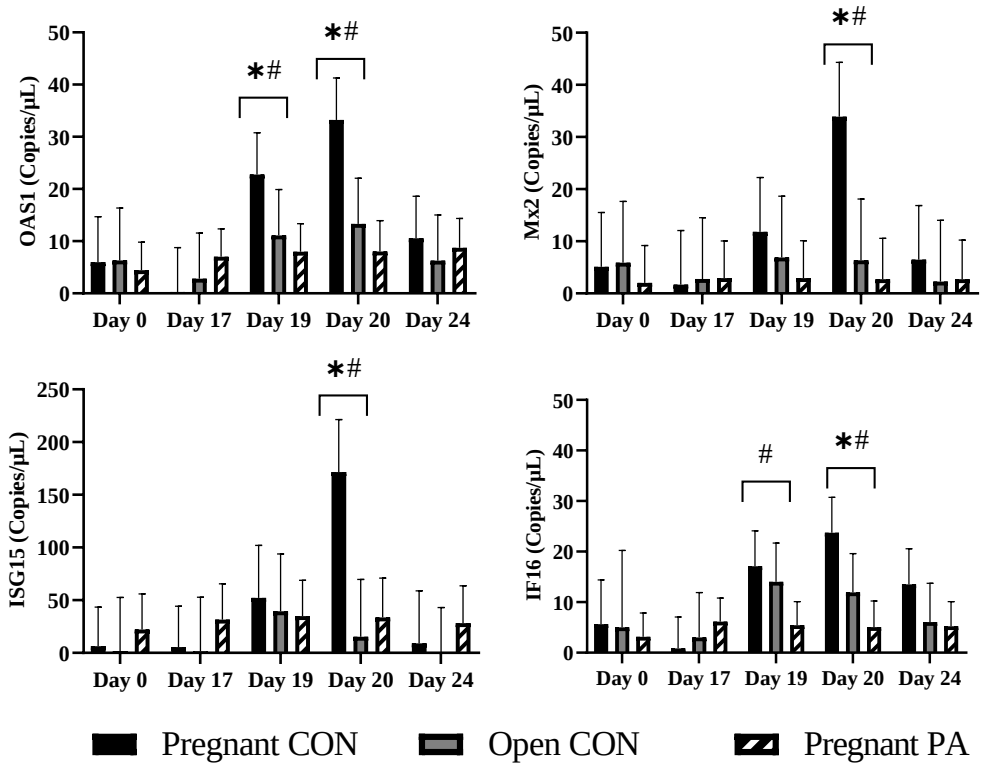

Figure 4-7 Absolute quantification of interferon-stimulated genes.
Expression of OAS1, Mx2, ISG15 and IF16 in peripheral blood leukocytes of cows pregnant with control embryos (Pregnant CON) compared to cows pregnant with parthenogenetic embryos (Pregnant PA) and to control non pregnant cows (Open CON). *P < 0.05 (Pregnant CON vs Open CON); #P < 0.05 (Pregnant CON vs Pregnant PA).

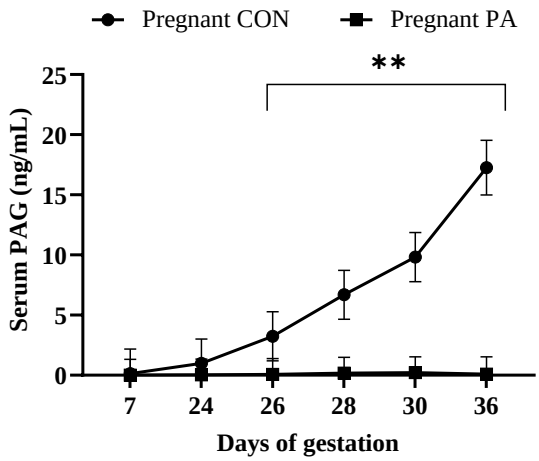

Figure 4-8- Serum concentration of pregnancy associated glycoprotein (PAG) Cows pregnant with parthenogenetic embryos (Pregnant PA) compared to cows pregnant with control embryos (Pregnant CON). Results are presented as mean ± 95% CI. *P < 0.05

5. CONCLUSIONS

Embryonic mortality in cattle will remain a significant problem until the mechanism that regulates conceptus formation are better understood and regulated. The first couple projects of this dissertation characterized the sire contribution to embryonic mortality in cattle, demonstrating that there is a male contribution to pregnancy loss during different periods of gestation. These results strongly suggest that we need to revisit our current methods to evaluate sire fertility to obtain better prediction of male genetics contribution to pregnancy success. The second part of this dissertation started to dissect the possible mechanism that paternal genetics regulates the conceptus formation and pregnancy maintenance in cattle. Uniparental embryos are a very powerful tool to understand genomic imprinting and parental dynamics into conceptus formation and is extensively used in rodents and other species. In cattle, however, most of the relevant studies with uniparental embryos were performed decades ago, without the advanced technologies that we have current. The results presented herein are a good initial characterization of paternal contribution to embryonic elongation and initial placentation. Next steps of this projects involve complete gene expression characterization to further elucidate the mechanism that regulates placenta formation and possibly develop fertility markers for pregnancy success.