Interleukin-6 Supplementation Improves Post-Transfer Embryonic and Early Fetal Development of in vitro Produced Bovine Embryos.

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Abstract

In vitro produced (IVP) bovine embryos are useful for investigating the mechanisms affecting early embryonic failure. The work in this thesis explored how interleukin-6 (IL6), an embryokine that increases inner cell mass (ICM) influences post-transfer embryonic survival and development of the embryo-proper and fetus. Four replicates of slaughterhouse-derived cumulus oocyte complexes underwent in vitro maturation and fertilization. On day 5 post-fertilization, embryos were treated with either 1% Bovine Serum Albumin (BSA) (CONT) or 100ng/mL recombinant bovine IL6 with 1% BSA (TRT). On day 7.5 post-fertilization, individual blastocysts were loaded into transfer straws. Beef and dairy cow recipients were synchronized with the day of in vitro fertilization using a 7-d CO-Synch protocol. A subset of cows from each group underwent fixed-time artificial insemination (AI) (n=37). The remaining cows underwent embryo transfer (ET) in the uterine horn ipsilateral to a corpus luteum (CL) (IL6 n=35; CONT n=51). Embryo and fetal measurements were performed via transrectal ultrasonography weekly from days 28-56 post-insemination, respectively. Overall pregnancy rates were 40.0% IL6; 19.6% CONT; and 32.4% AI. Crown-rump lengths (CRL) were reduced ($P<0.05$) in CONT pregnancies when compared with IL6 and AI at days 28, 35, 42, and 56. A tendency ($P=0.057$) for larger abdominal diameters was detected between IL6 and CONT groups. Also, IL6 had larger crown-nose lengths than CONT ($P<0.05$) and tended to be larger than AI ($P=0.07$). In summary, IL6 treatment produced pregnancies resembling AI-generated pregnancies more so than conventionally cultured embryos, supporting the hypothesis that IL6 improves developmental competency of IVP embryos.

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General Audience Abstract

The incidences of pregnancy loss in both beef and dairy cattle industries are profound and are upwards of 60 percent. Financial stability for both of these industries revolves closely around the ability of cattle to give birth to a live calf annually. While artificial insemination (AI) has been heavily adopted and utilized widely in the dairy industry, the use of in vitro produced (IVP) bovine embryos has shown promise in lessening some of the stresses placed on impregnating cattle. The IVP of bovine embryos serves as a strong model to understand how pregnancy losses occur. Briefly, IVP involves the collection of eggs from donor animals, and subsequent fertilization to mimic what occurs within the animal naturally. A disadvantage of in vitro produced embryos is their reduced likelihood to establish pregnancy after transfer into recipient animals. Interleukin-6 (IL6) was recently identified as a pro-developmental factor that may improve the quality and post-transfer competency of in vitro produced embryos. The objective of this work was to determine if IL6 supplementation during in vitro culture improves post-transfer fetal development. Oocytes (i.e. eggs) were retrieved from slaughterhouse-derived ovaries and subjected to in vitro maturation and fertilization. On day 5 post-fertilization, embryos were treated with either 0 (CONT; 1% BSA) or 100ng/mL recombinant bovine IL6. On day 7.5 post-fertilization, individual embryos (blastocyst stage) were loaded into transfer straws. Estrous synchronized beef (n =) and dairy (n =) cow recipients were allocated into treatment groups in the following manner. A subset of cows from each group underwent fixed-time AI (n=37). Remaining cows underwent embryo transfer (ET) in the uterine horn ipsilateral to a corpus luteum; 51 of these cows received a CONT embryo and the remaining 35 cows received an IL6 embryo. Thus, there were three treatment groups: AI, CONT, and IL6. Embryo and fetal measurements were performed via transrectal ultrasonography weekly from day 28 to 56, these included crown-rump length, crown-nose length, abdominal diameter, and amniotic vesicle. Pregnancies that remained throughout the entirety of the experiment 40.0% for IL6 (14/35); 19.6% for CONT (10/51); and 32.4% for AI (12/37). In summary, IL6 treatment of embryos produced pregnancies with characteristics more similar to the current industry standard of AI, rather than conventionally cultured embryos (CONT), supporting the hypothesis that IL6 supplementation to bovine embryos on day 5 post-fertilization improves developmental competency of in vitro produced embryos.

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List of Abbreviations

AD	Abdominal Diameter
AI	Artificial Insemination
ANOVA	Analysis of Variance
ART	Assisted Reproductive Techniques
BHB	Beta-Hydroxy Butyrate
BNC	Binucleate Cell
CAR	Caruncle
CL	Corpus Luteum
CNL	Crown-Nose Length
CRL	Crown-Rump Length
COCs	Cumulus Oocyte Complex
DMI	Dry Matter Intake
DPR	Daughter Pregnancy Rate
E2	Estradiol
EGA	Embryonic Genome Activation
ET	Embryo Transfer
FSH	Follicle-Stimulating Hormone
GH	Growth Hormone
GnRH	Gonadotropin-Releasing Hormone
GP130	Glycoprotein 130 kDa
HCR	Heifer Conception Rate
HPG	Hypothalamic-Pituitary-Gonadal
IC	Intercaruncular
ICM	Inner Cell Mass
IFN-α	Interferon-Alpha
IFN-γ	Interferon-Gamma
IFNT	Interferon Tau
IGF1	Insulin-Like Growth Factor-1
IGFBP-2	Insulin-Like Growth Factor Binding Protein-2
IL6	Interleukin-6
ISG	Interferon Stimulated Gene
IVC	In Vitro Culture
IVM	In Vitro Maturation
IVP	In Vitro Produced
JAK	Janus Kinase
LBF	Liver Blood Flow
LE	Luminal Epithelium
LH	Luteinizing Hormone
LOS	Large Offspring Syndrome

LPS	Lipopolysaccharides
MCR	Metabolic Clearance Rate
MET	Maternal to Embryonic Transition
MOET	Multiple Ovulation and Embryo Transfer
MRP	Maternal Recognition of Pregnancy
NEB	Negative Energy Balance
NEFA	Nonesterified Fatty Acids
OPU	Ovum Pick Up
P4	Progesterone
PGC	Primordial Germ Cell
PGF2α	Prostaglandin-F2-Alpha
PRID	Progesterone-Releasing Intravaginal Device
PSPB	Pregnancy-Specific Protein B
STAT3	Signal Transducer and Activator of Transcription 3
TE	Trophectoderm
TRUS	Transrectal Ultrasonography
TNF-α	Tumor Necrosis Factor-Alpha
ZP	Zona Pellucida

Chapter 1 – Literature Review

Introduction

The global population is estimated to reach 10 billion by the year 2050. While the need to feed the growing population could potentially be met solely through plant-based proteins, the demand for animal proteins remains. The need for animal protein has tripled in the past 30 years and is expected to double by 2050 [1, 2]. The ability of global agricultural industries to meet these steep demands requires the adaptation, development, and implementation of cutting-edge technologies. The diversity in animal agriculture industries gives rise to a paradoxical challenge in that no two industries are the same and each industry likely will require different technological approaches to meet these steep demands. Even within the cattle industries (dairy and beef) there lies drastic differences in management styles. Despite these differences, however, lies a unifying factor across both cattle industries, which is the ability of each cow to conceive and maintain a pregnancy to term.

In mammals, parturition is required to initiate lactation. The implications of this physiological occurrence are quite clear when examining how consumable products are originated from cattle. For dairy cattle, having one calf per year allows for these cows to stay within their most efficient points of lactation for a greater portion of their productive life. This milk produced is used for human consumption. In the beef sector, milk production has a secondary benefit, wherein nutrients in milk are provided to their offspring, which will eventually be used for meat. The bottom-line profitability for both industries hinges

around the establishment and maintenance of pregnancy, the initiation of lactation, and the achievement of having one healthy calf per cow, each year.

Reproductive performance, and more importantly, the prevalence of reproductive failure has become one of the most costly aspects of management on modern dairy operations [3]. The value of a lost pregnancy in dairy cattle in lost lifetime milk productivity and management-related costs has been estimated at $555 [4]. In beef cattle, the value of pregnancy failure is less clear and is estimated between $700-$1,110 per instance of pregnancy loss [5, 6].

With pregnancy losses having a substantial impact on profitability and sustainability further research on the causes of embryonic mortality is needed so that schemes to circumvent these losses may be devised. The aim of this literature review is to highlight the primary causes of pregnancy failure in cattle. While the primary focus of this review is centered around dairy cattle, the applicable information regarding beef cattle will be highlighted as available. Finally, it is the author's goal to introduce the potential utility of developing and incorporating assisted reproductive tools for dairy and beef industries that may improve overall reproductive efficiency.

Standard Reproductive Practices

Prior to discussing pregnancy loss, it is important to identify both the commonalities and divergences in reproductive management practices between beef and dairy operations. The most commonly discussed standard practices are artificial insemination (AI), estrous synchronization programs, and embryo transfer (ET).

Both industries benefit from using AI. In each industry, AI can improve herd genetics, reduce the risk of spreading venereal disease, and improve farm safety conditions [7]. In

dairy cattle, a result of genetic selection intensity in the past 20 years has caused a 5% improvement in daughter pregnancy rate while also improving milk production [8]. Cow-calf beef operations also benefit from the use of AI because weaned calves are heavier and generate a return of $25 to $40 due to improved genetic merit [9]. The dairy industry has capitalized on these benefits, and approximately 80% of dairy cattle in North America are bred using AI, whereas only 7.6% of beef herds currently use AI [10, 11]. This disparity in utilization of AI is likely due to the predominate difference in the labor required to implement these breeding strategies in each industry. With dairy cattle, the labor associated with detecting estrus or with implementing synchronization protocols is not as pronounced because dairy cattle are handled daily. Beef cattle, however, typically reside in pastures or rangeland and are handled less frequently. The application of estrus detection or an estrous synchronization program in the beef industry requires additional labor that would not normally be associated with simply using a bull for breeding.

These production schemes also fit well with the goals of each operation. In the U.S., most dairy farms use year-round calving; this roughly equates to 1/12th of the total mature herd calving every month. This strategy helps ensure near constant year-round milk production on a given dairy farm. Further, this strategy intends for each cow to calve a single time in a calendar year. Like the dairy industry, a goal of U.S. beef cow-calf operations is also for each cow to calve a single time in a calendar year. However, unlike the dairy industry, the beef industry typically uses seasonal breeding Discrete calving windows allow for calf crops to be born at a set time of the year. The implication of a narrow window of pregnancy success means that producers frequently use natural service to ensure pregnancy.

Estrous synchronization protocols are employed under both management schemes to maximize the effectiveness of reproductive strategies, although the beef and dairy industries use different approaches. The aims of various ‘synch’ practices are to regulate the stage of the estrous cycle and induce cyclicity in anestrous females [8]. Differences between beef and dairy estrous synchronization protocols center on reduced the number of handling events in beef protocols compared to dairy protocols. Dairy producers rely on Ovsynch protocols, where injections of gonadotropin-releasing hormone (GnRH) and prostaglandin-F2-alpha (PGF2α) synchronize the timing of ovulation [12]. The strategy is that GnRH induces a surge of luteinizing hormone (LH) and cause ovulation of any large, dominant follicles so that a new follicular wave begins. The newly formed dominant follicle is then induced to ovulate first by administering PGF2α to cause luteolysis of corpus luteum (CL), and then GnRH injection to time ovulation [13]. Beef producers also use these injectable hormones in a similar sequence, although a CO-synch protocol is often employed, where the second GnRH dose “coincides” if administered at the time of AI [14, 15].

Heat detection is crucial for improving reproductive success on dairy and beef operations [16, 17]. The number of dairy farms that milk over 500 cows becomes continues to grow, and the reliance on traditional visual heat detection is lessened and the use of timed AI is increased. It is estimated that solely using estrus detection for timing of insemination will identify less than 50% of cows that actually ovulate in most dairy herds [18]. Likewise, heat detection aids are being employed and can be attributed to the stabilization of fertility trends [19]. All estrous synchronization protocols involve the manipulation of endocrine and physiologic events to improve follicle synchronization and

growth, the timing of ovulation, and CL formation [14]. The use of estrous synchronization positively benefits producers through improved heat detection. Superior heat detection increases the likelihood of pregnancy success by allowing for optimal gamete interaction. Secondly, with improved heat detection and synchrony, producers can manage the variation in the number of days to first service and also decrease the total number of AI events needed to achieve pregnancy With synch programs, the ability to detect estrus increases from the former 50% to identifying nearly all cattle [20].

While AI is a widely adopted it is limited in proliferation of genetics because cattle are mono-ovulatory animals and typically only have one offspring per year. Despite this, ET allows for multiple calves to be born from one dam per year. Both industries use embryo technologies, and specifically superovulation and ET. The principle behind multiple ovulation and embryo transfer (MOET) is to collect numerous embryos from a donor, followed by transfer of embryos to recipient females of lesser value, increasing the number of offspring a donor can produce in her lifetime [21]. Using super-stimulatory injectable hormones, like follicle stimulating hormone (FSH), allows for more than one follicle to be ovulated and subsequently fertilized. In cattle, embryos can be collected surgically but are more commonly collected through nonsurgical means. According to the American Embryo Transfer Association, beef donors produce slightly more embryos than dairy donors [22]. In 2018, 469,967 MOET beef and dairy embryos were collected globally with 386,133 of these being transferred [23]. This suggest that the size of the global implementation of traditional embryo technologies is large. As MOET and other embryo technologies continue to grow, it becomes easy to justify the need for further research.

Emerging Assisted Reproductive Technologies in Global Cattle Industries

The implementation of in vitro production (IVP) of embryos and other assisted reproductive technologies (ART) has increased globally to account for the growing demand of offspring from genetically elite females [24]. Although MOET has allowed for quick transference of improved genetics, the major limitation is the use of individual bulls per flush. This disadvantage arises because producers can only use one select bull for each embryo flush attempt [21]. With this challenge, newer technologies, such as ovum pick up (OPU) and in vitro fertilization (IVF) are emerging to allow for greater diversity in parental selection. When comparing the number of offspring produced from either MOET or OPU-IVF, the latter has the clear advantage. Under the traditional MOET protocols, it is expected that cattle will average five embryos of transferrable quality, resulting in 50 embryos per donor per year. After freezing and embryo transfer, this would result in the birth of approximately 30 calves. When utilizing OPU-IVF, the total number of offspring produced per donor per year increases. This advantage arises by allowing for two OPU sessions per week throughout the year, potentially yielding 150 embryos per year. These embryos can be expected to result in approximately 70 calves per year [21]. While the post-transfer efficiency of OPU-IVF embryos is lower, the number of offspring potentially derived from this strategy are promising.

There are other prominent advantages of OPU and IVP, and these include the ability to collect oocytes from juvenile animals and animals who are pregnant. Harvesting oocytes from both of these group of animal can improve genetic selection intensity [21]. While there are many benefits to OPU and generated IVP embryos, one of the most notable is their use on farm to achieve pregnancy in problematic cows who fail to ovulate or are incapable of fertilization [24]. However, with the adaptation of new technologies,

there are also inherent disadvantages. The most notable is the cost with OPU-IVF embryos averaging $50 more per embryo than traditional flush embryos [25]. While drug costs are lower, the technical skills, overhead costs associated with equipment, and the current availability of expertise in OPU-IVF raise the price.

It is clear that OPU-IVF schematics currently are lower in efficiency and costs more than MOET derived embryos. Nonetheless, improvements in the OPU and IVP processes and training of OPU and IVF technicians is anticipated to continue to drive the use of this emerging technology, and this should eventually make this technology more cost-effective and available to the dairy and beef industries.

Contributors to Infertility

Milk Production

Intensification of various management practices focused on physiological performance have all taken a toll on reproductive efficiency in dairy cattle [19]. Problems that cause infertility are multifaceted, but one physiological event that is negatively associated with reproductive efficiency is milk production [19, 26, 27]. Current dairy management practices, if not monitored properly, are setting the high producing cow up for reproductive failure. While the initial correlation was made between the relationship of milk production and infertility, it is now understood that problems during the transition period are more the key contributors to fertility problems [19, 28]. The transition period is identified as the time when cows are shifting from the physiological state of being pregnant and non-lactating to when they are no longer pregnant, and lactation begins [29]. The endocrine expectations of cattle are pronounced during the transition period. There is a strong favoritism towards milk production, and this homeorhetic hierarchy

places additional stress on these transition cows. This hierarchy designates milk production as higher priority above body reserve stabilization and reproduction. Moreover, the metabolic associations with the initiation of lactation and the increase of fetal and uterine growth through the last phase of gestation are accompanied by increases in the prevalence of metabolic disorders during this peripartum transition period. Also, the severity of negative energy balance (NEB), instances of displaced abomasa, weight loss, and reduced feed intake all contribute negatively towards the reproductive success of lactating dairy cows [30, 31].

One of the most challenging aspects of achieving pregnancy in lactating dairy cattle stems from the desire to get cattle pregnant during their peak milk production. In most other mammals, pregnancy and lactation are independent physiological stages. In dairy cattle, current management strategies have made them concurrent events. In fact, on most U.S. dairy farms, the time of first AI after calving often coincides with the time of peak lactation. Postpartum physiological stressors are attributed heavily to infertility and anovulation [30]. While the high producing dairy cow is the most susceptible to the effects of modern dairy farming, this group of cows that are able to transition from nonlactating to lactating state without experiencing adverse metabolic or health problems are among the highest producing and most fertile cows in the herd [19, 28]. Rather, the challenge is to maintain fertility in transition cows that have faced adverse health and metabolic events. Genetics are also a contributing factor. The decline in first-service conception rate has been cited changing from 65% in 1951 to 40% in 1996 [32]. This reduction in conception rates likely stems from selecting cows that favor milk production over fertility.

One very important additional aspect of the second half of the transition period and is the prevalence of early lactation NEB. NEB occurs when the homeostatic requirements needed for maintenance and lactation exceed the dietary energy intake [33]. This involves dramatic mobilization and metabolism of body tissue reserves to meet the heightened energy demand for milk production [19]. This phenomenon occurs in a substantial portion of postpartum dairy cows, but the severity and magnitude of NEB is key to fertility status in lactating cows. Notably, NEB occurs immediately before and sometimes at the same time when AI is being attempted for the first-time post-calving. If cows are in NEB at the time of Ai, probability of pregnancy success is very low.

Negative energy balance has a substantial adverse impact on various physiological aspects of the lactating cow, but two of special note are the effects on the hypothalamic-pituitary-gonadal (HPG) axis and on secretion of factors from the liver and metabolites from the digestive system and other organs that control homeostasis. The HPG axis involves positive and negative signaling between the hypothalamus, pituitary gland, and the ovaries responsible for normal cyclicity. Also, the impacts of NEB affect the HPG axis by altering the signaling of hypothalamic GnRH and results in altered levels of FSH and LH [34]. Improper signaling has negative downstream effects, but most notably the altered production of steroid hormones estrogen and progesterone (P4)[30, 35]. The bottom line is that this endocrine disturbance results in reduced conception rates and elevated risks of embryonic mortality [36].

Negative energy balance also affects concentrations of liver derived insulin and IGF1, which limit the ovarian response to circulating LH and FSH, lessening the likelihood of ovulation. Insulin-like growth factor 1 regulates growth hormone (GH) secretion through

negative feedback [37]. During the period of NEB, blood glucose concentrations plummet and, likewise the levels of insulin decrease. Hepatic IGF1 synthesis is decreased because of the reduced passage of insulin [38]. IGF1 is crucial to optimal reproductive performance in the ovary, oviduct, and uterus [39]. The reduced presence of IGF1 negatively affects follicular development during the pre-recruitment phase [40]. Oviductal concentrations of IGF-1 are also altered during NEB. This occurrence reduces the efficacy of the oviduct to release proteins into the oviductal lumen that are associated with sperm survival and embryo competency [39, 41, 42]. Two other key players in the metabolic status of lactating cows are nonesterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB). Both are liver metabolites that arise because of elevated rates of lipolysis attempting to resolve the enormous energy demands. Both NEFAs and BHBs can be detected in follicular fluid that are comparable to those in serum which have severely toxic effects on oocyte maturation and the function of granulosa cells [43, 44].

Another event that compromises fertility in lactating dairy cows relates to the increase in dry matter intake (DMI) that is associated with lactation. Peak DMI follows shortly after peak milk yield in lactation. Increased DMI to meet the demands of greater milk production is correlated with increases in liver blood flow (LBF) [45], and this has a negative effects on steroid hormones in the circulation. This presents a set of problems with steroid clearance through the liver. Specifically, the metabolic clearance rate (MCR) of the steroid hormones P4 and estradiol-17β (E2), both of which are essential for estrous cyclicity and pregnancy, is increased when DMI is high. This increase in MCR can be pronounced. For example, when LBF is increased from 1000 to 2000 L/h, the concentration of circulating P4 will decrease by 50% [46]. This change in LBF is attributed

to increased DMI, and subsequently reduces the likelihood that cattle will be fertile postpartum.

It is important to recognize that these various negative consequences of lactation and metabolic status can increase the risk of peripartum disorders, and this also will impact subsequent fertility [29]. Improper rumen function is cited as being a key contributor of poor transition period health, as it is associated with the prevalence of postpartum metabolic disease [29]. The most notable of these problems for reproduction include abomasal displacement, laminitis, and ketosis. It is also important to mention that metabolic disorders increase the prevalence of systemic inflammation. Dysfermentation and dysbiosis causes metabolic alterations are in the rumen and these can result in the microbial release of cell-free lipopolysaccharides (LPS) from membranes of Gram-negative bacteria. If LPS permeates mammalian cell barrier and is recognized by immune cells, systemic inflammation typically results [29]. Systemic inflammation and ongoing metabolic disturbances at the time of AI both limit the probability of successful pregnancy in dairy cattle.

Immunological Stressors

Uterine infections and mastitis are the two prevalent immunological stressors that can impact fertility in dairy cattle. The effects of infection within the uterine environment of high producing dairy cows on endocrinology, fertility, and ultimately embryonic development is pronounced [47]. Nearly 40% of post-partum dairy cows develop clinical metritis [48]. Uterine infection has a threefold effect against the promotion of a viable pregnancy [47]. First, the presence of LPS within the uterus disturbs the delicate endocrine balance involved in the HPG axis. While not entirely clear, these disturbances

arise from presence of proinflammatory mediators [47]. The primary result decreases GnRH secretion from the hypothalamus and alters production of E2 and PGF2α [49, 50]. The disruption of these hormones impacts the entire animal's physiology and impairs ovarian cyclicity by extending the luteal phase of the estrous cycle [47, 51, 52]. Secondly, uterine infection contributes to ovarian dysregulation, which ultimately affects the quality of the oocyte being ovulated. In addition to the endocrine abnormalities such as reduced circulating estradiol, hindered ovulation, impaired follicular growth and increased length of the luteal phase; ovarian follicles experience changes in cellular and molecular signaling that reduce oocyte quality [47]. Oocyte competence is drastically decreased in the presence of LPS and promotes the rate of germinal vesicle breakdown [53]. Finally, the endometrium becomes less capable of supporting embryonic development and further implantation. The understanding for this impaired function is related to the presence of pro-inflammatory genes within the endometrium that recruit inflammatory mediators, neutrophils, macrophages, and antimicrobial factors to ward off the infection, but ultimately contribute to infertility because of the increased temperature and presence of immune cells [47].

Mastitis and other infections within the mammary gland are extremely prevalent within the population of lactating dairy cows. These localized infections can have negative effects on pregnancy rate and increase the total number of services required to attain pregnancy [54]. While the direct causative links between pregnancy loss and mastitis are difficult to elucidate, cytokines are believed to be the leading agents accounting for the shift of physiological normality [54]. Cytokines such as interferon-alpha (IFN-α), interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and PGF2α induce

hyperthermia, reduce the secretion of LH, decrease circulating P4, promote the presence of endometrial prostaglandins all of which interfere with oocyte maturation and ultimately embryonic development [54]. The implication is that by managing the incidences of mastitis, producers can reduce the likelihood of adverse embryonic mortality and optimize the entire physiology in preparation for pregnancy retention.

Genetics

In addition to stresses imposed by parturition and lactation, changes in genetic selection within the dairy industry have contributed to the increase in prevalence of unfavorable traits associated with infertility [30]. Specific genes or certain quantitative trait loci can affect fertilization capability and impact early, late and total embryonic mortality [55]. The relationship between the growing inbreeding coefficient and increased incidences of recessive genetic defects carrying haplotypes can negatively impact embryonic development and in utero interactions, thus resulting in embryonic mortality. Fanconi anemia (*FANCI),* apoptotic peptidase activating factor 1 (*APAF1),* structural maintenance of chromosomes 2 *(SMC2),* glycinamide ribonucleotide formyltransferase *(GART),* and solute carrier family 37 member 2 (*SLC37A2)* are heterozygous recessive genes that are associated with mutations and increase in prevalence with inbreeding. The ability to monitor for these traits in mating pairs can reduce the likelihood that embryonic mortality will be caused by recessive genetic traits. These haplotype mutations have been identified in most of the dairy breeds presently used in North America [55].

Critical Periods of Pregnancy Failure in Cattle

The largest proportion of pregnancy forfeiture in dairy and beef cattle occurs within the two months post-insemination. Differences amongst breeds and physiological status

affect the proportion of pregnancies will fail. For example, when transferring fresh and frozen embryos to lactating dairy cows, less than 50% of embryos are retained in pregnancy between days 27-30 [56, 57] while in beef cattle, 69 and 83% of embryos resulted in pregnancy by day 37 [58]. Pregnancy losses in this review are divided into four temporal periods including embryonic, conceptus, fetal, and placental development. The next several sections will link causal events to pregnancy loss as a means of explain possible reasons for pregnancy losses occurring at each time.

Early Embryonic Losses: Days 1-7

Embryonic losses within the first week post fertilization is the most prevalent time period of pregnancy failure in cattle. While fertilization rates are normally between 80 and 90%, the first seven days of pregnancy failure represent the largest portion of losses, with losses ranging from 10 to 50% in lactating dairy cattle [59]. Several key events occur during this early period. During normal fertilization, fusion of the male and female pronuclei, termed syngamy, results in a zygote. The zygote undergoes mitotic divisions to generate blastomeres results consisting of totipotent daughter cells, meaning each cell has the potential to give rise to a fully formed individual [60]. These cleavages continue to take place until the morula stage, which is denoted by the inability to count individual blastomeres. At the morula stage, two specific cell populations form. The inner-lying cells congregate, the outer cells of the morula are permeable and allow for fluid accumulation within the embryo giving rise to the blastocoel cavity, denoting the embryo as a blastocyst [60].

Embryonic death during the first week of pregnancy is difficult to diagnose. The challenges with identifying early embryonic loss are that cows experiencing embryonic

mortality during this time period often will experience a normal estrous cycle. Currently, there are very limited methods of accurately determining pregnancy failure for preimplantation embryos. Interestingly, although we normally consider that fertilization failures do not account for a large portion of pregnancy losses in cattle, there are various errors that occur before fertilization. These errors will not affect fertilization, per se, but will have detrimental effects on early embryonic development. Descriptions of these problems in both the male and female gametes is described further in the next two sections.

Spermatic Errors in Fertilization

Damage to spermatic macromolecules and can decrease the embryo's competency to sustain a pregnancy [61]. The damages to spermatozoa can occur in both the male reproductive tract and post-copulation in the female reproductive tract. Under normal fertilization events, the sperm head decondenses after penetrating the oocyte and releases the genetic material resulting in the formation of the male pronucleus. One such damage that has direct impacts on the ability of cleaved embryos to become blastocysts is the delay or reduction of pronuclear formation due to heat damage [62]. The damage associated with heat stress negatively affects the motility of the spermatozoon, and this ultimately impairs the formation of the male pronucleus [63]. The subsequent embryos derived from embryos fertilized with compromised motility have reduced competence for development [63, 64]. An experiment by Fatehi et al. illustrated that spermatozoa containing broad levels DNA damage were unaffected in their fertilization capability. However, DNA damage reduced developmental competency of fertilized embryos, whereas the levels of apoptosis were greater throughout the second and third cleavage

events. The fragmented sperm also were inhibited in their ability to achieve the blastocysts stage of development [65].

Oxidative stress also adversely impacts the efficacy of sperm to induce oocyte cleavage. Spermatozoa are at an increased risk of damage due to their relatively skewed ratio of polyunsaturated fatty acids to low antioxidant enzymes [66]. Excessive oxidative damage in the form of reactive oxygen species (ROS) to the portion of the sperm that are incorporated into the zygote results in decreased developmental competency [67]. ROS are a normal consequence of the metabolism of oxygen and present as superoxide, hydrogen peroxide, and nitric oxide and are required in small amounts for normal capacitation to occur [68, 69]. The exposure of spermatozoa to high levels of ROS decreased oocyte penetration but was countered with the addition of catalase [70]. The exposure of frozen-thawed semen increased apoptotic cell numbers and decreased embryonic cleavage rates, both of which ultimately reduced embryo viability [71].

Oocyte Quality Issues

Prior to ovulation, follicular size and function and hormonal dynamics are major determinants of oocyte quality. Oogenesis is the process by which these primordial germ cells (PGCs) undergo mitotic divisions resulting in the furthered development into primary oocytes. A select few oocytes are recruited and subsequently contribute to the next generation [72]. Nuclear maturation of primary oocytes is halted from birth until puberty. This is often designated as nuclear arrest.

The process by which oocytes mature is closely related to folliculogenesis and due primarily to the cellular communication between the oocyte and surrounding follicular environment [72]. Folliculogenesis involves the passing of primary follicles through

several different stages including secondary, tertiary, and antral stages. Each of these stages contain distinctive morphological characteristics. Within the antral follicle, granulosa and theca cells have corresponding receptors for FSH and luteinizing hormone LH. These two hormones are key regulators within the HPG and are responsible for the growth of follicular waves and subsequent LH surge which induces ovulation. Inadequacy of preovulatory follicular development can result in decreased developmental competency and increase the likelihood of early embryonic mortality.

Postovulatory oocyte quality is a key determining factor in whether oocytes will be fertilized and reach the blastocyst stage [73]. In cattle, roughly 90% of cumulus oocyte complexes (COCs) recovered via OPU are immature oocytes. However, after their removal from the follicle, COCs are capable of both meiotic and nuclear maturation [74, 75]. Of these matured COCs, only 30-40% are capable of forming a blastocyst [64]. These instances of oocyte immaturity can be applied in vivo when investigating the timing of ovulation relative to AI. Improper maturation of oocytes increases the likelihood that pregnancy will fail.

Cumulus cells are somatic cells originating from the follicular granulosa. These cells are involved in the communication and development of bovine oocytes through gap junctions [76, 77]. Cumulus cells have been shown to be beneficial for developmental competency. When removed before in vitro maturation (IVM), development is hindered. Likewise, removing cumulus cells immediately before IVF reduces fertilization, cleavage and blastocysts rates, when compared to zygotes denuded from cumulus 20h after IVF [76]. Lack of cumulus cells can result in the decreased rates at the time of fertilization.

Just as the size of the ovulatory follicle impacts the quality of the oocyte, the secretion of P4 from the residual follicle also has a significant role in pregnancy outcomes. P4 secretion is involved in multiple roles by promoting the endometrial secretions needed for the preimplantation embryo and preventing the return of cyclicity [78, 79]. Delays in post-ovulatory concentrations of P4 result in the inability of cows to maintain a pregnancy [80].

Methylation Changes During Early Embryogenesis

There are many speculations as to why embryos do not divide and form viable blastocysts, but one set of biological processes that has received heavy attention as key players in embryo competency in maintaining pregnancy are epigenetic factors. Epigenetic changes such as DNA methylation, modifications to the histone tail, and the binding of non-histone proteins to chromatin are thought to be the regulatory mechanism by which multicellular organisms acquire differences in gene expression [81]. Following fertilization, gamete-specific epigenetic “marks” are removed and exchanged with embryonic marks that identify cells as either totipotent or pluripotent [82].

The 6-8 cell stage marks the period where methylation begins and corresponds with embryonic genome activation (EGA) [83]. Improper DNA methylation effectively contributes to the modulation of key pregnancy genes within the uterus and is associated with early embryonic loss because of improper gene expression [84, 85]. As zygotes undergo cellular growth in the form of cleavages and extensive changes in methylation, they rely less on the maternal transcripts and begin the maternal-to-embryonic transition (MET) [86]. The successful completion of MET is considered to be the most crucial event in the developmental period of mammalian preimplantation embryos [77]. MET consists

of several crucial steps that involve the breakdown and translation of maternal transcripts, a shift from oocyte stored, maternally derived transcripts to embryonic transcripts, and ultimately the synthesis of new embryonic specific transcripts, coined EGA [77]. Failure to undergo EGA increases the likelihood of embryonic mortality.

Conceptus Losses; Days 8-27

Although pregnancy loss is most pronounced during the first seven days, ample pregnancy failures also occur during the next three weeks of pregnancy. Instances of pregnancy loss have been reported between 19 and 41% by days 28 and 32 of gestation [59]. Several crucial events must occur during this period for pregnancy to be maintained. The newly formed blastocyst at day 8 will begin hatching from the zona pellucida (ZP) as a result of blastulation, enzymatic breakdown, and penetration of trophectodermal cells [87-90]. The initial shape of bovine embryos following hatching between days 12 to 14 is ovoid, but it continues undergoing extensive growth throughout the following days prior to implantation [91]. From days 12-14 the rapidly elongating conceptus extends the entire length of the uterine horn ipsilateral to the dominant CL and progresses to the contralateral horn [92]. The filamentous structure of the developing embryos is now referred to as a conceptus because it contains all of the embryonic and extraembryonic tissues needed for pregnancy [93].

One crucial event that must occur soon after the conceptus has elongated is maternal recognition of pregnancy (MRP) [94, 95]. Maternal recognition of pregnancy is the process by which the developing conceptus communicates with the maternal endometrium to allow for further development. The specific factor secreted by ungulate the conceptus is interferon-tau (IFNT). This Type 1 interferon is released by the

mononuclear trophoblast cells and induces several changes in the maternal reproductive tract [96]. One crucial modification to the uterus that is needed for pregnancy to be retained is in the release of PGF2α, the agent responsible for CL regression. Interferon-tau alters PGF2α release by blocking oxytocin receptors responsible for uterine production of PGF2α. This action prevents luteolysis and subsequent CL regression [97]. If this cascade of events does not occur, pregnancy failure will result from the loss of CL function and return to cyclicity.

There are several other actions of IFNT that are important for sustaining pregnancies. Interferon-tau induces the expression of endometrial transcripts known as interferon stimulated genes (ISGs). These transcripts are responsible for the remodeling of maternal immune cells to allow for the allogenic conceptus to continue development [98].

Optimization of the Uterine Environment

While IFNT is crucial for MRP, other factors also must be in place for pregnancy to succeed. The importance of having a strong CL is clear when looking at the modulatory role it has on the production of histotrophe, ultimately supporting elongation, and subsequently MRP. The adequacy of the uterine environment is correlated to the secretion of ovarian P4 and the presence of histotroph. Several studies have demonstrated the negative relationship that decreased levels of P4 from days 10-12 post AI have on pregnancy outcomes [79]. Histotroph is secreted by the endometrial epithelia and contains pro-developmental factors such as growth-promoting proteins, lipids, sugars, ions, and microvesicles intended for conceptus survival and development [99]. The mechanism by which the embryotrophic factors within histotrophe direct elongation

is through proliferation of embryonic trophectoderm [96, 100]. The importance of an optimal uterine environment can be highlighted in the fact that bovine blastocysts do not elongate in vitro, and that the cellular signals and nutrients secreted by the endometrium are crucial in this process of elongation [95]. New literature also suggests that the presence of the embryo within the uterus also initiates signaling that secretes crucial growth molecules into the uterine histotroph [100].

Implantation of Bovine Conceptus

The implantation process can be divided into three pivotal events [101]. Following hatching and elongation, the blastocyst must correctly orient itself within the uterus, deemed apposition and begin to loosely contact the uterine epithelium. By day 17, binucleate giant trophoblast cells begin to interact with uterine epithelium. The apposition phase of implantation in ruminants involves two specific endometrial tissue types, caruncular and intercaruncular tissue [102]. Microvilli within the uterine luminal epithelium enter the plasma membranes of the conceptus' trophectoderm are crucial for the linkage between the two tissues and ultimately result in the privileged uptake of nutrients from the uterus [101, 102]. Apposition initiates centrally surrounding the embryonic region and spreads outward towards the ends of the conceptus [101]. Likewise, during this period, the genetic competency of trophoblast cells is changed, and the expression of IFNT comes to a halt. At day 19 in cattle, a now fully elongated conceptus begins central implantation. The adhesion stage involves the initial fusion of the cellular structures between the embryo and endometrium [101]. The uterine microvilli insert themselves into the plasma membrane folds of the trophoblast cells and form the adherence that remains constant throughout pregnancy [101]. Finally, the formation of trophoblast giant

binucleate cells (BNC) that arise from the invasion of BNC into the luminal epithelium to form multinucleated syncytial plaques [96, 99, 101]. Additionally, BNCs migrate through the tight junction to fuse uterine epithelial cells to the chorion, which is the outermost covering of the embryo [101, 103].

The presence of BNCs makes the ruminant placenta somewhat unique among mammals because it cannot be classified as a syndesmochorial or epitheliochorial placenta because of the interrelationships it has between the uterine epithelium and fetal trophectoderm [103]. Rather, ruminant placentas are identified as distinctively synepitheliochorial, with the term ‘syn-‘ highlighting the cell fusion between the uterine epithelial cells, and BNCs [103]

Late Embryonic and Early Fetal Losses: Days 28-60

Pregnancy losses also are observed during the second month of gestation. These losses range from 12.5 to 17.5% in dairy cattle [59]. In beef cattle, late embryonic failures are less pronounced than in dairy, however, losses during this period are reported between 4.8 to 6.9% [104]. There are two key developmental events that occur with the placenta at this time that likely contribute to pregnancy losses during this period.

During the late embryonic period of development, bovine conceptuses rely on nutrients from the yolk sac prior to further placentation [105]. Failure of the fetus to appropriately transition from the choriovitelline to the chorioallantoic placenta is listed as one of the contributing factors associated with pregnancy loss during the placental stage of pregnancy [59]. Additionally, failure of placentome formation is detrimental to fetal development because of the decrease in exchange of nutrients and waste.

Placentome Development and Vascularization

Throughout this period of pregnancy, there are fundamental changes in the architecture of the placenta that are uniquely ruminant. Additionally, the embryonic source of nutrition shifts initially from histotroph to a choriovitelline origin, and then eventually will adapt a chorioallantoic placenta. The choriovitelline placenta utilizes the embryonic yolk sac as the functional unit of nourishment for the developing embryo from days 18-23, peaking at day 25, and begins regressing to complete absence by day 60 [106].

The development of placentomes and the chorioallantoic placenta allow for a principle shift in the exchange of nutrients and waste between the maternal and embryonic systems [59]. Maternal endometrial caruncles that jut into the lumen of the uterus interact with embryonic cotyledons, post implantation, and give rise to create each placentome [107]. This location allows for the maternal-fetal exchange of gasses, nutrients and metabolic waste throughout pregnancy through high surface area exposure of the maternal blood system with feto-placental blood [107]. Aires et al describe the cellular formation of the placentome in four phases. First, the S1 where the caruncular (CAR) and intercaruncular (IC) surfaces respond similarly to the presence of the chorionic membrane. The S1 phase occurs between 20-26 days of gestation and involves both mono and binucleate TE cells that reside in a sheet over the CAR and IC surface. The S2 phase occurs at days 28-33 of pregnancy and is characterized by the chorionic-uterine epithelial cell layer beginning to rupture into the CAR and IC. There is only a minor support of embryo development during this phase. S3 and S4 which correspond to day 35-40 and 50-60, respectively, and involve further cellular remodeling, where endometrial epithelia and subepithelial stratum proliferate rapidly. During these two phases, juxtaposition

occurs between two adjacent blood supplies of the CAR and the villi of the embryonic trophectoderm to allow for nutrient exchange [107]. Errors in these key timed events or improper stretching in the endometrial environment can result in implantation failure, and ultimately pregnancy loss [107].

Placental Hormones and Secreted Factors

The placenta, while extremely important in its main contribution to fetal growth, also acts in a multitude of temporary autocrine, paracrine, and endocrine fashions [108]. The placenta acts as a true endocrine organ, where it releases hormones and other molecules into the maternal circulation. The impairment of specific placental factors may be resultant of abnormal fetal development and be indicative of a compromised pregnancy.

Placental hormone production is not studied vastly in cattle, but it is known that BNCs are the source for the pregnancy-specific hormone termed chorionic somatomammotropin hormone 1, which is better known as placental lactogen. Also, BNCs and mononucleated trophectoderm produce several steroid hormones, including E2 and P4, although the importance of these steroids during pregnancy remains largely unstudied [103].

Although hormone production is not well-studied in cattle, another group of placental secreted proteins that enter the maternal bloodstream have been extensively studied. One of the first placental-specific products identified in the maternal circulation was termed pregnancy-specific protein B (PSPB). This and other proteins that fall into this family of factors are now often referred to as pregnancy-associated glycoproteins (PAGs) [109]. These proteins are aspartic proteases that originate from BNCs and are released

into the uterine lumen as early as 15 days after fertilization [110]. They can be detected in the maternal circulatory system beginning around 24 days of gestation [111, 112]. The functions of PAGs remain speculative. Some have proposed they support pregnancy by sequestering both P4 and PGE_2 from the CL and endometrium, respectively [113-115] while others have proposed they may facilitate pregnancy by controlling the release of the alpha chemokine granulocyte chemotactic protein-2 [110]. However, the importance of PAGs in cattle lies in the ability to use these placental proteins to diagnose pregnancy in cattle and other ungulates.

In cattle, PAG concentrations in serum or plasma will change according to the stage of gestation as well as parity, number of services, milk production, and the prevalence of metabolic diseases [28, 116]. On days 30-60, cows that are expected to maintain their pregnancy will have greater concentrations of plasma PAGs than those cows that are at an elevated risk of late embryonic failure [28, 117]. This difference is confounding when comparing cows that experience late fetal loss, which have relatively normal PAG levels throughout the embryonic period. PAGs that are greater than normal can also be used as a tool to identify animals that are at an increased risk of embryonic mortality [116]. The current understanding is that elevated PAGs are reflective of an overcompensatory pathway because of placental inefficiency [28].

Late Fetal Losses: Days 60- Parturition

Instances of pregnancy failure from day 60 onward are less common and range from 1 to 3% in dairy and 5.8% in beef cattle [59, 104]. Several underlying causes for pregnancy failures have been noted throughout this time of gestation. The two most

prominent causes of fetal losses after day 60 are twinning-induced fetal expulsion and uterine infection.

Twin Development

The presence of twins during the early fetal period, starting at day 45, is associated with increased risk of fetal mortality in high yielding dairy cattle [118]. The occurrences and magnitude of losses associated with twins are attributed to two leading causes. First, there is a 3.45-fold increased risk for pregnancy loss when twins are within the same gravid horn ipsilateral to the dominant CL when compared to bilateral twin pregnancies [118]. This suggests that fetal overcrowding of the uterine horn is less conducive to the maintenance of pregnancy. The secondary factor attributed to twin fetal loss are the result of the regression of one or more functional CL ipsilateral to the gravid horn. This luteal regression is attributed to maternal stressors that compromise pregnancy, but it also can happen spontaneously [118]. A study comparing the location and number of CLs to the death of a twin pregnancy noticed that 26% of embryonic reductions were associated with the regression of an ipsilateral CL [118].

Pathogenic Factors

The presence of pathogenic viruses, bacteria, protozoa, mycoplasma and other pathogens and can increase the instances of fetal death [119]. Non-infectious environmental conditions account for approximately 70% or more instances of prenatal loss. While most environmental factors are lethal at the embryonic period, brucellosis and other pathogenic bacteria are attributed to fetal death [119]. There are two modes that environmental impacts work to cause fetal mortality; direct and indirect [119]. Direct effects are connected with impairment of either the fetus itself or the supporting fetal

environments, namely the uterus. Hematogenous pathways, artificial insemination, and natural service are the routes that pose the highest likelihood of compromise to the endometrial epithelia [119].

Toxoplasma gondii and *Neospora caninum* are two prevalent protozoa that are capable of traveling through maternal blood circulation, affecting the uterus, crossing the placental barrier and ultimately causing abortions in cattle [119, 120]. These are examples of indirect pathogens. *N. caninum* in pregnant cattle is associated with frequent repeat abortions and is dependent on the stage of gestation in which the infection occurs; however, abortions can occur as early as 3 months post-fertilization throughout to gestation [120].

Campylobacter fetus can be introduced to the vaginal environment during natural service. This gram-negative bacterium is found in the crypts of the prepuce and can be transmitted to the maternal reproductive tract during copulation [121]. *C. fetus* is attributed to an increased prevalence of abnormal estrous, infertility, and abortions [121]. The pathogenic nature of the *C. fetus* genome is attributed to its ability to affect endometrial epithelial cells and disrupt adhesion, secretion systems and antiphagocytic layers [121].

The mechanisms that cause these various pathogens to induce abortions are numerous, but most of these mechanisms are indirect, where they affect the entire maternal system and cause systemic effects through septicemia, viremia or toxemia and fetal loss is associated with increases in body temperature due to the persistent infection. Fever associated with various infectious agents is known to cause denaturation of embryonic proteins. Inflammation within the body also draws the response of

supplementary prostaglandins, which can induce luteolysis and result in pregnancy loss [119].

Overcoming Infertility

With pregnancies being extremely valuable in both the dairy and beef sectors, it becomes easy to justify the need for further research to explain how pregnancy failures occur. The use of ART has been heavily adopted to minimize the impact that certain factors, such as postpartum metabolic stress and anovulation have in respect to pregnancy [24, 122]. As mentioned in this review, reproductive efficiency faltered until the mid 1990s [19]. In the dairy industry, the use of synchronization protocols, genomic analyses, estrus detection aids, and ET have been attributed to alleviating the decline in reproductive performance [123, 124].

Arguably the best technology to emerge to combat infertility in dairy cattle in the past two decades has been estrous synchronization protocols. These protocols aim for achieving time AI, where ovulation can be pharmacologically induced and timed without the need for estrus detection [19]. Ovsynch protocols are expected to increase pregnancy rates to 30-40% [15]. Many variations build off of the premise of the Ovsynch protocol with variations in the number of injections intended to improve anovular animals [123]. The most notable adaptations include the utilization of “presynch” strategies. Of the available presynch approaches, the most common in dairy cattle is the Double-Ovsynch [125]. This protocol adds GnRH 10 days and a $PGF_{2\alpha}$ 3 days before the standard Ovsynch protocol and induces ovulation in cattle who were not cyclic prior to synchronization [125].

Emphasis on reproduction traits in genetic merit indices have worked to improve the decline in fertility. Genomic evaluations focused on the reproductive traits daughter

pregnancy rate (DPR) and heifer conception rate (HCR) have increased in relevance in the dairy industry. These traits are being used to correlate positive reproductive outcomes with genetics. Heifer conception rate is intended to measure the probability that the offspring of a specific bull will become pregnant when compared with herd mates, while DPR is the likelihood a bull's daughter will become pregnant after calving within a 21-day time period [124].

Estrus detection aids have also been attributed to the improvement of fertility in cattle. Marker aids vary in complexity. The most simple examples are paint or adhesive patches placed on the rump of potential animals that are rubbed off during estrus expression by herd mates [123]. The disadvantage of these tools is the labor required to apply the markers to animals in the herd. As technology becomes more readily available, so does the automation of estrus detection systems. In dairy cattle, pedometers are used to observe natural mating behavior associated with estrus. Additionally, activity tags that monitor estrus-related behaviors have been investigated to have near 90% accuracy [123, 126]. AccuBreed is an electronic estrus detection system for beef cattle that attaches above the tail and transmits mounting events to software for analysis by the producer [127].

Lastly, but arguably most importantly for the purposes of this MS thesis research project is the continued rise in the use of IVP embryos to meet the production demands in cattle. More than 1 million IVP embryos were transferred globally in 2018 [23]. Although these embryos are primarily being used to improve calf production from elite genetics, these embryo technologies are also finding a niche in for cattle facing challenges that would be embryonic lethal prior to the normal transfer period of 6-8 days [122]. This

benefit is pronounced in stressed animals. The use of ET works in three probable ways. ET minimizes problems associated with oocyte incompetency, uterine and oviductal factors, and problems with AI [122].

Limitations of In Vitro Pregnancies

As mentioned above, there are several benefits of using IVP embryos. The development of embryos in vitro allows for some natural selection barriers to be avoided but exposes preimplantation embryos to other stressors unique to the IVP process. The pregnancy success rate of IVP embryos is markedly less than their superovulated in vivo counterparts [24]. There have been noticeable adverse differences in embryos, fetuses, and the associated pregnancy membranes derived from IVP pregnancies [128]. To follow is an overview of differences observed between IVP and in vivo-generated embryos.

Competency of IVP vs In Vivo Blastocysts

Arguably, differences in embryo morphology is the most definitive difference identified between IVP and in-vivo generated embryos [129]. Differences are detected at the morula and blastocyst stages, with morulae compacting less prominently, smaller perivitelline space, variable coalescence between blastomeres, and a grainy appearance. Blastocysts also appear darker, contain increased numbers of granules and lipid droplets and have incomplete junctional connections between the inner cell mass (ICM) and trophectoderm (TE) [130]. The ICM of IVP blastocysts certainly appears to be different from those of in vivo-produced blastocysts. Transferring IVP embryos into rabbit oviducts produced ICMs with greater cell numbers [131]. Additionally, the embryonic disk, which forms from the ICM during conceptus formation, is smaller in IVP embryos [132, 133]. Therefore, while preimplantation embryos are considered extremely flexible in their ability

to develop in culture systems that are less than ideal, future work is needed to improve IVC to more closely match conditions of in vivo development [73].

In Vitro vs In Vivo Placental Development

The treatment of IVP on bovine embryos can affect the size of the bovine placenta. In a study comparing placental tissues from IVP and in vivo embryo transfers at day 222 of gestation, placental tissues differed in weight, with IVP being heavier [134]. Despite the difference in weight the total number of placentomes did not differ. The surface area of placentomes was less for the IVP group and increased the ratio of blood vessels to placentomes [135]. This change can be attributed to a compensation effort in the vascularization of placentas happening throughout the later portions of gestation [128]. The changes in placental size and altered nutrient perfusion between IVP and in vivo pregnancies are thought to contribute to this compensation despite a decreased number of fetal villi and binucleate cell volumes [128].

To summarize thus far, the underlying cause for the reduced competency of IVP embryos remains largely unknown. The manipulation of embryos through a pipette, variations in temperature and pH, limits of the nutrients supplemented in medium and contact with metabolic end products have all been identified as potential stressors for the preimplantation embryo that may hold lasting effects for later development [135]. Alternatively, the absence of certain oviductal and uterine derived factors likely also prevents IVP embryos from reaching their full competency. The use of bovine IVP embryos as models for embryogenesis in cattle and other mammals, including humans, has offered opportunities to explore how to improve IVP embryo competency to survive

in the uterus of surrogates after ET. The remainder of this review will discuss some of the factors that appear to be primary determinants of IVP embryo competency.

Embryonic Culture and Metabolism

Culture environments are multifaceted and include the growth medium, the embryo:medium ratio, the temperature of incubation, and the gas atmosphere [73]. Both culture medium and the uterine environment need to provide the appropriate amount of energy and specific metabolites required for proper cell division and ultimately be conducive to growth [136]. Commonly defined additives to culture medium include amino acids, pyruvate, and fatty acids [137]. The conditions of culture are intimately related to the fact that ruminant embryos exhibit a particular sensitivity to their culture environment [73]. Amino acids are central ingredients in the development of culture media because of their uses in protein synthesis, energy, osmoregulation, pH balance, protection from oxidative stress and excretion of ammonium [138].

It is hypothesized that the modifications to the preimplantation embryo occur at the level of the embryonic epigenome, where cell-signaling molecules either in culture or in the uterus play substantial roles in the alteration of DNA methylation status [139]**.** One major concern are the effects that culture environment can have on the epigenetic status of the developing fetus and subsequent neonate. The addition of fetal calf serum (FCS) to culture medium has shown to clearly increase the speed of development from the morula to blastocyst stage [140]. While FCS will increase blastocyst rate, it also has converse effects on the ICM:TE ratio [141]. This increases the possibility of the developmental defect known as large offspring syndrome (LOS). One of the main contributing factors associated with LOS is the preferential allocation of cells to the TE

during differentiation, which results in a larger placenta. The consequence of these changes is uncharacteristic fetal growth and enlarged development which can negatively affect pregnancy outcomes [142].

Throughout the early cleavage stages of development, cellular energy is generated primarily from pyruvate by means of the Krebs cycle and oxidative phosphorylation [137]. Glucose is a key metabolite involved in the development of cleavage stage embryos into blastocysts because of the increased production of ATP. At the time of blastocyst formation, there is a shift in oxygen consumption due to the increase in glycolysis [137]. The ATP derived from embryonic metabolism is necessary for the process of cavitation [138]. When comparing in vivo and IVP embryos, the latter utilize a greater rate of aerobic glycolysis and produce more lactate [137, 143].

Antioxidants are supplemented to IVF media to combat the presence of ROS by protecting from DNA damage. The supplementation of antioxidants can improve embryonic quality and increase developmental competency [144]. Antioxidants are beneficial during IVM and IVC but reduce the proportion of COCs that achieve the blastocyst stage of development when supplemented during IVF [145]. This is likely due to the need of ROS by sperm for capacitation.

Embryokines

Throughout this literature review, a common, recurring theme identified is the current lack of performance of IVP embryos after ET. This decreased function is attributed to the shortfall of in vitro culture media to match maternal environments. Researchers have sought ways improve the culture conditions to more closely resemble the temporal molecular signals originating from within either the oviduct or the uterus [146].

Embryokines are a group of maternally secreted regulatory molecules that effect development of the embryo [146]. A limiting factor in the consideration of utilizing embryokines in in vitro culture systems is the lack of knowledge of their relative abundance in situ and the timing that these regulatory factors play roles on the developing embryo [146].

Known Embryokines

Many potential embryokines have been identified by isolating genes within the endometrial and oviductal epithelium that are associated with the expression of hormones, growth factors, chemokines, cytokines and WNT-related molecules [146]. A common thread across the realm of embryokines that deserves special consideration for improving IVP embryo competency is their ability to increase the percentage of embryos developing to the blastocyst stage of development as well as improving the total cell number of blastocysts. With this in mind, there are two particular embryokines that have been studied by other groups that are of particular interest because of their positive role on embryonic growth. These are colony-stimulating factor 2 (CSF2), and insulin-like growth factor 1(IGF1) [146].

Colony-stimulating factor 2 is secreted by oviductal luminal epithelium and the endometrium. The secretion of CSF2 is understood to be in response to seminal factors. The expression of CSF2 also follows a uniquely time-based pattern being lower throughout estrous and increasing throughout days 13 to 17 post-ovulation [146]. CSF2 has shown a tendency to increase not only the proportion of oocytes that become blastocysts, but also the number of ICM cells and ICM to TE ratio [147]. When transferred, embryos treated with CSF2 show improved pregnancy retention [147].

Insulin-like growth factor 1 is a crucial signaling molecule that is responsible for inter and intracellular functions [148, 149]. The roles of the IGF family of signaling molecules have been well investigated, and their involvement with pregnancy [150]. Unlike other embryokines, IGF1 has a role predominantly involving the development of TE cells and subsequently placental development [149, 151]. Insulin-like growth factor 1 has illustrated its pro-developmental benefits in bovine blastocysts by increasing the proportion of zygotes reaching the blastocyst and hatched blastocyst stages with the inclusion of IGF1 in culture medium [152]. Insulin-like growth factor 1 cultured blastocysts have shown positive results when transferred into cattle underheat stressed conditions [153]. Additionally, these blastocysts resulted in greater pregnancies per transfer [153].

These embryokines however, are not exclusive in their ability to yield positive changes to preimplantation embryos. As aforementioned, many transcripts code for the secretion of necessary growth factors needed for normal growth [146]. As further understanding of embryonic needs arise, the formulation of in vitro culture (IVC) medium can be tailored to best supplement the preimplantation embryo. Our laboratory uncovered a new embryokine that we think deserves considerable attention for its ability to alter the morphology of the embryo in ways that may make it more closely resemble an in vivo-generated embryo. This new embryokine is interleukin-6 (IL6).

Interleukin 6 as an Embryokine

Interleukin-6 is a member of the IL6 family of cytokines, which consists of several family members that function through a common receptor subunit, termed glycoprotein 130 kDa (gp130) [154]. This family of cytokines has many physiological functions that lie beyond their actions as immune cell regulators and controllers of inflammation. There has

been an interest in examining IL6 as an embryokine ever since transcripts for IL6 were identified in oviductal, endometrial, and conceptus tissues [146, 155].

Two hallmark discoveries of IL6 activity in bovine embryos were recently identified by our laboratory [156, 157]. While supplementing IVP embryos with recombinant bovine IL6 had no impact on improving blastocyst formation when cultured in groups, an increased proportion of embryos formed blastocysts when embryos were cultured individually [156]. Embryos under low density culture conditions lack important inter-embryonic signals that would normally be present in group culture. This has far-reaching implications because it suggests that IL6 improves embryonic development during culture, and this action may promote pregnancy retention after IL6-treated embryos are transferred [156].

The second notable discovery made by the laboratory was the ICM numbers were increased by nearly 2-fold in IL6-supplemented IVP blastocysts [156]. As mentioned earlier in this review, reduced ICM numbers may be a cause for reduced IVP embryo competency after ET. However, it remains unstudied whether IL6 treatment can actually improve IVP embryo competency after ET.

Summary and Concluding Remarks

While the knowledge and improvement in reproductive efficiency has improved remarkably in the past century, there are still vast amounts of knowledge regarding the mechanisms of pregnancy failure that remain unknown. The future is also likely to present new challenges as the management of cattle changes. Perhaps in the future, technologies will arise that allow for the circumvention of pregnancy failure as understandings of the mechanisms are expanded. Finally, the role of IVP of embryos and

the function of embryokines in the future application for producers and as a research tool is apparent to investigate the underlying shortcomings in bovine reproduction.

Objectives and Hypothesis

The objective of this thesis is to extend on recent findings made in our laboratory. The implications of supplementing IVP embryos with uterine derived factors is intended to improve embryonic competency and post-transfer outcomes without resulting in deleterious development. This thesis research tested the hypothesis that IL6 supplementation will produce pregnancies that contain embryos, fetuses, and placentae that more closely resemble in vivo-generated pregnancies than IVP-generated pregnancies.

Chapter 2

Interleukin-6 Supplementation Improves Post-Transfer Embryonic and Early Fetal Development of in vitro Produced Bovine Embryos.

Introduction

Causes of pregnancy loss are not completely understood in cattle, and it is imperative that we learn more about this problem because of the detriment it has on productivity and efficiency of cattle operations. The value of a lost pregnancy has been estimated at $555 in dairy cattle due to reduced lifetime milk production and additional management expenses [4]. In beef cattle, the value of pregnancy failure is estimated at approximately $94 per exposed cow to service. This value may be higher due to the fact that income is reliant on the total number of calves sold [5, 6]. The most prevalent time period of pregnancy loss is within the first week of gestation [59], however, it is evident

further losses occur within the following weeks after fertilization. The need to understand the mechanisms that cause pregnancy failure becomes clear when considering the potential to circumvent these problems.

Embryo transfer is an approach that has the potential to overcome several reproductive challenges in dairy and beef cattle. Since most pregnancy failures occur during the first week of pregnancy, bypassing this period by transferring an embryo that is morphologically normal may improve opportunities to maintain pregnancies in the face of suboptimal uterine environments and other physiological imbalances [122]. OPU-IVF is a newer strategy that has gained particular interest in avoiding potential pitfalls of modern management systems and maximizing the number of transferrable embryos that can be received from donors. This laboratory utilizes an IVF-ET model for two purposes; first to study the etiology of embryo development in cattle and second to develop schemes that maximize post-transfer pregnancy retention in cattle.

This work focused on examining whether IL6 supplementation during IVP improves post-transfer pregnancy outcomes in cattle. This newly identified embryokine is produced by endometrial and conceptus tissues [146, 155], and recent work within this laboratory revealed several benefits that IL6 may cause in embryos to make them more competent at retaining pregnancy after transfer [156, 157]. However, IL6-mediated changes in embryo morphology may also have unintended consequences that may be a detriment to pregnancy outcomes. This work explored how the developing pregnancy, and specifically the embryo-proper and fetus, develops from embryos that were supplemented IL6 before transfer. Specific markers were used to evaluate fetal and placental

competency including crown-rump length (CRL), crown-nose length (CNL), abdominal diameter (AD), and amniotic vesicle size (AV).

Materials and Methods

A supplemental timeline of procedures is provided in Figure 2-1.

Animal Use

All animal procedures and experimentation were completed with the approval of the Virginia Tech Animal Care and Use Committee (protocol #19-032). This experiment was designed to utilize three defined treatment groups of AI, IL6-ET and CONT-ET. The study was completed over an 18-month period in 2019 to 2020. In the four replicates utilizing beef animals, 25, 17, 26, and 34 animals were used in each of the four replicates. The beef cattle were housed at Kentland Farm and were supplemented with unsampled, ad libitum hay. 21 non-lactating, mature Holstein dairy cows were used in a secondary replicate of the study. These cows were housed at Kentland Dairy and were supplemented with ad libitum hay and occasional weigh backs of lactating cow ration. Nutrient contents were not evaluated. Some beef cows were used in multiple replicates.

Estrous synchronization

A 7-day CO-Synch + CIDR protocol was used to synchronize mature beef and dairy cattle [158, 159] The synchronization was initiated by administering GnRH (100 µg; Cystorelin, Merial, Duluth, GA) and an intravaginal device to release progesterone (Eazi-Breed CIDR™ containing 1.38 g P4, Zoetis Animal Health, New York). After 7 days, the CIDR™ was removed and a luteolytic dose of PGF2α was provided (25 mg; Lutalyse, Zoetis Animal Health). After another 60-66 h, a second dose of GnRH was administered and FTAI was performed in one-third of synchronized cows (37/123), allowing the other

two-thirds of cows to become ET recipients (86/123). Straws of Holstein semen collected from bulls (n=4) was used for IVF and AI (generously donated by Select Sires Inc, Plain City, OH).

In Vitro Bovine Embryo Production

The IVP of bovine embryos was completed as previously described [156, 157]. Oocytes were collected from ovaries originating from Brown Packing (Gaffney, SC). Cumulus oocyte complexes were isolated and incubated at 38.5ºC in 5% CO_2 in groups of 25-20 in 500 µl TCM-199 containing Earle's salts and supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA), 25 µg/ml bovine follicle stimulating hormone (Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada), 2 µg/ml estradiol (Sigma0Aldrich; St. Louis, MO), 22 µg/ml sodium pyruvate, 1 mML L-alanyl-L-glutamine (Glutamax) and 25 µg/ml gentamicin sulfate. After 21 to 24h, COCs were transferred to fertilization medium, and exposed to BoviPure gradient purified (Nidacon, Spectrum Technologies, Healdsburg, CA) bovine spermatozoa derived from a pool of frozen semen from 4 Holstein bulls (same semen used for AI). After 18 h at 38.5 C in 5% CO_2 in humidified air, cumulus was removed by pipetting, and groups of 25-30 presumptive zygotes were placed in 50 µl drops of SOF containing 20 µg/ml essential amino acids (Sigma-Aldrich), 10 µg/ml nonessential amino acids (Life Technologies), 4 mg/ml fatty acid free bovine serum albumin (Sigma-Aldrich), and 25 µg/ml gentamicin sulfate (Life Technologies), and were cultured at 38.5ºC in 5% O_2, 5% CO_2, 90% N_2. At day 5 post-fertilization, embryos were supplemented either with 100 ng/ml recombinant bovine IL6 (Kingfisher Biotech, St. Paul, MN) or carrier control (1% bovine serum albumin [BSA]). At day 7.5, embryo stage and quality were assessed as previously described

[160]. Only embryos categorized as regular blastocyst or expanded blastocysts and containing quality scores of 1 or 2 were selected for ET.

Embryo Transfer

On day 7.5 after the start of fertilization, transfer-quality blastocysts were placed in Holding Medium (Vigro™; Vetoquinol, Fort Worth, TX) and loaded into ET straws. Straws containing embryos were maintained at 38.5ºC in a shipper incubator while being transported to Virginia Tech Beef Center. Prospective transfer candidates underwent transrectal ultrasonography to determine the presence and location of a CL. Cows lacking a CL were excluded from the study. Table 2-1 illustrates the number and stage of embryos transferred for each treatment group. Cows were prepared for ET by administering epidural anesthesia (5 ml lidocaine hydrochloride, 2% [w/v] solution, VEDCO, St. Joseph, MO) into the lumbosacral foramen. An ET straw was loaded into an ET applicator, plastic sheath, and secondary plastic sheath. The vulva was cleaned and manually spread by an assistant while the applicator was kept warm prior to insertion by the ET technician. Embryos were deposited within the upper one-third of the uterine horn ipsilateral to the CL.

Differential Cell Counting

A representative sample of regular and expanded embryos from the BSA and IL6 treatment groups were selected randomly at each replicate study (IL6; n=49 embryos) (CONT; n=32 embryos) and processed for differential cell counting as described previously [156]. In brief, embryos were fixed with 4% [w/v] paraformaldehyde for 15 minutes, permeabilized with 0.25% [v/v] Triton X-100 (Thermo-Fisher Inc, Waltham, MA), and blocked with 10% [v/v] horse serum (Gibco) before incubation with a ready to use

CDX2 antibody solution (mouse monoclonal anti-CDX2 IgG1, Biogenex: AM392-5M). After room temperature incubation for 1h, embryos were incubated in the secondary antibody (1:200 dilution of donkey anti-mouse IgG1 in antibody buffer) (ThermoFisher AlexaFluors) for 1 h at room temperature. After washing, embryos were stained with DAPI (1 µg/ml) (ThermoFisher) and loaded onto slides containing 10% [v/v] slowfade solution (ThermoFisher). An Eclipse Ti-E inverted microscope with an additional X-Cite 120 epifluorescence illumination system was used to visualize DNA and immunoreactive complexes. Images were taken using a DS-L3 digital camera with supplemental NIS-Elements Software (Nikon Instruments, Melville, NY). Cells were counted using ImageJ software (U.S. NIH, Bethesda, MD). CDX2 positive cells were determined to be TE while DAPI positive, CDX2 negative were ICM.

Progesterone Analysis

Blood samples were collected via the coccygeal vein from animals enrolled in each replicate of the experiment. Following sample collection, blood tubes were placed on ice and centrifuged (1500 x g for 20 min; 4ºC) for serum. Samples were aliquoted and stored at -20ºC until the time of analysis. Serum was thawed at 24 hours prior to analysis by chemiluminescent immunoassay (IMMULITE 1000; Siemens, Malvern, PA).

Pregnancy Diagnosis

Pregnancy was diagnosed on day 28 post-ovulation by utilizing a portable transrectal ultrasound from Ibex EVO2 ultrasound with a linear 8-5 MHz multifrequency transducer (E.I. Medical Imaging, Loveland, Colorado). Diagnostic procedure involved verifying the presence of the functional CL, the presence of an amniotic sac with associated fluid accumulation within the uterus, and presence of an embryo with a

heartbeat. Pregnancy status was reassessed weekly thereafter until day 70-post ovulation.

Fetal Measurements

Video loops (8 s) were used to capture the best representative images for measuring crown-rump length (days 42-56), crown-nose length (days 56-70), abdominal diameter (days 49-70), and amniotic vesicle area (days 56-63). All measurements were performed by utilizing the caliper tool installed on the portable ultrasound. Crown-rump length was taken by measuring from the top of the fetus' head to the base of the tail in a linear manner, while CNL was determined from the top of the head to the end of the nose [161]. The AD measurement was taken at the linear bisection at the point of attachment at the umbilicus. The section of the AD was used for the measurement. The AV measurement was completed as described previously [133, 161]. Fetal sex was determined on day 56 as described previously [162].

Pregnancies were terminated at day 70 by administering PGF2α (25 mg; Lutalyse). Between 6 and 7 d later, transrectal ultrasonography was completed to verify the absence of a fetus and amniotic or allantoic fluids. If still present, a second dose of PGF2α was administered and verification of pregnancy loss was determined 6 to 7 d later. All pregnancies were successfully terminated using this scheme. Recipient cows were then reused again for a second ET after >60 days of recovery. Seven cows were used twice, eleven cows three times, and five cows were used in all four replicates. No attempts were made to distribute cows to different treatment groups upon reuse. Treatment assignment was random for each replicate. Also, no attempts were made to adjust the pregnancy rate data based on multiple ET usage for cows.

Statistical Analyses

Repeated Measurements analysis using the Statistical Analysis System (SAS, Cary, NC) was completed initially to examine embryo and fetal measurements over time. The main effects of treatment, day of pregnancy and replicate and their interactions were included in the model. Cow (treatment) was used as the error term for treatment. Within timepoint comparisons were completed by using Analysis of Variance (ANOVA) with the General Linear Model (GLM) of SAS for main effects of treatment and their interaction. Fetal sex was excluded from all final analysis because it was found to be non-significant (P=0.19). Beef and dairy pregnancies were analyzed solely based on the replicate of the study and not based on breed. Significance was set at ($P \leq 0.05$) and tendency was determined as ($P \leq 0.1$). Blastocyst cell counts were analyzed by ANOVA. Pairwise comparisons were completed using the Probability of Difference (PDIFF) option in SAS. Binary data were analyzed by Chi-square analysis using GraphPad-Prism 7 (San Diego, CA).

Results

No differences in blastocyst development were detected between CONT and IL6-treated embryo groups at day 7.5 post-fertilization, the time of ET (46 $\pm$ 2.24% for IL6; 38 $\pm$ 1.24% for CONT). Greater ($P<0.05$) ICM cell numbers and ICM:TE ratios were observed in IL6-treated regular and expanded blastocysts (Fig. 2-2). No effects were noted on TE numbers, but total cell numbers were greater ($P<0.05$) for IL6-treated blastocysts (Fig. 2-2).

Table 2-1 shows the distribution of regular and expanded blastocysts used for each treatment group in each replicate. A representative sample of regular blastocyst and expand blastocysts populations was utilized for each replicate. No difference in pregnancy rates were detected at day 28 post estrus (Fig 2-3). However, after accounting for pregnancy losses between day 28-70, there was a tendency (P=0.09) for improved pregnancy retention in IL6 group when compared to CONT transfers.

Various embryonic measurements were taken during the embryonic (days 28 and 35) and fetal (days 42, 49, 56, 63, 70) periods of development. The first measurement was CRL (Fig. 2-4). Repeated measurement analysis detected an effect of day on CRL (P<0.0001). Also, a treatment effect was present (P=0.01) with the of IL6 fetuses to resemble AI (P<0.05). No treatment by day (P=0.4), fetal sex (P=0.3), or sex by day (P=0.2) interactions were detected. When examining CRL outcomes within each week, there was a treatment effect (P<0.05) with IL6 and AI embryos being larger than CONT on days 28, 42, 56, and 63 but not on days 35 and 49 (Fig. 2-4). The IL6 and AI group were not different at any timepoint.

Fetal CNL measurements were taken on days 56, 63, and 70 (Fig. 2-5). In the repeated measures analysis, IL6 fetuses were longer than CONT (P=0.0002) and had a tendency to be larger than AI (P=0.07). There was an effect of day (P<0.0001) and of treatment (P=0.0003). No effect of fetal sex or sex by day interactions were observed. When examining the effect of treatment within each day, IL6 had longer CNL than CONT on days 56, 63, and 70 (P<0.02). The IL6 and AI groups were not different from one another on days 56 (P=0.1) or 70 (P=0.2) nor was there a difference between AI and CONT. IL6 had a tendency (P=0.06) to have larger CNL than AI on day 63.

Abdominal diameter measurements were performed on days 49, 56, 63, and 70 (Fig. 2-6). An overall effect of day (P<0.0001) was observed. Additionally, there was a was a tendency for a treatment difference between IL6 and CONT (P=0.07) with IL6 being larger. There was no difference between AI and CONT or AI and IL6 on days 49, 56, or 63. When observing the effects of treatment within day, IL6 had a tendency for larger AD than CONT on days 56 (P=0.09) and 70 (P=0.07). There also was a tendency for CONT to have larger AD than AI on day 70 (P=0.10). There was no difference between AI or IL6 on any day of measurement.

The volume of the uterine horn ipsilateral to confirmed pregnancies was evaluated using a scale of high, medium and low on days 28 and 35 (Fig. 2-7). On day 28, AI and IL6 had more fluid (*P*<0.0001) than CONT whereas no difference in volume was observed on day 28 between AI and IL6. On day 35, only AI had more fluid than CONT (*P*=0.02), but no differences were detected between AI and IL6 or IL6 and CONT. Amniotic vesicle diameter was measured on days 56 and 63 (Fig. 2-8). An effect of day was present for the repeated measurement analysis (*P*<0.0001), but no effect of treatment (*P*=0.65) or treatment by day (P=0.91). No difference was observed amongst treatments within either day 56 or 63.

Finally, the concentrations of progesterone were analyzed for all animals enrolled in all treatments throughout the study (Fig. 2-9). Blood samples were collected on days 14, 17, 21, 28, 35, 42, 49, 56, 63, and 70. The data was examined using a repeated measure analysis. There was no effect of treatment and no treatment by day interaction. An effect of day was observed (*P*<0.0001).

Discussion

Pregnancy success in cattle is multifaceted. For pregnancies to succeed, the embryo must go through crucial developmental periods involving distinct changes to the embryo proper and extraembryonic membranes. Although the advancements in technology relating to bovine reproduction have greatly improved in the past century, it remains clear that many problems are unanswered. Pregnancy loss represents a major source of financial strife in both beef and dairy cattle industries. The mechanisms behind pregnancy loss are not entirely understood, and further work is needed to better understand and eventually circumvent instances of pregnancy failure.

The in vitro production of bovine embryos has revealed variances in developmental competency both during the embryonic and fetal stages [24]. These negative changes include greater incidences of early pregnancy failures, heavier birth weights, alterations in placental development, and increased risk of dystocia [134, 163]. The differences in embryonic quality between IVP and in vivo embryos is hypothesized to be from apparent changes in signals during culture [146]. The use of embryokines has several benefits. In the cattle industry, embryokines attempt to improve in vitro preimplantation embryonic fitness, particularly after transfer to result in more viable pregnancies [146]. The addition of embryokines also allow for a broader understanding for the roles that signaling molecules have during mammalian embryonic development.

Interleukin 6 is known for its pro-inflammatory roles systemically [154]. The work completed for this thesis was beneficial in further understanding the effects of supplementing in vitro produced preimplantation bovine embryos with embryokines. The growing global demand for post-transfer performance of OPU-IVF embryos, and apparent

hindered functioning justifies the need for further investigation [24]. The supplementation of IL6 has been previously explored by this lab [156, 157], but this was the first study observing embryonic and fetal growth in cattle.

Embryonic and fetal CRL, CNL, AD, and AV measurements were observed to examine how IL6 supplementation may influence early embryonic and fetal development over time [164]. Pregnancies derived in vivo through AI are typically larger and more competent at pregnancy retention [129]. We hypothesized that the increase in ICM cell number from IL6 treatment would result in fetuses that more closely resembled AI originated pregnancies than IVP pregnancies. Because instances of LOS are more prevalent in populations of IVP derived pregnancies [165], this work intended to observe if alterations in ICM cell number would affect the occurrences of LOS.

Embryonic and fetal CRL were larger for IL6 pregnancies than CONT on days 28, 42, 56, and 63. Fetal CRL measurements could not be completed on day 70 because fetuses were larger than the ultrasound image allowed for accurate measurements. There was no difference between the AI and IL6 groups on these days, suggesting that IL6 cultured pregnancies were more similar in regard to overall growth than CONT, but not different from AI. This is positive in suggesting that IL6 was also not over stimulating development in a pattern of LOS. There were no differences on days 35 or 49 for CRL. Crown-nose lengths were evaluated on days 56, 63, and 70. There was a tendency for IL6 to be longer than AI and CONT on all three days. This supports our hypothesis that IL6 would be larger than CONT, however, IL6 having longer CNL than AI should be further investigated. Abdominal diameters were evaluated as previously described at the widest portion of the fetus where the umbilicus connects [165]. Interleukin 6 fetuses had larger

AD than CONT but did not differ from AI. There was a tendency for CONT to have larger AD than CONT on day 70. This is promising to suggest that pregnancies originating from IL6 culture did not experience organ overgrowth.

Uterine volume differences were observed during initial pregnancy diagnosis. At these stages of development, differentiation between uterine fluid and extraembryonic membranes could not be made. Pregnancies from the AI and IL6 groups had more fluid on days 28 and 35. The reasoning for why AI and IL6 derived embryos had more fluid is yet to be investigated, as IL6 has no known effect on the TE of IVP embryos. To further investigate these changes, amniotic vesicle diameters were analyzed at later stages of development in an attempt to determine if placentae were different between treatment groups. There were no differences in amniotic vesicle diameter at days 56 or 63.

Progesterone concentrations were sampled and plotted to observe relative concentrations according to the day of gestation. Animals were grouped according to when decreases in P4 occurred. Animals that had elevated P4 at day 14 and a sharp decrease at between days 17 and 21 were non-pregnant and continued estrous cyclicity. Cows with elevated P4 at day 14 but were diagnosed open at day 28 potentially experienced pregnancy loss. There also were several cows with elevated P4 through days 42, 49 and 56. These cows most likely experienced extended luteal phases but were not pregnant. Our data suggest that animals were not different in concentrations of P4 between treatment groups. This is important when considering the populations of animals utilized in the study were most likely culled due to lower reproductive performance relative to their herd mates.

In conclusion, IL6 resulted in embryos that were larger than control embryos derived from similar culture conditions. There were few instances of difference between IL6 and AI, which is promising for ensuring that IL6 pregnancies are not experiencing large offspring syndrome. The work in this thesis was completed during early gestation, and because fetal growth follows an exponential curve [33] as gestational days proceed, further work is needed to ensure that IL6 embryos will not experience overgrowth in later portions of pregnancy.

Chapter 3 - Interpretive Summary

This thesis has highlighted the multifactorial nature of pregnancy loss and discussed the environmental and physiological factors that add to increased risk of pregnancy failure. The magnitude of pregnancy loss is clear when evaluating the detrimental effects it has on productivity and economic security of cattle operations. Pregnancy failure can occur throughout the entirety of gestation; however, profound losses occur during the preimplantation embryonic period undoubtedly because of the detrimental effects of environmental and physiological imbalances that occur with lactation. The use of ART has clearly benefited the efficiency of reproduction in the cattle industries, and this trend is likely to continue as more knowledge is uncovered. The use of ET technologies is promising in avoiding the side effects of inadequate maternal and environmental conditions. Likewise, as the understanding of in vitro culture systems improves, it is anticipated that embryo competency will also increase.

The goal of this work was to evaluate the effects of improved early embryonic development from the addition IL6 on late embryonic and fetal growth. This permitted us to examine 1) whether pregnancies produced from IL6-treated embryos resemble

pregnancies from non-treated IVP-embryos or pregnancies from AI (i.e. normal pregnancies), and 2) whether pregnancies from IL6 treated embryos show any abnormalities in fetal and placental development.

The supplementation of IL6 improved the quality of pregnancies derived from in vitro culture systems. IL6 treated fetuses more closely resembled AI originated fetuses than CONT counterparts. The objective was to improve IVP embryo quality post transfer without adverse consequences. These results suggest that IL6 accomplished this objective and did not give rise to unfavorable conditions. Further studies are needed to fully understand the implications of IL6 on neonatal calf development as well as productivity in adulthood.

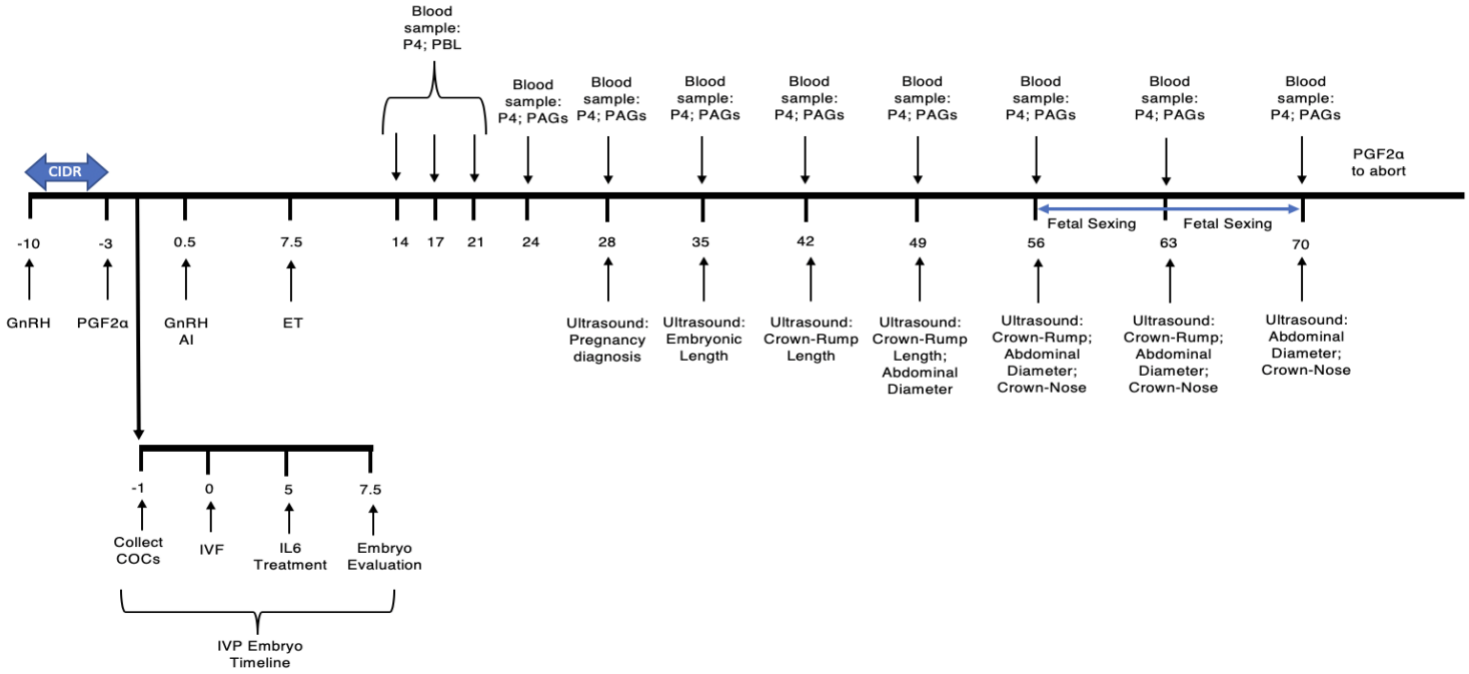
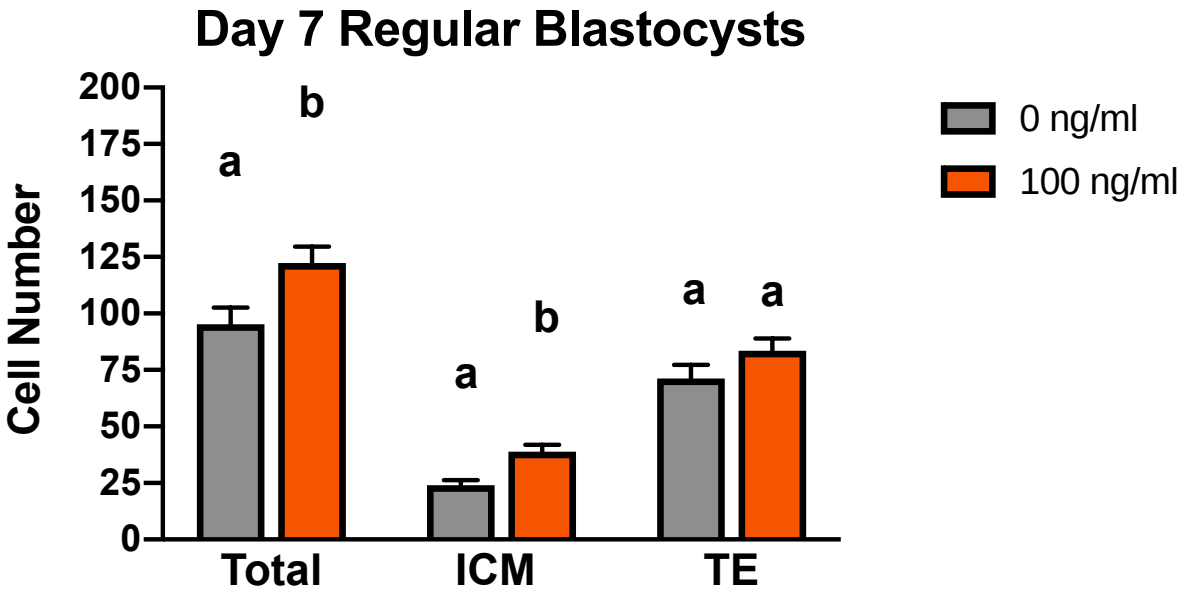

Blood sample: P4; PBL
Blood sample: P4; PAGs
Blood sample: P4; PAGs
Blood sample: P4; PAGs
Blood sample: P4; PAGs
Blood sample: P4; PAGs
Blood sample: P4; PAGs
Blood sample: P4; PAGs
Blood sample: P4; PAGs
CIDR
PGF2α to abort
Fetal Sexing
Fetal Sexing
-10
-3
0.5
7.5
14
17
21
24
28
35
42
49
56
63
70
GnRH
PGF2α
GnRH AI
ET
Ultrasound: Pregnancy diagnosis
Ultrasound: Embryonic Length
Ultrasound: Crown-Rump Length
Ultrasound: Crown-Rump Length; Abdominal Diameter
Ultrasound: Crown-Rump; Abdominal Diameter; Crown-Nose
Ultrasound: Crown-Rump; Abdominal Diameter; Crown-Nose
Ultrasound: Abdominal Diameter; Crown-Nose
-1
0
5
7.5
Collect COCs
IVF
IL6 Treatment
Embryo Evaluation
IVP Embryo Timeline

Figure 2-1 Experimental design. Cattle were synchronized using a 7-Day CO-Synch+CIDR initiated on day -10. Tail paint was applied. A subset of cows displaying estrus were artificially inseminated (AI) on day 0.5. The remaining cows were candidates for embryo transfer on day 7. Blood samples were taken on selected days for determining circulating progesterone (P4) and pregnancy-associated glycoprotein (PAG) concentrations. Transrectal ultrasonography was completed weekly from day 28 to 70 to measure embryo and fetal development.


Day 7 Regular Blastocysts
Cell Number
200
175
150
125
100
75
50
25
0
a
b
a
b
a
a
Total
ICM
TE
0 ng/ml
100 ng/ml

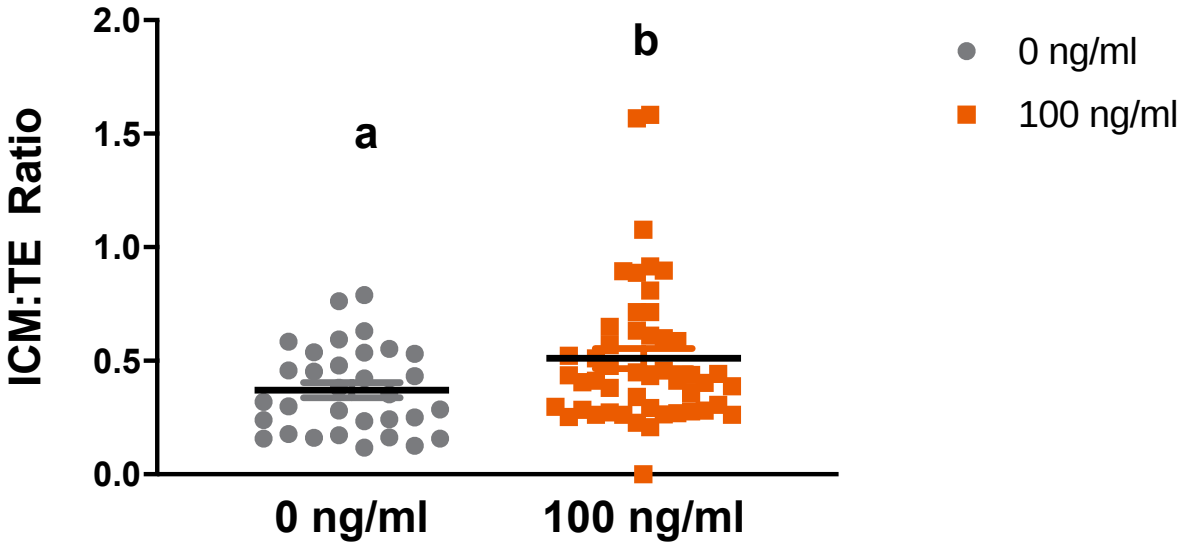
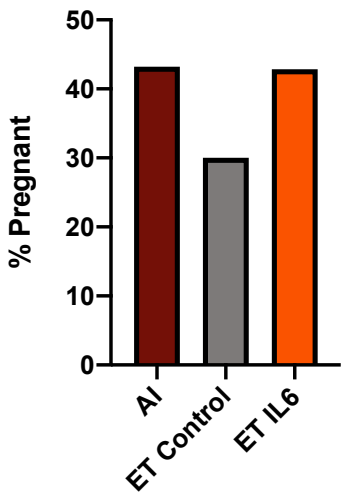
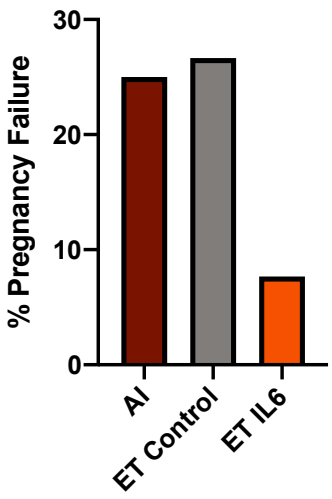

ICM:TE Ratio
2.0
1.5
1.0
0.5
0.0
a
b
0 ng/ml
100 ng/ml
0 ng/ml
100 ng/ml

Figure 2-2 Number of inner cell mass (ICM) and trophectoderm (TE) cells in blastocysts following treatment. Embryos were cultured from day 5 to 7.5 in medium containing 0 or 100 ng/ml IL6. A representative sampling of blastocysts were processed for differential cell counting [156] to distinguish ICM and TE cells. Panel A: Depicts total, ICM, and TE cell numbers. Panel B: The ICM:TE ratio. Different superscripts within each data set represent differences ($P < 0.05$).


% Pregnant
50
40
30
20
10
0
AI
ET Control
ET IL6


% Pregnancy Failure
30
20
10
0
AI
ET Control
ET IL6

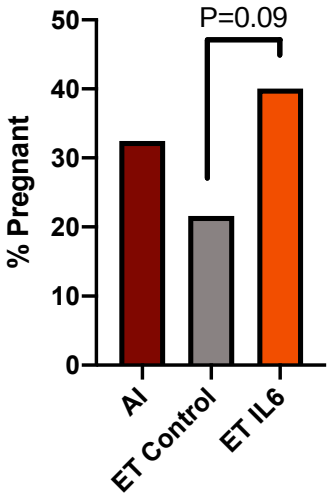

P=0.09
% Pregnant
50
40
30
20
10
0
AI
ET Control
ET IL6

Figure 2-3 Effect of embryo treatment on pregnancy outcomes at day 28 and 70 of pregnancy. Pregnancy was diagnosed at days 28 and 70 with transrectal ultrasonography for cows that were artificially inseminated (AI) or underwent embryo transfer (ET) with IVP embryos that were cultured in 0 (ET-CONT) or 100ng/ml IL6 (ET-IL6). Panel A: represents all animals that were diagnosed pregnant and includes animals that experienced pregnancy loss. Pregnancy failure was determined by the lack of embryonic or fetal heartbeat and the absence of fetal membranes. Panel B: depicts the percentage of animals that experienced pregnancy loss between days 28-70. Panel C: shows the adjusted pregnancy rate percentages for animals that were diagnosed pregnant on day 28 and remained pregnant through to day 70.

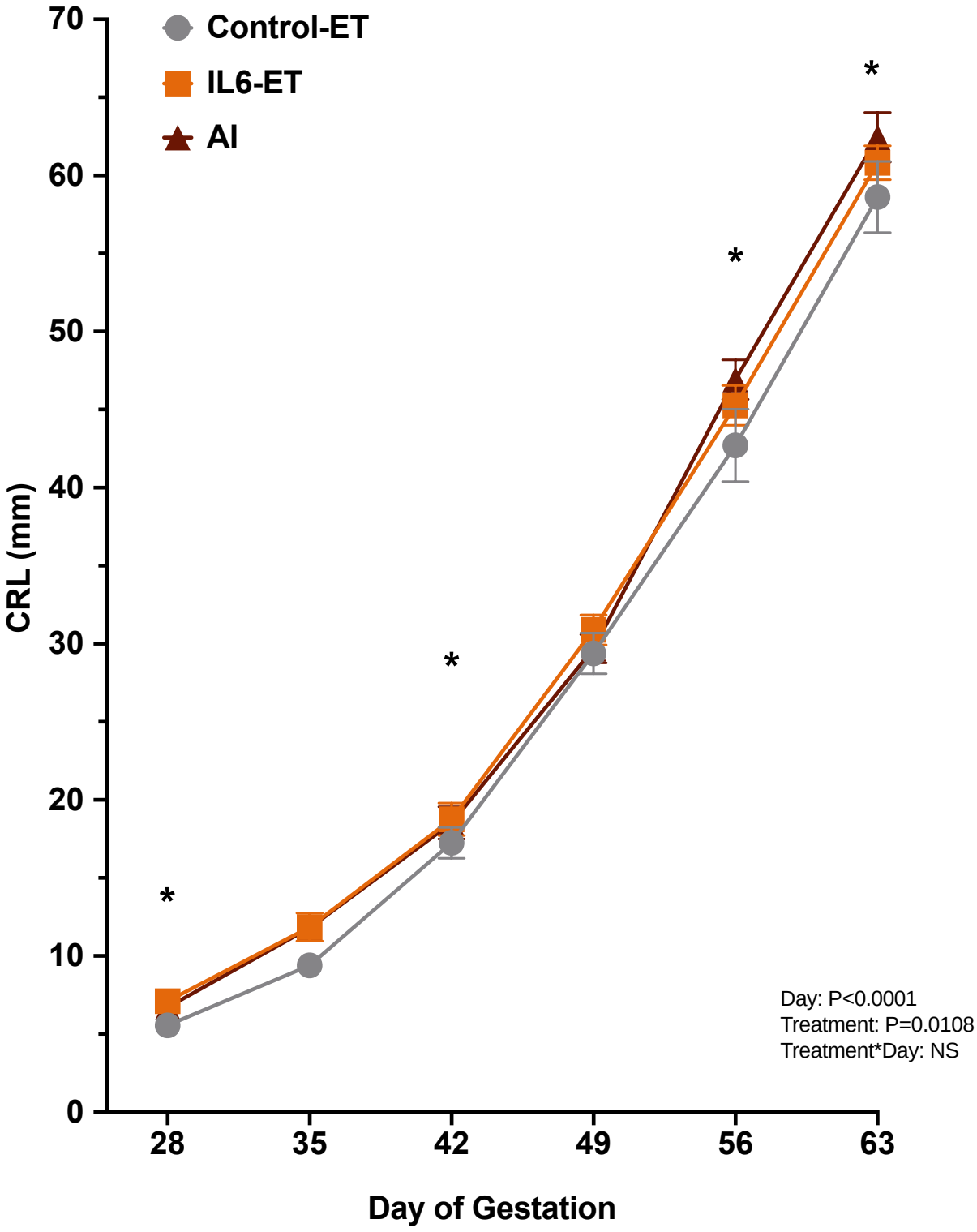

Control-ET
IL6-ET
AI
CRL (mm)
Day of Gestation
0
10
20
30
40
50
60
70
28
35
42
49
56
63
*
*
*
*
Day: P<0.0001
Treatment: P=0.0108
Treatment*Day: NS

Figure 2-4 Effect of treatment on crown-rump length (CRL) from days 28 to 63 of pregnancy. Cows were either artificially inseminated (AI; n=12) or received an in vitro produced (IVP) embryo with either interleukin-6, IL6-ET (100 ng/ml IL6; n=14) or control BSA, CONT-ET (0 ng/m; n=10l). CRL measurements were assed with transrectal ultrasonography weekly. A repeated measurement analysis was completed to examine overall effects (P values are included in the figure). An ANOVA was performed to examine the effects within each day. Differences within each day are designated with an asterisk [*].

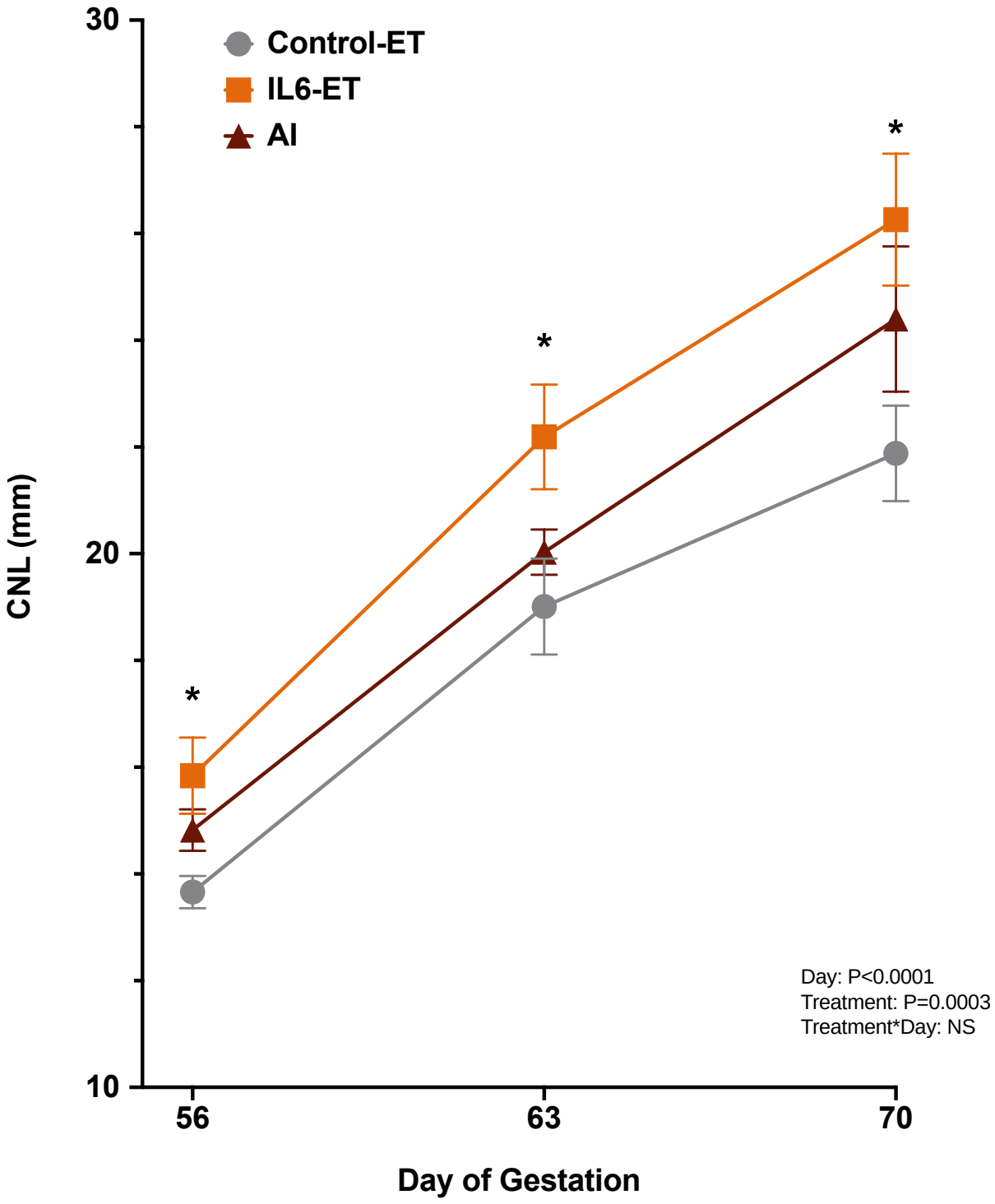

30
20
10
CNL (mm)
Control-ET
IL6-ET
AI
*
*
*
Day: P<0.0001
Treatment: P=0.0003
Treatment*Day: NS
56
63
70
Day of Gestation

Figure 2-5 Effect of treatment on crown-nose length (CNL) from days 56 to 70 of pregnancy. Cows were either artificially inseminated (AI; n=12) or received an in vitro produced (IVP) embryo with either interleukin-6, IL6-ET (100 ng/ml IL6; n=14) or control BSA, CONT-ET (0 ng/m; n=10l). CNL measurements were assed with transrectal ultrasonography weekly. A repeated measurement analysis was completed to examine overall effects (P values are included in the figure). An ANOVA was performed to examine the effects within each day. Differences within each day are designated with an asterisk [*].

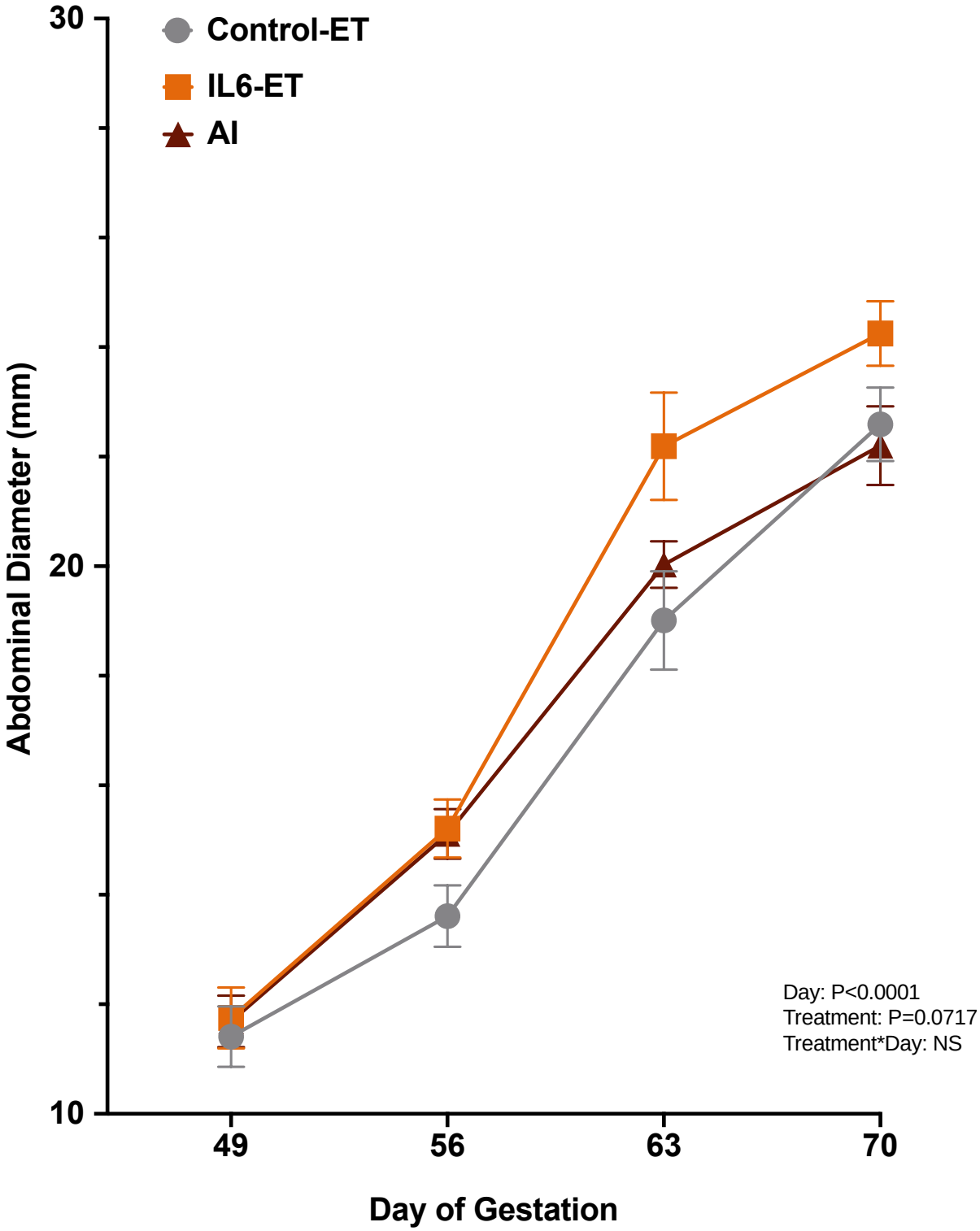

Control-ET
IL6-ET
AI
30
20
10
Abdominal Diameter (mm)
49
56
63
70
Day of Gestation
Day: P<0.0001
Treatment: P=0.0717
Treatment*Day: NS

Figure 2-6 Effect of treatment on abdominal diameter (AD) from days 56 to 70 of pregnancy. Cows were either artificially inseminated (AI; n=12) or received an in vitro produced (IVP) embryo with either interleukin-6, IL6-ET (100 ng/ml IL6; n=14) or control BSA, CONT-ET (0 ng/ml; n=10). AD measurements were assed with transrectal ultrasonography weekly. A repeated measurement analysis was completed to examine overall effects (P values are included in the figure). An ANOVA was performed to examine the effects within each day.

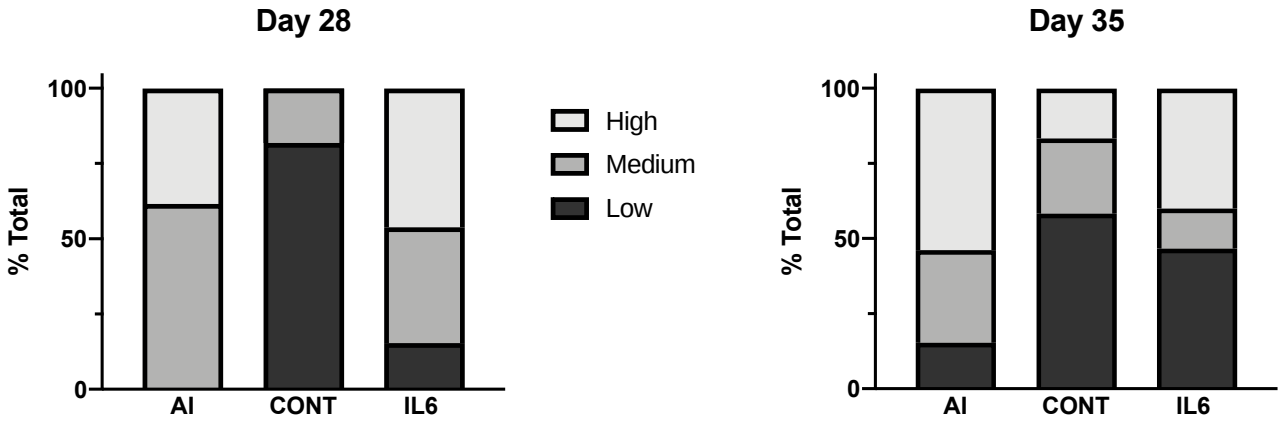

Day 28
Day 35
% Total
100
50
0
AI
CONT
IL6
High
Medium
Low

Figure 2-7 Uterine area. A difference in uterine volume was noted through visual observation of transrectal ultrasound clips on days 28 and 35. A scale of high, medium, and low were established to rank each uterine volume according to the amount of fluid present.

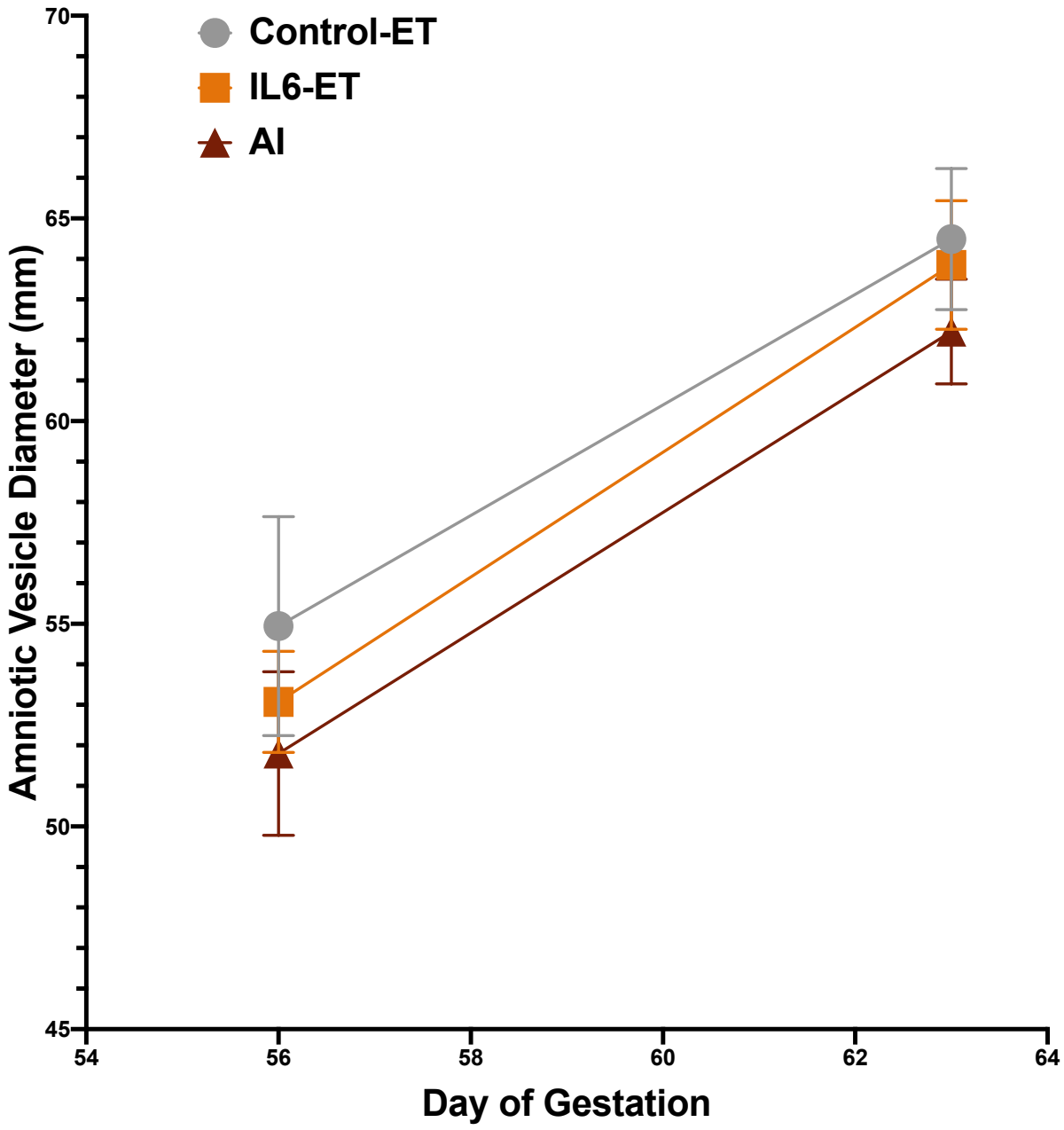
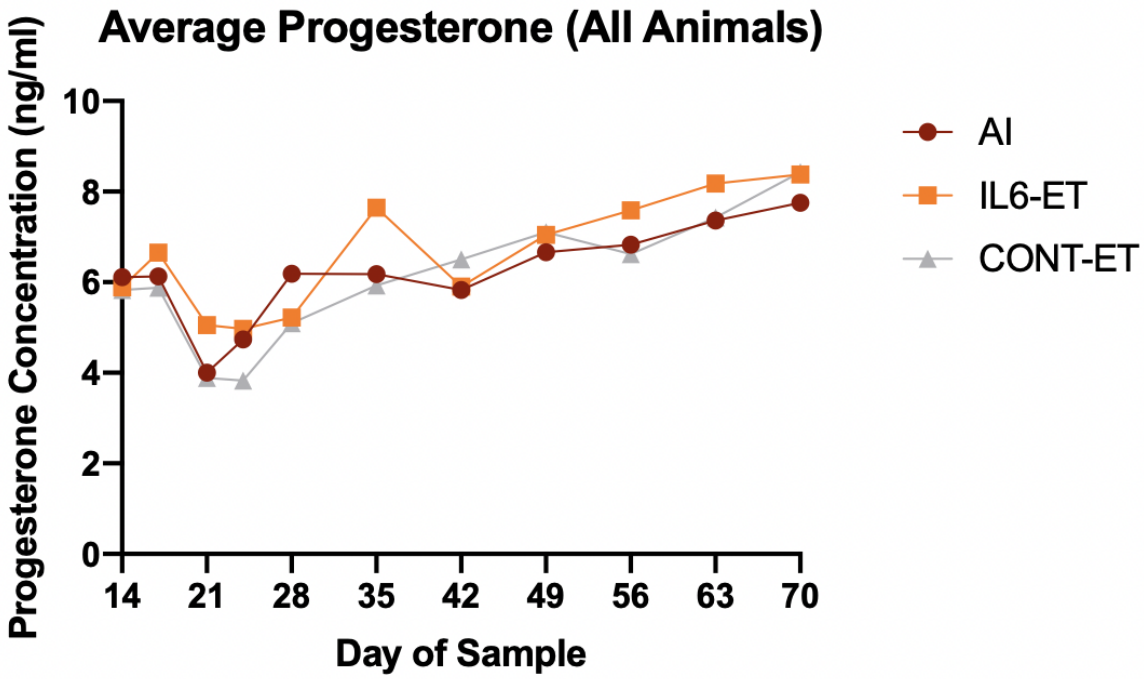

Control-ET
IL6-ET
AI
Amniotic Vesicle Diameter (mm)
70
65
60
55
50
45
54
56
58
60
62
64
Day of Gestation

Figure 2-8 Amniotic vesicle diameter. The diameter of the amniotic vesicle was measured on days 56 and 63. Cows were either artificially inseminated (AI) or received an in vitro produced (IVP) embryo with either interleukin-6, IL6-ET (100 ng/ml IL6) or control BSA, CONT-ET (0 ng/ml). AV measurements were assed with transrectal ultrasonography. No differences were detected among treatments within either day.


Average Progesterone (All Animals)
Progesterone Concentration (ng/ml)
10
8
6
4
2
0
14
21
28
35
42
49
56
63
70
Day of Sample
AI
IL6-ET
CONT-ET

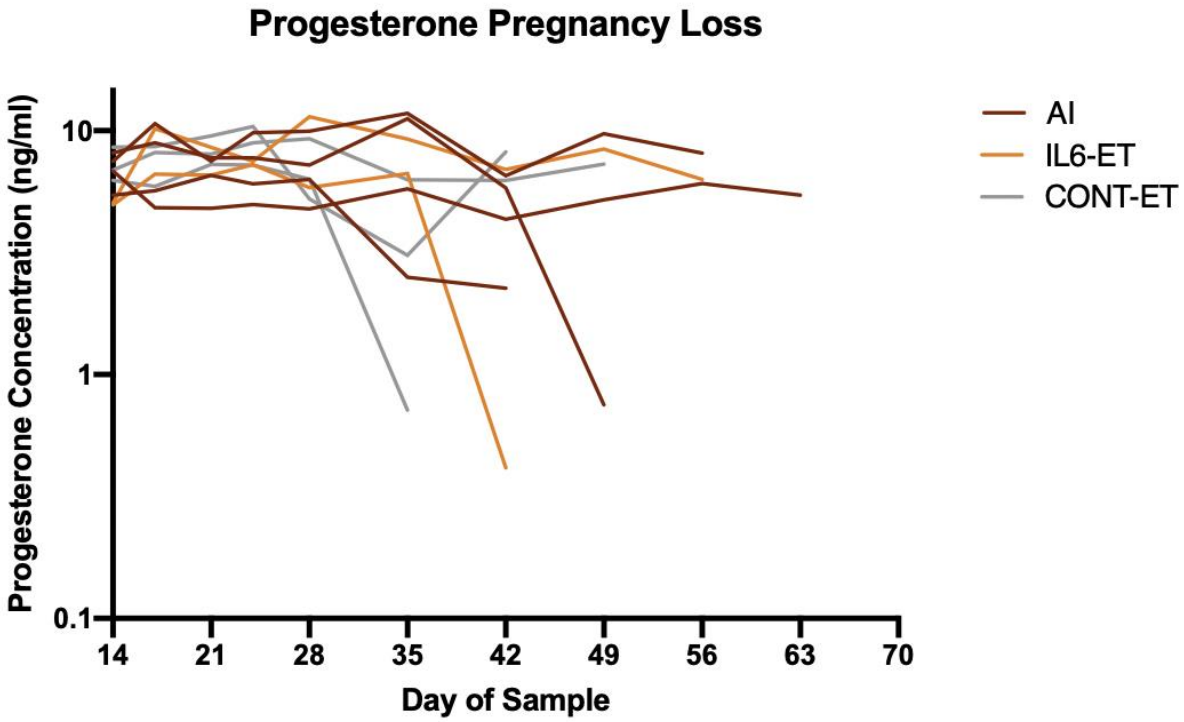

Progesterone Pregnancy Loss
Progesterone Concentration (ng/ml)
10
1
0.1
14
21
28
35
42
49
56
63
70
Day of Sample
AI
IL6-ET
CONT-ET

Figure 2-9 Progesterone profiles do not differ between pregnant and lost pregnancies. Blood samples were collected via coccygeal vein from treatment animals to be analyzed by IMMULITE chemiluminescent immunoassay. Concentrations of progesterone were plotted for each animal according to sampling day (Panel A). Panel B indicates animals that were diagnosed pregnant on day 28 and experienced pregnancy loss prior to day 70.

Table 2-1 Number of cows used in each experimental treatment for each replicate of the study.

	Replicate 1			Replicate 2 (Beef)			Replicate 2 (Dairy)			Replicate 3			Replicate 4			
	n[1]	BL[2]	EB[3]	n	BL	EB	n	BL	EB	n	BL	EB	n	BL	EB	
AI	10	-	-	5	-	-	9	-	-	8	-	-	8	-	-	**37**
Control	8	5	3	6	3	3	6	2	4	12	7	8	19	9	10	**51**
IL6	7	4	3	6	2	6	6	1	5	6	3	3	7	3	4	**35**

1Number of cows that were inseminated or used for ET in each replicate.

2Number of regular blastocysts used for each treatment in each replicate

3Number of expanded blastocysts used for each treatment in each replicate

Table 2-2 Number of male and female pregnancies in each experimental treatment for each replicate of the study.

	Replicate 1		Replicate 2 (Beef)		Replicate 2 (Dairy)		Replicate 3		Replicate 4		**Total**	
	M	F	M	F	M	F	M	F	M	F	M	F
AI	1	-	2	-	1	1	2	-	4	2	**10**	**2**
CONT	-	-	-	2	1	-	-	2	3	3	**4**	**7**
IL6	-	2	1	1	-	2	-	3	2	3	**3**	**11**

Chapter 4 - Appendix

Ultrasonographic Image Collection

Embryonic and fetal measurements were collected weekly using a portable transrectal ultrasound from Ibex EVO2 ultrasound with a linear 8-5 MHz multifrequency transducer (E.I. Medical Imaging, Loveland, Colorado). The Ibex EVO2 has a variety of settings to maximize the ease of use for the operator. Firstly, utilizing the “Repro Workflow” setting allows for animal identifications to be grouped collectively under a single scanning session. The “Repro Workflow” option can be activated under the “Misc Options” selection tool. To access the “Misc Options” table, press the “S key” (super key), followed by F1 (options), and F3 (Misc). Once on the table, select the tick for “Repro Workflow” to on. After the “Repro Workflow” is enabled, scanning sessions can be labeled by pressing the “Patient” key. This will open an interface allowing to type the desired identifier for the session in the top row and individual identification on the second. The length of video loops saved can also be changed under the “Misc Options” table. Ultrasonographic clips can range from 2-8 seconds, as well as a “start/stop” function. Simply select the desired length of the scan session.

It is recommended that the ultrasound technician have one to two available assistants for scanning pregnant animals. Individual animal identification should be typed prior to beginning the scan session. While the technician is scanning the reproductive tract, the assistant should stand beside the chute to allow for ease of saving video files. The screen should face the scanning technician, and when the desired image appears on the screen, the technician should notify the assistant. For 2-8 second video clips, continually scan over the desired structure for the length of the clip.

To save ultrasound cineloops, the assistant should press the “Freeze” key, which will freeze the clip. If the clip is desirable, the assistant should then select the “F1” key. This will save the clip with the identification number and scan session for the animal currently in the chute. If only an image is desired, simply pressing “Freeze” will store the current image on the screen as a .jpeg file, rather than an .avi file.

Analysis of Ultrasound Files

Analysis of ultrasound files for this study utilized the built-in caliper feature of the Ibex EVO2. To access previously saved cineloops or images, press the “Review” key on the keyboard. This will load the internal storage interface. From here, the desired scanning session and cow identifier will be selected. After location of the desired file, select the “F6” key on the keyboard to load the clip or image.

For video clips, the technician can slowly review the entire clip by clicking the right and left arrows on the keyboard. Once the desired image is present and frozen on the screen the caliper can be used. The caliper tool on the Ibex EVO2 utilizes the trackpad below the keyboard. To operate the caliper, simple select the beginning point of the measurement and pull the trackpad to the end of the measurement and click. This will present the measurement. Increments of measure can also be changed under the “Misc” table and changed between millimeters, centimeters, and inches.

Storage of Ultrasound Files

Once measurements are collected, files should be offloaded from the ultrasound to an external hard drive or flash drive. An USB port is located on the back of the Ibex EVO2 and can be exposed by removing the tab. Insert the USB flash drive into the port. To save files onto an external source, select the “S” super key on the keyboard, then

select the “F2” setup key, and finally the “F1” USB key. From this menu, “Export Files” should be selected, and the port with the USB will be selected.

Once on a flash drive, ultrasound clips can be uploaded to computer by dragging the folder from the flash drive to the computer’s desktop. The Ibex EVO2 saves video files in .avi form, which is not compatible with Quicktime player on Mac computers. For Mac users, a third-party application should be downloaded to view .avi files. This study utilized VLC. To view video clips on VLC, drag the video clips into the “Library” that opens when the application is started. Videos will begin playing automatically.

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