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Effects of Maternal Plane of Nutrition on Early Embryo Development and Offspring Performance

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EFFECTS OF MATERNAL PLANE OF NUTRITION ON EARLY EMBRYO DEVELOPMENT AND OFFSPRING PERFORMANCE

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Animal Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions by the candidate are necessarily the conclusions of the major department.

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"And I will bring the blind by a way that they knew not; I will lead them in paths that they have not known: I will make darkness light before them, and crooked things straight. These things will I do unto them, and not forsake them." *~Isaiah 42:16*

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ABSTRACT

EFFECTS OF MATERNAL PLANE OF NUTRITION ON EARLY EMBRYO DEVELOPMENT AND OFFSPRING PERFORMANCE

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Nutritional changes immediately after insemination can result in increased embryonic mortality, but the mechanisms that cause this increased mortality and the impact on the embryos that survive are not known. Therefore, two experiments were conducted to evaluate the impact of a nutritional change immediately following AI on calf performance, global DNA methylation, estrus expression and interval to estrus in heifers, peripheral metabolites, uterine luminal fluid metabolites, and day 6 embryo quality. In the first study (chapter 2) Angus-cross heifers (n = 142) were allotted into two developmental treatments: drylot or range. All heifers were fixed-time inseminated to a single sire each year and were turned out to pasture together and managed as a single group. Pregnancy success to AI was determined via ultrasonography, and calving data (calving date, birth weight, sex, and weaning weight) were collected. Samples of DNA were obtained from calves at weaning and analyzed for global methylation (total methylation and 5-hmC methylation). Results from this study indicate that heifer development and sire can impact future performance of the calf that is in utero as determined by BW and WW, and this change in performance may be regulated through DNA methylation.

In the second study (chapter 3) Angus-cross heifers (n = 60) were allotted into two pre-AI treatments: low or high. Low treatment heifers were limit-fed ground cornstalks and mineralized soybean meal and urea to achieve 64.1% maintenance. High

treatment heifers had ad libitum access to ground cornstalks and supplemented mineralized soybean meal, urea, and corn to achieve 139% maintenance. Heifers remained in their respective treatments for 33 to 36 days and were then inseminated upon estrus expression from a single collection of a single beef sire. Following AI, 30 heifers were randomly reassigned within treatment, creating four nutritional treatments: low treatment remaining on low (LL), low treatment moving to high (LH), high treatment remaining on high (HH), and high treatment moving to low (HL). Heifers remained on treatments post-AI for six days and were then flushed for embryo and uterine luminal fluid (ULF) collection. Blood samples were collected daily from AI to embryo collection. Results from this study indicate that the early stages of embryo development are sensitive to maternal plane of nutrition even though ULF metabolite concentrations are not representative of peripheral metabolite concentrations during the first six days of gestation. Therefore, proper nutritional management is critical around the time of AI to ensure heifers are not placed in a negative energy balance nor adversely impacting embryo survival and early conceptus development. Breeding season pregnancy success of heifers after this study was impacted by nutritional treatments from the study's feeding trial. Although heifers were fed at a positive plane of nutrition for 30 days after the study prior to AI and throughout the breeding season, nutrient restriction of heifers six days after AI may be equally as detrimental in pregnancy success compared to 33-36 days of nutrient restriction prior to AI.

In summary, nutritional management of heifers prior to and immediately following AI has the potential to impact embryo development and the critical window of

DNA methylation, which may mediate future impacts on post-natal development of the offspring.

CHAPTER I

REVIEW OF LITERATURE

INTRODUCTION

Reproductive efficiency is critical to maintaining females in the beef herd. Young beef females require additional management for successful establishment and longevity within the herd, as reproductive failure is the primary reason for heifers leaving the herd (Short et al., 1990). Reproductive failure also imposes a major economic loss to beef enterprises, netting approximately $502 million annually for beef producers (Bellows et al., 2002). While fertilization rates among beef cattle are at 90%, calving rates from a single insemination are at 50%, suggesting that embryonic mortality accounts for 44% of reproductive failure (Diskin and Sreenan, 1980). The majority of this embryonic mortality occurs between eight to 16 days post insemination (Diskin et al., 2011) and may be a result of several factors, including genetic defects, disease, environmental stress such as heat, and nutrition (Bridges et al., 2012). Thus, nutrition and maternal energy balance play a pivotal role in embryo and fetal development, especially during conception and early embryo development (Diskin et al., 2011).

The purpose of this chapter is to examine the relationship between nutrition and reproductive functions. This review will briefly describe the bovine estrous cycle and embryo development and then go into further details about maternal nutrition's impacts on the estrous cycle, uterine environment, embryogenesis, and how method of heifer development influences the epigenetic mechanisms that dictate in utero and postnatal development.

BOVINE ESTROUS CYCLE

The bovine follicular phase typically consists of two or three follicular waves. A follicular wave consists of the recruitment of small growing follicles that respond to an increase in follicle stimulating hormone (FSH). The follicle that will eventually ovulate is selected from these growing follicles and secretes increasing concentrations of estradiol under the influence of FSH and luteinizing hormone (LH). Dominant follicles that emerge when a corpus luteum (CL) is present become atretic (Hansel and Convey, 1983); however, the dominant follicle that is present following luteal regression continues to grow and further increases in the secretion of estradiol. High concentrations of estradiol stimulate a period of sexual receptivity in females during the time of estrus (d 0 of estrous cycle), which lasts on average between 12 – 16 h (Allrich, 1994). Estradiol concentrations peak around 36 h prior to ovulation, with ovulation occurring 25 – 29 h following the onset of estrus (Chenault et al., 1975; Bernard et al., 1983). High concentrations of estradiol during the period of low progesterone increases the anterior pituitary's sensitivity to gonadotropin releasing hormone (GnRH) and results in a preovulatory surge of LH. This LH surge triggers a cascade of events resulting in ovulation of the dominant follicle.

Following ovulation, the female enters the luteal phase. Progesterone concentrations increase in conjunction with the development of the CL. Minimal estradiol concentrations are reached by d 2 of the estrous cycle and remain low until d 11 (Wettemann et al., 1972). The high concentrations of progesterone block expression of estrus and GnRH receptors from releasing GnRH, thereby inhibiting LH (Nett et al., 2002).

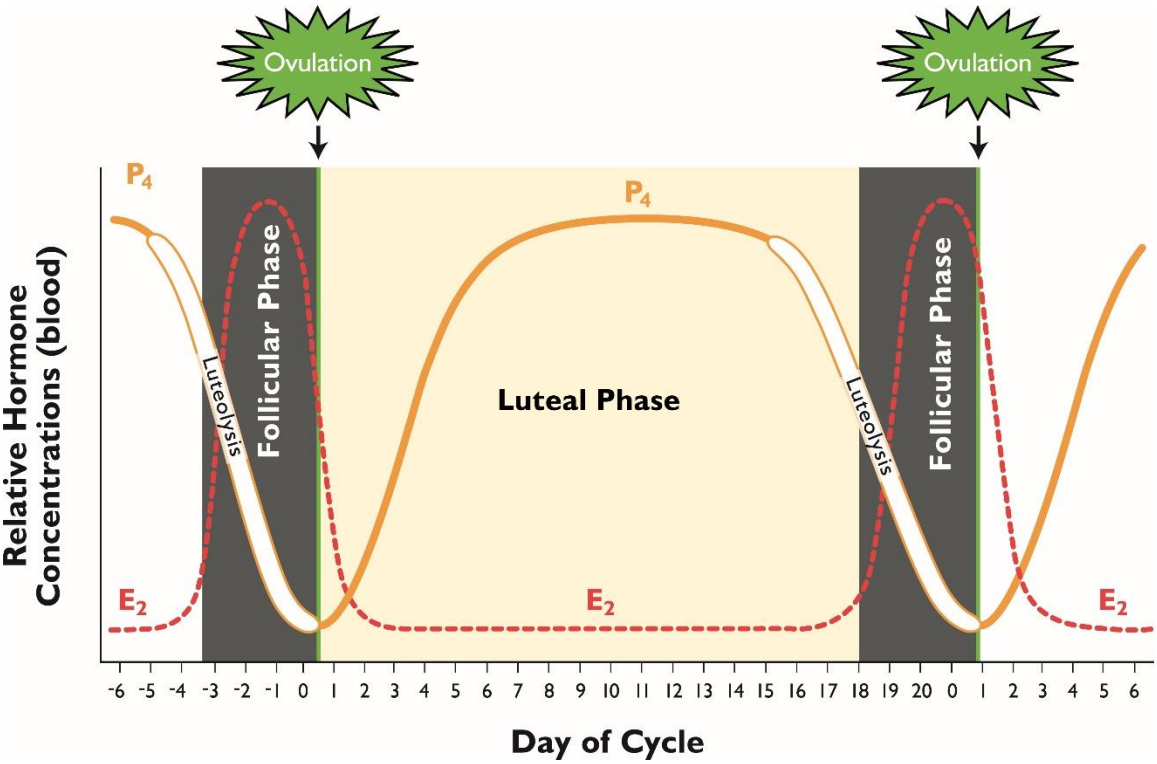

Figure 1. Hormonal profiles associated with the varying stages of the bovine estrous cycle (Senger, 2003).

BOVINE EMBRYO DEVELOPMENT

Fertilization of the oocyte by the sperm occurs within the oviduct following ovulation of the dominant follicle on d 1, with the embryo entering the uterus on approximately d 3–4 (Black and Davis, 1962). The embryo undergoes a series of cell divisions to form a morula at the 16-cell stage before developing into a blastocyst comprised of the inner cell mass and an outer cell layer (trophectoderm). The inner cell mass will further differentiate into the embryo proper, whereas the trophectoderm will progress into the placenta (Forde and Lonergan, 2012). The blastocyst hatches from the zona pellucida on d 9 (Forde and Lonergan, 2012) and experiences a phase of exponential growth as it develops from a spherical to elongated shape from d 12–16 (Grealy et al., 1996). During the elongation phase the bovine blastocyst secretes increasing levels of

interferon-τ from d 8 through d 17–19 to inhibit proliferation of oxytocin receptors in the endometrium and signal maternal recognition of pregnancy (Goff, 2002). The blastocyst floats freely in the endometrium during this period and is reliant on maternal uterine secretions known as the histotroph for survival (Forde and Lonergan, 2012). Contact with the uterus is not necessary until attachment occurs on d 19 with actual adhesion to the uterus occurring d 21–22, followed by placentation on d 25 (Chavatte-Palmer and Guillomot, 2007).

Synchrony between the uterine environment and blastocyst is critical during the preimplantation period for embryo survival and development (Goff, 2002) and requires an endometrial environment specific to the evolving nutritional demands of the rapidly growing blastocyst. Most species have an implantation window of 1–2 days, and asynchrony between the blastocyst and uterine environment will result in impaired implantation, embryo development, and possible abortion (Chavatte-Palmer and Guillomot, 2007). Embryo development is impeded during embryo transfer if the uterus is not as mature as the blastocyst, while development is expedited when embryos are placed into a more mature uterus (Lawson et al., 1983; Wilmut and Sales, 1981). The postovulatory rise in progesterone regulates synchrony between the embryo and uterine environment by acting on the endometrial epithelium to secrete factors necessary for embryogenesis. The timing of the progesterone rise is vital for embryo development, whereas sufficient progesterone concentrations are needed to prevent luteolysis (Goff, 2002). Maternal plasma progesterone concentrations are also positively correlated with conceptus production of interferon-τ; low maternal progesterone concentrations altered the histotroph and thereby impaired the embryo's ability to secrete interferon-τ to prevent

prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion and luteolysis while also reducing conceptus growth (Kerbler et al., 1997; Mann et al., 2006).

Elongation and development of the conceptus is dependent on the uterine environment. The endometrium consists of luminal epithelial, superficial epithelial, deep glandular epithelial, and fibroblast-like stromal cells that provide secretions for conceptus growth and development (Forde and Lonergan, 2012). Secretions from these cells and molecules transported into the lumen create a composite of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, electrolytes, and glucose, and changes in these secretions play a pivotal role in the determinant survival and development of the embryo (Gao et al., 2009a). Therefore, factors impacting the uterine environment indirectly influence embryogenesis.

IMPACTS OF MATERNAL NUTRITION ON REPRODUCTION

Successful conception and establishment of pregnancy are dependent on adequate nutrition. In circumstances when nutrition is restricted, the body allocates energy to bodily functions determined most important during periods of limited energy availability. The classic partitioning of nutrients is outlined as follows: 1) basal metabolism and activity, 2) growth, 3) basic energy reserves, 4) pregnancy, 5) lactation, 6) additional energy reserves, 7) estrous cycles and initiation of pregnancy, and 8) excess reserves (Short et al., 1990). Therefore, if nutrient intake is not meeting energetic demands of the female, initiation and maintenance of normal estrous cycles and successful establishment of pregnancy will be one of the first functions terminated. Young females are particularly susceptible to changes in nutrient management during pregnancy and lactation, as they are still partitioning nutrients towards growth.

Nutritional Impacts on Estrous Cycle

Nutritional restriction has various impacts on the bovine estrous cycle that inhibit the female from properly cycling. Changes in ovarian activity are likely modulated through changes in circulating gonadotrophins. Concentrations of FSH are greater in animals during nutrient restriction compared to non-restricted animals (Bossis et al., 1999), particularly during pre-emergence of a follicular wave (Mackey et al., 2000). Changes in the activin-inhibin-follistatin axis on the anterior pituitary may be responsible for increased FSH concentrations (Mackey et al., 1999). Restricted feed intake in cattle will also suppress LH secretion and reduce LH pulse frequency (Rhodes et al., 1996). While depressed levels of LH are not noticeable during short periods of acute nutrient restriction (Mackey et al., 2000; Mani et al., 1996), reduced LH concentrations are evident during chronic nutrient restriction and are prominent two cycles before animals enter anestrous (Bossis et al., 1999). Without sufficient circulating concentrations of LH, the dominant follicle will fail to mature and ovulate.

Functionality of the CL and Progesterone

Functionality of the CL may be compromised due to feed restriction. Diameter and weight of the CL decreased in response to feed restriction (Bossis et al., 1999; Rasby et al., 1991), which might explain why some studies have also reported decreased progesterone during dietary restriction (Villa-Godoy et al., 1990). However, high-intake diets have also reported a reduction in circulating progesterone concentrations (Parr et al., 1987). In sheep, plasma progesterone concentrations of ewes fed at 200% maintenance are consistently diminished, likely due to increased hepatic blood flow and higher progesterone metabolism (Williams and Cumming, 1982). However, plasma

progesterone concentrations in feed-restricted cattle are more variable, with reports of increased or no difference in progesterone concentrations due to nutrient restriction (Nolan et al., 1998b; McCann and Hansel, 1986; Murphy et al., 1991). These differences may be dependent on severity of dietary restriction and sampling protocol.

Interval to Estrus

Interval to estrus may also be impacted by nutrient manipulation. Minimal data has been collected in this area for cattle; however, onset of estrus after $PGF_{2\alpha}$ administration among goats fed at 25% maintenance tended to be longer compared to goats fed at 100% maintenance (Mani et al., 1992), and does that were assigned to a low energy diet also expressed estrus later compared to does assigned to a moderate or high energy diet (Kusina et al., 2001). Although the mechanism dictating longer interval to estrus is not determined, nutrient restriction resulted in smaller dominant follicle size (Mackey et al., 2000) and reduced concentrations of LH (Bossis et al., 1999). Smaller dominant follicles may produce less estradiol due to reduced LH pulses and follicular growth rate (Mackey et al., 1999), thereby extending the female's interval to estrus. While Bossis et al. (1999) did not report detectable differences in estradiol concentrations in nutrient restricted heifers until the cycle preceding anestrous, minimal changes in estradiol concentrations in response to nutrient restriction may be enough to delay the onset of estrus.

Anestrus

Anestrus is defined as lack of luteal activity (Richards et al., 1989). Long-term nutritional deprivation will result in loss of body weight and body condition, eventually arresting estrous cycles (Imakawa et al., 1986). The mechanisms signaling anestrus have

been under scrutiny. Contrary to previous hypotheses, reduced insulin concentrations do not appear to signal onset of anovulation, as heifers still ovulated despite a 50% reduction in insulin concentrations (Bossis et al., 1999). Although the exact mechanisms resulting in anestrus in cattle are still being examined, reduction in LH pulse frequency and suppressed GnRH secretion may induce failure to ovulate (Rhodes et al., 1996). Ovarian estradiol appears to decrease LH secretion from the pituitary by suppressing GnRH secretion from the hypothalamus during anestrus (Imakawa et al., 1986).

Nutritional Impacts on Follicular Development

While nutrient restriction of the dam may alter hormone secretions and impair CL functional as previously described, follicular dynamics may also change in response to maternal nutrient changes.

Small Follicle Count

Short-term undernutrition does not appear to impact follicle count (Gutiérrez et al., 1997), but increasing the plane of nutrition may have beneficial impacts on follicular development. Feeding heifers 200% of maintenance requirements elevated plasma insulin concentrations and was correlated with increased recruitment of small ovarian follicles, which indicates that insulin may assist in mediating nutritional changes on follicular development (Gutiérrez et al., 1997). Insulin may also increase sensitivity of follicles to gonadotropins and enhance steroidogenesis from granulosa cells *in vitro*, although these mechanisms are still under scrutiny (Downing and Scaramuzzi, 1991).

Follicle Diameter and Size

Several studies have reported a decrease in growth rate and maximum diameter of dominant follicles due to limited nutrient intake (Bergfeld et al., 1994; Mackey et al.,

2000; Murphy et al., 1991; Rhodes et al., 1996). Cattle with restricted dietary intake exhibited smaller dominant follicles and more three-wave estrous cycles compared to animals with higher dietary intake (Murphy et al., 1991). Heifers that underwent acute nutritional restriction from 120% maintenance to 40% maintenance exhibited decreased follicular growth rate and maximum follicle diameter at time of CIDR removal and during the subsequent dominant follicle, with 60% of heifers becoming anovular (Mackey et al., 1999). Failure to ovulate may have been due to a lack of LH pulsatility, resulting in a lack of androgen production needed to prime the hypothalamus for a GnRH surge and subsequent ovulation (Bossis et al., 1999; Rhind et al., 1985). Decreased diameter of dominant follicles has also been associated with reduced estradiol concentrations, mainly during the cycle preceding anestrous (Bossis et al., 1999; Rhodes et al., 1996).

Supplementing animals with dietary fat may improve follicle number and size of the preovulatory follicle (Beam and Butler, 1997). Leptin may be a potential mediator; although thecal cell viability remained unaffected, leptin increased the proliferation of insulin-induced thecal cells (Spicer and Francisco, 1998), thereby enhancing follicle number.

Steroidogenesis

Estradiol and androstenedione production of granulosa and theca cells may be antagonized by physiological concentrations of leptin (Armstrong et al., 2003; Spicer 2001). Leptin had no effect on granulosa cell number, but leptin concentrations inhibited insulin-induced progesterone and estradiol production of granulosa cells in both small and large bovine follicles (Spicer and Francisco, 1997). Furthermore, leptin inhibited

insulin, IGF-I, and LH-induced androstenedione production from thecal cells. Therefore, high concentrations of leptin may inhibit ovarian function by restricting granulosa and thecal cell steroidogenesis and lead to decreased estradiol concentrations.

Recruitment of small follicles and development of the dominant follicle are impacted by both undernutrition and overnutrition, and this sensitivity may be mediated by fluctuations in hormones, gonadotropins and steroids.

Nutritional Impacts on Oocyte Development

While follicular development appears to be stimulated to a certain extent by increased maternal nutritional intake, oocyte competence may benefit from short-term nutrient restriction. In superovulated animals, a high plane of nutrition may result in degraded oocyte quality and subsequent blastocyst (Freret et al., 2006). Postpartum dairy cows fed a diet stimulating increased insulin production until resumption of cyclicity were then fed a diet inducing decreased insulin production during the mating period, which resulted in increased pregnancy rates compared to cows in opposing treatments (Garnsworthy et al., 2009). These results suggest that follicular development and oocyte/embryo competence may benefit from two different nutritional strategies (Ashworth et al., 2009). A recent study also reported that both cumulus cells and oocytes have insulin receptors, and cumulus cells also undergo insulin-stimulated glucose uptake, which may explain how the effects of high dietary intake and insulin are communicated to the oocyte (Purcell et al., 2012).

Protein

High levels of rumen degradable protein are also detrimental to oocyte quality. Elrod and Butler (1993) reported conception rates reduced by at least 30% in heifers fed

diets exceeding ruminally degradable protein by 50% and attributed this to hostile alterations within the uterine environment as evidenced by decreased uterine pH levels in heifers fed excess protein (Elrod and Butler, 1993). Protein catabolism produces ammonia and urea, which may be toxic to both the oocyte and subsequent embryo (Leroy et al., 2008). These metabolites impair the growth and metabolism of the surrounding granulosa cells, which lose their ability to support oocyte maturation *in vitro* (Rooke et al., 2004) and ultimately result in impaired fertility (Elrod and Butler, 1993).

Cholesterol

Maternal cholesterol concentrations have also been reported to affect oocyte competence. Oocytes acquire fatty acids from their environment, thus altering their lipid configuration (Leroy et al., 2005). Oocytes containing a greater lipid content result in embryos with reduced viability, which may be related to compromised metabolism and impaired mitochondrial activity in the oocyte (Igosheva et al., 2010).

Glucose and NEFA Concentrations

Glucose and non-esterified fatty acid (NEFA) concentrations are additional indicators of oocyte quality. Suppressed glucose concentrations inhibit cumulus expansion *in vitro* and may further impair oocyte maturation in cattle (Leroy et al., 2006). Hyperglycemia is also responsible for altered oocyte maturation and, although not yet studied in cattle, may be responsible for impaired oocyte competence (Sutton-McDowall et al., 2010).

Ensuring animals are not in a negative energy balance is also crucial for oocyte development. Placing animals in a negative energy balance resulted in heightened circulation of NEFA concentrations, which has been reported to reduce oocyte quality *in*

vitro and result in apoptosis of cumulus cells (Diskin and Morris, 2008; Leroy et al., 2005). Oocyte maturation and preimplantation embryogenesis are critical periods when epigenetic reprogramming, such as DNA methylation, occurs, making the oocyte and early embryo susceptible to changes in the follicular fluid and uterine environment (Tang and Ho, 2007). Oocytes exposed to elevated NEFA concentrations *in vitro* resulted in blastocysts that exhibited altered DNA methylation patterns, affecting pathways related to cell death, survival, and cellular metabolism (Desmet et al., 2016). The negative impact of elevated NEFA concentrations may be less prominent on oocyte maturation compared to blastocyst development, as oocytes are protected by cumulus cells, which incorporate lipids that protect the oocyte from in vitro induced lipotoxic effects (Lolicato et al., 2015).

Leptin

Leptin is a peptide involved in the regulation of food intake and energy expenditure and has also been identified as a factor in reproductive functions (Gonzalez et al., 2000). Leptin has been associated with enhanced oocyte maturation, and when supplied at increasing physiological concentrations, leptin enhanced oocyte maturation, exerted anti-apoptotic effects on cumulus cells surrounding the oocyte and further improved subsequent blastocyst quality (Boelhauve et al., 2005). These anti-apoptotic effects indicate that leptin may enhance survival of the oocyte and minimize cellular damage.

Although not yet reported in cattle, *in vitro* and *in vivo* studies in rats have reported that leptin at high concentrations has been linked to ovulation failure (Ricci et

al., 2006). Leptin may have inhibitory impacts on prostaglandins, nitric oxide and steroids, thereby hindering follicle rupture.

Oocyte competency can be altered due to changes in metabolites, hormones, and steroids of the follicular fluid. These impacts can further influence subsequent blastocyst cleavages and preimplantation development of the embryo.

Nutritional Impacts on Embryo Survival and Development

Embryo survival and development are sensitive to maternal nutritional changes via the uterine environment, which are mediated through alterations in circulating components of the histotroph, including metabolites, hormones and steroids (Ashworth et al., 2009). Both low and high planes of nutrition can alter the uterine environment and impair embryogenesis. Cumming et al. (1975) reported that embryo survival was greatest among ewes fed at 100% maintenance compared to ewes fed at 200% maintenance and ewes fed at 25% maintenance days 2 – 16 after breeding. Therefore, achieving nutritional balance is vital for optimal reproductive success.

Undernutrition: Alterations in Histotroph

Nutrient restriction alters uterine secretions by reducing insulin-like growth factor I (IGF-I) and glucose concentrations while increasing growth hormone and NEFA concentrations (Bossis et al., 1999). Undernutrition may place animals in a negative energy balance, causing maternal weight loss and mobilization of body lipids, thereby creating an increase in NEFA concentrations (Vizcarra et al., 1998). Elevated NEFA concentrations significantly reduced the number of oocytes capable of reaching the blastocyst stage by d 7 post-insemination due to an increase in the apoptotic cell ratio, altered metabolic functions and gene transcription that contributed to oocytes' decreased

developmental capacity (Van Hoeck et al., 2011). Embryos exposed to elevated NEFA concentrations *in vitro* have been reported to exhibit altered pathways associated with lipid and carbohydrate metabolism as well as down-regulated genes related to embryonic cell growth, cell differentiation, and cell-cell interaction (Desmet et al., 2016). Energy restriction may also increase endometrial production of $PGF_{2\alpha}$, creating a hostile environment for embryo survival and development (Lozano et al., 2003). Increases in $PGF_{2\alpha}$ in nutrient-restricted ewes may be due to the conceptus producing less interferon-τ (Abecia et al., 1999); however, as minimal animals were used in the study, results are not conclusive.

Undernutrition: Embryo Survival and Viability

Embryo development in response to maternal nutrient restriction may vary, depending on if the embryo is cultured via *in vitro* fertilization (IVF) or within a physiological environment. A six-week period of dietary restriction enhanced *in vitro* blastocyst production and embryo quality in overfed dairy heifers (Freret et al., 2006). Nutrient restriction also increased the number of transferable embryos when cultured *in vitro* for 24 hours, resulting in an increase in total cell number per blastocyst (Nolan et al., 1998a).

Although embryos *in vitro* demonstrate increased viability, IVF may not be a consistent representation of metabolic parameters within the uterine environment. Physiological conditions of nutrient restriction can have detrimental effects on embryo survival and development. Dunne et al. (1999) reported a reduction from 65 to 38% in embryo survival measured from d 14–16 when decreasing heifers' intake from 2.0 to 0.8 times maintenance immediately following insemination. Heifers fed at 85% of

maintenance requirements for energy and protein exhibited reduced pregnancy success, with a reduction in fertilization rate for heifers that underwent nutrient restriction (Hill et al., 1970). Furthermore, embryos flushed six days post-AI from heifers undergoing nutrient restriction after breeding exhibited reduced quality and stage of development compared to embryos from heifers on a control diet (Kruse et al., 2017). These studies suggest that post-AI nutrition can have significant impacts on embryo development even when pre-breeding nutrition is adequate. Several processes by which nutrient restriction may alter embryo development have been suggested, although the precise mechanisms are not yet clearly elucidated.

Overnutrition: Embryonic Mortality

Maternal overnutrition has also been associated with embryonic mortality, specifically within sheep (Brien et al., 1981; Cumming et al., 1975; El-Sheikh et al., 1955). Ewes that were fed high energy rations exhibited significantly reduced pregnancy rates compared to ewes fed low or medium rations, except for ewes given progesterone inserts d 8–14 following mating (Parr et al., 1987). These results are less clear in bovine embryo studies; however, *in vitro* studies indicate that overnutrition appears to be more harmful for blastocyst development than undernutrition (McEvoy et al., 1995; Nolan et al., 1998a). Whether these results can be replicated in physiological conditions has yet to be fully demonstrated.

Overnutrition: Glucose

Oversupplying glucose may have detrimental effects on bovine embryogenesis, as early embryonic development is sensitive to glucose (Wrenzycki et al., 2000). High concentrate diets typically lead to high propionate production. Propionate is the main

source for gluconeogenesis and increases both circulating glucose and insulin levels (Istasse et al., 1987). The embryo and endometrium are not capable of generating glucose and are therefore reliant on peripheral glucose concentrations, especially as metabolic needs for glucose increase throughout embryogenesis (Gao et al., 2009b); thus, glucose availability is critical for adequate embryogenesis. However, *in vitro* studies have reported that high glucose concentrations negatively affect embryo development as morulas progress into blastocysts (Jiménez et al., 2003; Kimura et al., 2005). Absence of glucose during the first 24 h of culture did not hinder blastocyst quality or cell number, whereas increasing glucose concentrations impaired embryo viability (Furnus et al., 1997), and physiological increases of glucose due to high propionate diets resulted in degraded embryo quality (Yaakub et al.,1999) due to retarded metabolic development (Moley et al., 1996). This indicates that before blastocyst formation, glucose may not be a preferred substrate for early preimplantation development (Leese and Barton, 1984).

Overnutrition: Insulin

Insulin has stimulatory effects on follicular development, but its influence on embryo development is more variable. Elevated insulin concentrations appeared to enhance early embryogenesis in beef heifers with no impact on maternal progesterone concentrations (Mann et al., 2003). Dairy cows fed diets to increase insulin production before the first rise in progesterone postpartum and then moved to a diet that resulted in decreased insulin production exhibited significantly higher pregnancy rates compared to cows fed diets resulting in increased insulin production after the progesterone rise, suggesting that insulin benefits follicular development but hinders embryo survival (Garnsworthy et al., 2009). Insulin receptors have been observed in the bovine embryo

from the 1-cell zygote to the blastocyst stage (Schultz et al., 1992). Embryos are sensitive to an overdose of insulin, as embryos supplemented in maturation media with insulin at 10 or 0.1 μg mL^{-1} (physiological dose) exhibited decreased blastocyst rates compared to insulin-free controls (Laskowski et al., 2017). Overdosing maturing oocytes with insulin may also lead to long-lasting repercussions in blastocyst development *in vitro* (Laskowski et al., 2017), indicating that the blastocyst may have decreased tolerance compared to the oocyte for insulin.

Overnutrition: Protein

Conceptus metabolism may also be impacted by changes in maternal nutrition. Embryos recovered from ewes fed supplementary urea for 12 weeks showed retarded development compared to control embryos and exhibited greater glucose metabolism when cultured *in vitro* (McEvoy et al., 1997), indicating that increased urea intake may alter the metabolic efficiency of the conceptus in response to excess ammonia (Ashworth et al., 2009). Metabolic byproducts of high crude protein diets (ammonia and urea) appear to initiate conception failure/embryonic mortality and may interfere with normal inductive actions of progesterone within the endometrium (Mondal et al., 2015). Furthermore, high urea concentrations may be responsible for alterations in the ionic composition of uterine fluid, increased secretions of $PGF_{2\alpha}$, and a more hostile environment for sperm motility and viability (Butler, 1998; Elrod and Butler, 1993; Leroy et al., 2008). These studies suggest that avoiding excessive crude protein intake is essential for successful blastocyst development and resulting pregnancy success.

However, an increase in uterine pH in response to excess protein intake has also been reported in cattle (Grant et al., 2013), indicating that impaired fertility and embryo

survival in response to excess protein intake may be through other mechanisms than increased ammonia concentrations. A review by Adams (1995) reported that legumes high in phytoestrogens are commonly incorporated into high protein diets to elevate nitrogen content. These phytoestrogens, specifically isoflavones, are similar in chemical structure to estrogen, enabling them to bind to estradiol receptors in the endometrium to produce estrogenic activity (see review by Adams, 1995) and may be responsible for reported infertility in animals fed high protein diets with legumes (Canfield et al., 1990; Elrod and Butler, 1993).

Synchrony Between Uterus and Embryo: Progesterone

Adequacy of the uterine environment for embryogenesis is dependent on maternal progesterone production (Mann et al., 2003). Nutritional intake of the dam may impact circulating progesterone concentrations and influence conceptus development. Maternal peripheral progesterone concentrations are necessary to stimulate interferon-τ from the conceptus (O'Callaghan et al., 2000). Without sufficient and timely increases in progesterone from the dam, the conceptus will fail to produce adequate concentrations of interferon-τ to prevent luteolysis and support additional embryonic growth, potentially leading to embryonic mortality (Kerbler et al., 1997; Mann et al., 2006). Nutrition and circulating progesterone concentrations may be inversely related (Williams and Cumming, 1982). Overfeeding has been associated with reduced progesterone levels and lower pregnancy rates in sheep (Parr et al., 1987), which suggests increased progesterone metabolism in animals consuming a high plane of nutrition may be a mechanism for mediating a less favorable uterine environment for embryo survival (Nolan et al., 1998b). However, circulating progesterone concentrations do not appear to be as imperative in

beef heifers, as progesterone concentrations did not differ between pregnant and non-pregnant heifers (Dunne et al., 1999; Hill et al., 1970; Mann et al., 2003). Some studies differ in their reports of progesterone's response to maternal malnutrition. Hill et al. (1970) attributes low fertilization rates in underfed heifers to be a potential result of reduced progesterone concentrations when compared to control heifers. As CL sizes and weights did not differ between underfed and control heifers, the mechanism behind this occurrence was not fully determined. Lozano et al. (1998) reported that while underfed ewes had increased peripheral concentrations of progesterone, endometrial progesterone concentrations were reduced in comparison to overfed ewes, while no differences were seen pertaining to ovarian or uterine venous progesterone concentrations. The mechanism dictating differences in progesterone concentrations throughout the body are not understood, but peripheral progesterone concentrations may not be an accurate representation of endometrial progesterone and may have minimal relevance when determining the impacts of progesterone on conceptus development.

Although the complex pathways by which metabolites impact embryogenesis are not fully understood, evolving research is demonstrating that maternal malnutrition alters histotroph secretions and detrimentally influences embryo development.

Impact of Heifer Development on Pregnancy Success

Additional factors may influence maternal diet aside from direct manipulation of dietary energy density. These factors are important when considering nutritional needs during heifer development and consequent impacts on pregnancy success and offspring performance. Heifers need to calve by 24 months of age to ensure maximum life-time productivity, and the earlier heifers conceive and calve, the more likely those heifers will

breed back as two-year olds and continue calving early in subsequent calving seasons (Cushman et al., 2013). Females that calve early as two-year olds have sufficient time to nutritionally recover during the postpartum period and begin cycling for the upcoming breeding season compared to heifers that calve late in the breeding season. Females that calve early during subsequent calving seasons will also wean heavier calves and be more profitable for producers (Dzuik and Bellows, 1983). Heifers need to wean between three to five calves to compensate for their development costs (Clark et al., 2005). Enabling heifers to attain this minimum longevity in the beef herd is dependent on proper nutritional management and ensuring heifers achieve their first pregnancy. Heifer pregnancy success may be impacted by method of heifer development post-weaning. Method of heifer development can impact how heifers adapt to management after AI in a grazing setting, which may affect embryo survival and development (Perry et al., 2009).

Replacement Heifer Development

Grazing is a learned behavior that animals acquire from adults prior to weaning (Provenza and Balph, 1987), and grazing experience early in life plays a fundamental role in developing livestock's future foraging and harvesting skills, as livestock's willingness to try novel foods declines during the first year of life (Lobato et al., 1980).

At weaning, heifers are typically moved to a confined feeding situation to maintain proper nutritional management (Olson et al., 1992). Alternatively, heifers may be developed in a forage setting to ensure that heifers maintain familiarity with the grazing environment. At weaning heifers exhibit increased forage consumption, as they are no longer reliant on their dams for the majority of their dietary intake through milk (Lyford, 1988). The development period between weaning and the following breeding

season is critical to ensure that heifers reach an appropriate percentage of mature body weight as well as maintain adequate body condition. Heifers that achieve 65% of their mature body weight and sustain a body condition score of six by the beginning of the breeding season should have attained puberty and be capable of maintaining a successful pregnancy (Hall et al., 1995; Patterson et al., 1991). Funston and Larson (2011) have reported that heifers reaching 56% of mature body weight by the beginning of the breeding season tended to have reduced AI conception rates compared to their counterparts reaching 65% of mature body weight, but neither final pregnancy rates nor percentage of heifers calving in the first 21 days differed between groups, indicating that heifers may be developed below 65% of mature body weight without compromising the number of animals bred in the first cycle.

Effects of Heifer Development on Pregnancy Success

During the breeding season heifers are commonly placed in a forage grazing setting. Prior grazing experience impacts heifers' capability to successfully select a diet to meet their nutritional needs. Heifers that grazed between weaning and breeding exhibited better retention of grazing skills gained prior to weaning and had increased average daily gains the following summer compared to heifers placed in a drylot (Olson et al., 1992). Heifers developed in a drylot do not utilize grazing skills acquired during the pre-weaning period; therefore, when they are introduced to a new grazing environment, they will expend more time and energy in foraging (Osuji, 1974) but will ingest less nutrients (Arnold and Maller, 1977; Curll and Davidson, 1983) compared to heifers developed on range. Average daily gains (ADG), activity level, and pregnancy success have been compared between heifers developed in a drylot versus grazing setting.

Heifers developed in a drylot and subsequently turned out to pasture exhibited reduced ADG (Perry et al., 2013) during the first 27 days and increased activity level (Perry et al., 2015) during the first three days compared to their range developed counterparts. When moved to a grazing situation immediately following AI, drylot-developed heifers also had reduced pregnancy success (Perry et al., 2013), indicating that lack of grazing experience resulted in heifers in a negative energy balance and impacted pregnancy rates (Perry et al., 2016).

Heifers will also experience a shift in microbial rumen populations and VFA production in response to diet change (Boland et al., 2001). As reduced pregnancy success has been observed for heifers moved from a drylot to grazing situation, Perry and coworkers investigated if this reduction in pregnancy success was indeed caused by grazing behavior and not a change in diet and microbial population shift alone. In a recent study Perry et al. (2016) developed heifers on a forage diet and then allocated heifers to 1) spring forage, 2) spring forage with DDGS supplementation, or 3) continued in feedlot for 42 days following AI. No differences were observed in pregnancy success among treatments, and heifers maintained or gained BCS across all treatments. Thus, reduced pregnancy success from previous studies of heifers transitioned from drylot to pasture at AI was not due to sudden alterations in diet and rumen microbial populations but was most likely a result of decreased nutrient intake while learning how to forage.

Weight change and body condition during heifer development may also impact reproductive performance. Heifers developed to gain < 0.45 kg/day from weaning until the subsequent spring had reduced pregnancy success and maintained fewer pregnancies between August and October pregnancy diagnoses compared to heifers developed to gain

≥ 0.45 kg/day (Short and Bellows, 1971). However, excess body condition also hinders reproductive efficiency (Patterson et al., 1992) and increases incidence of dystocia at time of parturition (Arnett et al., 1971). Heifers with a greater BCS before anestrus required increased weight gains to resume cyclicity (Cassady et al., 2009), and heifers at a lower BCS prior to anestrus began cycling at a lower body weight. Therefore, heifers with a greater BCS prior to anestrus will require greater nutritional intake in order to resume cyclicity compared to heifers at a lower BCS. Developing heifers to appropriate body weights and BCS for the breeding season will ensure increased pregnancy success and minimize dystocia during the calving season.

Method of heifer development may prompt changes in diet type, activity level, and body weight. These maternal shifts may impact pregnancy success and subsequent embryo survival, embryogenesis, fetal development, and postnatal development of the offspring (Barker et al., 1993; Muñoz et al., 2009). Therefore, it is critical to properly manage nutrition during heifer development for optimal reproductive success and progeny performance.

IMPACTS OF MATERNAL NUTRITION ON OFFSPRING EPIGENETICS

Evidence now points towards the long-lasting influence maternal nutrition during gestation has on postnatal development of the offspring in addition to embryo and fetal development. Maternal nutrient restriction and overnutrition throughout pregnancy can result in alterations in offspring metabolism, physiology and structure which permanently affect progeny development, a phenomenon known as fetal programming (Barker, 1997).

Maternal nutrition impacts offspring development from the earliest stages of pregnancy, as embryonic and trophoblast growth are dependent on concentrations of

specific nutrients and are particularly susceptible to nutrient changes during the 1-cell stage prior to implantation (Barker et al., 1993). Nutrient requirements during the embryonic stage appear limited in comparison to the later stages of fetal development, where 75% of fetal growth occurs during the last two months of gestation (Funston et al., 2010); however, meeting the embryo's nutrient requirements during the early embryonic stages of development are crucial, as the individual's entire genome is programmed during this period and thereby determines the maximum growth and development that animal will achieve under optimal circumstances postnatally (Barker, 1997). As all cells within the embryo are totipotent, any nutritional insult at this stage will impact development of all future tissues (Gicquel et al., 2008). Thus, meeting nutritional requirements of the embryo is critical for genomic programming in the embryo and subsequent development.

Rapid growth and cell division occur during the fetal stage. Different tissues of the body undergo periods of rapid cell division at different time points throughout gestation; therefore, insults to fetal growth at specific intervals during gestation will alter the development of specific tissues in utero and thereby program their development postnatally (Du et al., 2010). Maternal malnutrition may alter lean-to-fat composition, muscle fiber type, and marbling content in offspring carcasses, which may result in reduced production efficiency of the offspring (Du et al., 2010). Muñoz et al. (2009) concluded that offspring adiposity in sheep may be determined in utero due to maternal nutrient restriction during early pregnancy. Furthermore, restricted maternal plane of nutrition during late gestation in beef cattle resulted in downregulation of microRNA and target gene expression in offspring longissimus muscle (Moisá et al., 2016), providing

further evidence that maternal nutrition impacts postnatal offspring development in utero through regulation of gene expression.

Mechanisms Mediating Epigenetic Alterations

Developmental processes in an organism are determined by the genome and phenotypic expression of specific genes (Holliday and Pugh, 1975). Therefore, understanding the mechanisms that mediate changes in gene expression in utero is fundamental to improving animal performance (Gicquel et al., 2008).

Although the study of epigenetic alterations in animal agriculture is relatively new, these mechanisms are gaining interest in relation to livestock production. Epigenetic mechanisms occur via modifications in DNA or associated proteins and result in altered gene expression while the DNA coding sequence undergoing modification remains unaffected (Huang et al. 2014; Moore et al., 2013). Epigenetic modifications are essential to reprogramming gene expression and are critical during developmental processes such as embryogenesis and genomic imprinting (Huang et al., 2014). These alterations in gene expression result in heritable silencing (Egger et al., 2004) and may be seen in response to environmental factors such as maternal nutrition (Holland and Rakyan, 2013).

Systems that mediate epigenetic changes include DNA methylation, histone modification and RNA-associated silencing. These mechanisms are capable of influencing one another and reinforcing silencing effects on heritable gene expression (Egger et al., 2004; Gicquel et al., 2008).

DNA Methylation

The mechanism of DNA methylation as a form of gene control was first defined in 1975 by Holliday and Pugh, in which methyl groups are adjoined to cytosine residues

within CpG dinucleotide sequences. These CpG dinucleotides represent the majority of methylation sites in mammals, excluding clusters of CpGs known as CpG islands, which reside in the promoter regions of genes (Plass and Soloway, 2002) and contain a minimum 500 base pair sequence with a GC content greater than 55% (Takai and Jones, 2002). These CpG islands tend to remain unmethylated (Cedar and Bergman, 2009), a characteristic which protects these regions from 5-methylcytosine deamination (Jones and Takai, 2001). Methylation of the small but significant fraction of CpG islands inhibits initiation of transcription in mammalian somatic cells (Plass and Soloway, 2002) through changes in DNA and protein interactions, which modifies chromatin structure and alters transcription rate (Jones and Takai, 2001), leading to stable silencing of the associated promoter sequence (Bird, 2002). Methylation of DNA has been proven as a heritable epiegenetic mark (Paszkowski and Whitham, 2001) and is crucial for normal development, gene expression patterns, and genomic stability (Plass and Soloway, 2002). However, environmental influences may lead to abnormal DNA methylation, thereby affecting chromosome structure and resulting in diseases such as cancer (Jones and Takai, 2001).

Epigenetic reprogramming via DNA methylation occurs in two phases (Figure 2), with the initial stage of epigenetic modifications taking place during gameteogenesis. Primordial germ cells, originating from somatic tissue, mature into gametes and undergo DNA demethylation, in which the genome is stripped of methylation markers (Morgan et al., 2005). New methylation patterns and imprints are subsequently established in gametic genomes by *de novo* methyltransferases (DNMTs; Plass and Soloway, 2002).

Fertilization triggers the second stage of epigenetic reprogramming during preimplantation. The paternal genome undergoes complete DNA demethylation, which ensures that gamete specific regulatory marks are removed to prevent interference with embryo development (Jenkins and Carrell, 2012). Paternal chromosomes of the zygote are actively demethylated by enzymatic activity shortly after fertilization in the male pronuclei (Plass and Soloway, 2002) and is completed by the first cellular division (Jenkins and Carrell, 2012). In contrast, maternal chromosomes undergo passive demethylation during later cleavage stages, in which several rounds of cell division occur to reduce DNA methylation in the absence of a demethylating enzyme; histone modifications on maternal chromosomes may be responsible for preventing this active demethylation (Jenkins and Carrell, 2012). The embryonic genome is passively demethylated throughout early cell cycles prior to blastulation, but imprinted genes maintain their specific methylation throughout this reprogramming period (Morgan et al., 2005). Methylation of DNA is facilitated by DNMTs around the time of implantation (Cedar and Bergman, 2009), and although the process that determines which portions of the genome receive methylation are not understood, certain methods have been suggested. Global methylation may occur, in which by default all parts of the genome are initially subjected to *de novo* methylation (Bird, 2002). However, not all regions of the genome are equally accessible by DNMTs, which deters the definition of global. Another mechanism might be explained by the potential for some DNA sequences to possess higher affinity for methylation compared to other regions of the genome (Bird, 2002). This is further supported by evidence suggesting that DNA methylation does not occur to repress active promoters but rather affects genes that are already silenced to effectively

ensure permanent silencing (Bird, 2002). The majority of methylation occurs within the inner cell mass compared to the trophectoderm during *de novo* methylation of the blastocyst (Morgan et al., 2005). This stage of epigenetic reprogramming occurs during totipotency and determines the initial genetic regulation for future development of the offspring.

The conversion of 5-methylcytsoine (5-mC) to 5-hydroxymethylcytosine (5-hmC) has recently been discovered as a mechanism regulating DNA methylation's control on gene expression (Murrell et al., 2013). Studies have reported that 5-hmC is an intermediate in the removal of 5-mC in the DNA demethylation pathway, and 5-hmC may also relieve the inhibitory effect of 5-mC by impeding the binding of methyl-binding proteins (Branco et al., 2012). The presence of 5-hmC is also associated with promoters and gene expression (Branco et al., 2012), which may provide further insight into how gene expression is regulated.

Methylation of DNA is susceptible to environmental impacts, including shifts in maternal plane of nutrition (Holland and Rakyan, 2013). These environmental factors may result in long-term alterations to offspring development. Impacts of maternal diet on offspring performance have been studied in rodents and have reported that levels of 5-mC are dependent on nutritional factors and are therefore susceptible to dietary changes (Cooney et al., 2002).

Insufficient methylation appears to hinder animal health and longevity (Cooney et al., 2002). However, the association between offspring methylation patterns and performance data in response to maternal nutrient manipulation in beef cattle has not been examined, although numerous studies have reported the impacts of maternal plane

of nutrition on offspring performance and carcass data. Furthermore, minimal research has examined the impacts that maternal plane of nutrition during demethylation and *de novo* methylation in early embryogenesis have on offspring methylation patterns and postnatal performance in beef cattle. Therefore, additional research is necessary to determine how environmental factors such as maternal nutrition impact offspring performance through epigenetic mechanisms in livestock production.

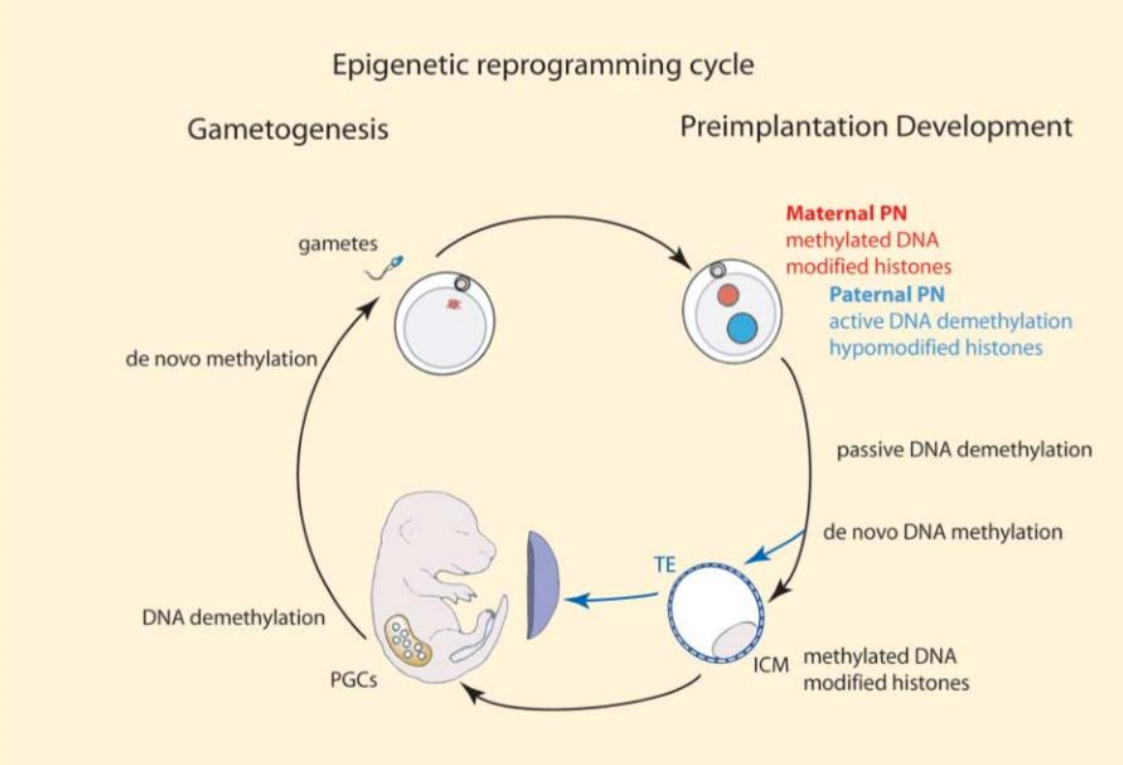

Figure 2. Epigenetic reprogramming cycle (Morgan et al., 2005).

Histone Modifications

Histone modifications are an additional mechanism determining gene expression. Histones are post-translationally modified in the genome via acetylation, phosphorylation, or methylation; these modifications regulate chromatin structure by recruiting enzymes to reposition nucleosomes, thus influencing transcription and repair,

replication and recombination of DNA, and may also moderate binding of chromatin factors (Bannister and Kouzarides, 2011). Regulation of these specific binding proteins subsequently modifies gene expression (Munshi et al., 2009; Murrell et al., 2013).

These mechanisms act cooperatively to direct DNA methylation patterns, with DNA methylation also providing a template for histone modifications after DNA replication (Cedar and Bergman, 2009). Histone modifications may serve to provide signals for *de novo* methylation after the erasure of epigenetic marks during demethylation as well as protect the maternal pronuclei from active demethylation (Morgan et al., 2005).

Although histone modifications are involved in epigenetic expression, their level of heritability is still in question (Bird, 2002). Therefore, when studying heritable epigenetic marks and changes within genome expression, DNA methylation is the primary mechanism examined.

RNA Silencing

The alternative method of RNA associated silencing has also been suggested as a mechanism regulating gene expression. Although not as extensively studied in mammals, RNA silencing as a regulator of gene expression is becoming more prevalent in plant epigenetics. Long and short non-protein-coding (nc) RNAs, including microRNA, Piwi-interacting RNA and antisense RNAs, are responsible for recruiting chromatin factors and may generate short interfering RNAs that direct chromatin modifications such as DNA methylation, thereby directing gene silencing (Matzke et al., 2009).

Carcinogenesis in mammals has been associated with RNA silencing, as antisense RNA may signal heterochromatin formation and DNA methylation in tumor suppressor

gene silencing and result in long-term heritability of gene expression, as evidenced in embryonic stem cells (Yu et al., 2008). This mechanism supports the hypothesis of cancer, in which tumors originate from epigenetic modifications and result in massive tissue development. However, the heritability of RNA silencing and its role in the early embryonic genome has yet to be demonstrated in mammalian development. Further research needs to be conducted to further clarify the function of RNA silencing in these areas.

CONCLUSIONS

Extensive research has been conducted on the detailed interactions involving malnutrition in beef cattle and reproductive outcomes on the estrous cycle, folliculogenesis, follicular fluid, embryogenesis, and uterine environment. Method of heifer development creates an additional layer of complexity and requires careful nutritional management to ensure that lack of grazing skills for drylot-developed heifers does not reduce dietary intake, decrease ADG, and result in lower pregnancy rates. Dietary changes that occur in heifers at the time of AI when transitioned from a drylot to grazing scenario coincide with the establishment of epigenetic regulation in the offspring genome during early embryogenesis. As epigenetic markers are sensitive to changes in the external environment, changes in maternal nutrition during this period have the potential to impact both embryonic and postnatal development of the offspring. However, minimal research has been conducted relating to maternal impacts on offspring epigenetic regulation at the time of establishment and resulting postnatal effects. Therefore, the succeeding chapters will discuss field trials examining 1) the impacts of heifer development on offspring gene expression and postnatal performance characteristics and

2) the effects of pre- and post-breeding nutrition on uterine fluid composition and embryo development six days post-AI in heifers.

CHAPTER II

EFFECTS OF POST-INSEMINATION DAM NUTRITION ON CALF PERFORMANCE AND DNA METHYLATION

ABSTRACT

Nutritional changes immediately after insemination can result in increased embryonic mortality, but the impact of a nutritional change on the embryos that survive is not known. Therefore, the objective of this study was to evaluate the impact of a nutritional change immediately following AI on calf performance and global DNA methylation. Calving records were obtained for 147 heifers over two years that were allotted into two heifer development treatments: drylot or range. All heifers were fixed-time inseminated following the 7-day CO-Synch plus CIDR protocol to a single sire each year and were turned out to pasture together and managed as a single group. Pregnancy success to AI was determined via ultrasonography, and calving data (calving date, birth weight, sex, and weaning weight) were collected. Samples of DNA were obtained from calves at weaning and analyzed for global methylation (total methylation and 5-hmC methylation). Data were analyzed as a 2 x 2 factorial design using the GLM procedure in SAS with sex and replicate included in the model. Bull calves were heavier at birth compared to heifer calves ($P = 0.04$; 34.9 ± 0.45 vs 34.3 ± 0.60 kg) and both natural service-sired (NS-sired) calves ($P < 0.003$; 35.7 ± 0.40 kg) and calves from range-developed heifers ($P < 0.004$; 35.3 ± 0.50 kg) were heavier at birth compared to AI-sired calves (32.9 ± 0.50 kg) and calves from drylot-developed heifers (33.3 ± 0.50 kg), respectively. However, there was no replicate ($P = 0.99$) or development by sire

interaction ($P = 0.70$). Weaning weights were impacted by both heifer development ($P = 0.04$) and sire ($P < 0.0001$), with AI-sired calves (221.8 ± 5.10 vs 189.1 ± 2.70 kg) and calves from range-developed heifers (210.6 ± 3.60 vs 200.2 ± 3.40 kg) being heavier. Sex tended to influence weaning weight ($P = 0.09$), but there was no development by sire interaction ($P = 0.99$). Offspring ADG was not impacted by calf sex ($P = 0.44$), sire ($P = 0.65$), or heifer development ($P = 0.30$), but NS-sired calves from drylot-developed heifers (0.78 ± 0.02 kg) tended to have reduced ADG ($P = 0.07$) compared to AI-sired calves from drylot-developed heifers (0.83 ± 0.04 kg) and NS-sired calves from range-developed heifers (0.83 ± 0.04 kg). Total DNA methylation was not affected by development ($P = 0.55$), sex ($P = 0.35$), sire ($P = 0.32$), or any interactions ($P = 0.92$). For 5-hmC methylation there was a tendency for both heifer development and sex of the calf to impact methylation, with both bull calves and calves from range-developed heifers tending ($P = 0.10$) to have decreased 5-hmC methylation compared to heifer calves and calves from drylot-developed heifers. In addition, there was an interaction between heifer development and sire type ($P = 0.003$), with NS-sired calves from drylot-developed heifers having greater 5-hmC methylation than all other treatment groups. In summary, heifer development and sire type can impact future performance of the calf that is in utero as determined by BW and WW, and this change in performance may be regulated through DNA methylation.

INTRODUCTION

The primary reason young females are culled from the beef herd is due to reproductive failure (Short et al. 1990). While reproductive failure may occur from a variety of factors, maternal plane of nutrition during gestation has the potential to impact pregnancy success and is a factor that can be manipulated to the producer's benefit.

Managing replacement heifers' plane of nutrition is critical to ensure their longevity within the herd, and this management begins at the time of weaning when heifers are removed from their dam. Heifers are typically placed in either a confined feeding situation, where they are supplied feed on a daily basis, or a forage grazing setting, where heifers spend the majority of their time grazing. Heifers are targeted to reach a minimum body weight proportionate to their mature size during this time (Patterson et al., 1992).

At breeding heifers are generally managed on grass, and prior grazing experience impacts heifers' capability to successfully select a diet to meet their nutritional needs during the breeding season. Heifers developed in confinement do not utilize grazing skills acquired from their dams during the pre-weaning period; thus, they will expend more energy foraging when initially introduced to a grazing environment (Osuji, 1974), hence ingesting less nutrients (Arnold and Maller, 1977; Curll and Davidson, 1983) compared to heifers developed in a grazing scenario.

Perry and coworkers have reported distinct differences among heifers relating to average daily gains, activity level, and pregnancy success based on method of development between weaning and breeding. Heifers developed in a drylot and subsequently turned out to pasture exhibited reduced ADG (Perry et al., 2013) during the

first 27 days and increased activity level (Perry et al., 2015) during the first three days compared to their range developed counterparts. Drylot developed heifers also had decreased AI conception rates when moved to a forage-grazing setting immediately after AI (Perry et al., 2013), indicating that lack of grazing experience placed heifers in a negative energy balance and decreased pregnancy success (Dunne et al., 1999; Hill et al., 1970; Perry et al., 2016).

Changes in maternal nutrition during pregnancy impact not only embryo survival and development but also postnatal development of the offspring (Barker, 1997) and can be seen in carcass characteristics of the progeny (Muñoz et al., 2009). Understanding the mechanisms that influence these long-lasting epigenetic changes throughout postnatal development is of growing interest. Alterations in DNA methylation patterns occur during early embryogenesis (Bird, 2002), and it is possible that changes in maternal nutrition during early embryogenesis may impact DNA methylation patterns and alter which genes are silenced within the offspring genome, thereby impacting offspring performance (Wu et al., 2004). Therefore, the objective of this study was to assess the impacts of heifer development method and sire on subsequent offspring performance and further determine if differences observed in progeny performance could be explained by alterations in DNA methylation patterns of the offspring.

MATERIALS AND METHODS

Experimental Design

All treatments were approved by the South Dakota State University Institutional Animal Care and Use Committee. Angus-cross heifers (n = 147) at the South Dakota State University Antelope range and livestock research station were allotted over a two-year period into one of two treatments at weaning: 1) development in a drylot or 2) development on range. Heifers assigned to the drylot treatment were kept in a confined feeding situation and given *ad libitum* access to hay (grass-alfalfa mix; 8.1% CP, 66% NDF; DM basis) and a conventional supplement based on wheat middlings and sunflower meal (Table 1) fed at a rate of 1.2 to 1.6 kg/d per animal (DM basis), adjusted as needed throughout the winter to ensure they reached 65% of their mature body weight by the start of the following breeding season. Heifers assigned to the range treatment were placed on native range, where heifers met their nutritional needs through grazing and were supplemented with dried distillers grains plus solubles (Table 1) at a rate of 0.82 to 2.9 kg/d per animal (DM basis), adjusted as needed to develop them to 65% of their mature body weight by the beginning of the next breeding season. The feeding rate was adjusted over the winter to account for heifer size, weather conditions, expected forage quality, and observed interim performance. Heifers were synchronized for breeding following the 7-day CO-Synch + controlled internal drug release (CIDR) protocol, which consisted of an injection of GnRH (100 µg of Factrel; Pfizer Animal Health, Madison, NJ) and insertion of a CIDR (CIDR; 1.38 g progesterone; Zoetis, Florham Park, NJ) and seven days later an injection of $PFG_{2\alpha}$ (25 mg of Lutalyse; Zoetis, Florham Park, NJ) at time of CIDR removal. During the synchronization period heifers remained in their initial

respective treatments. All animals received an injection of GnRH and were fixed-time inseminated 54 ± 2 h after CIDR removal (d 0) by one technician to a single sire. After insemination, heifers were all moved to pasture and managed as a single group. Pregnancy success to AI was determined by transrectal ultrasonography, using fetal crown-rump length to determine fetal age 30 to 40 d post-AI. Heifers were exposed to clean-up bulls 14 d following AI for a 60-d breeding season, and breeding-season pregnancy success was determined 40 to 50 d following the breeding season. Heifers were subsequently divided into four treatment groups based on embryo conception date: 1) AI-sired range, 2) AI-sired drylot, 3) natural service-sired (NS-sired) range, and 4) NS-sired drylot. Heifer weights were collected at AI and at time of pregnancy determination. Calving data (calving date, sex, birth weight [BW], and weaning weight [WW]) were also collected, and average daily gain (ADG) was calculated as the difference between WW and BW divided by the age in days of the calf.

DNA Extraction

Samples of DNA were obtained from calves during year 1 of the study from blood spots on Whatman FTA cards (Flinders Technology Associates; GE Healthcare Life Sciences, Pittsburgh, PA). Isolation of DNA from FTA cards was completed using a DNeasy blood and tissue kit (QIAGEN, Venlo, Netherlands) and put through a series of centrifugations, ethanol precipitation and washes according to the DNeasy protocol with slight modifications. Modifications included a longer initial incubation period for complete lysis of the sample and eluting DNA twice during the final step with half the amount of required buffer each elution for increased DNA yield. Eluted DNA was then concentrated to 12 – 20 ng/uL for global methylation. Concentrations of DNA were

analyzed on a NanoPhotometer (IMPLEN, Munich, Germany). Concentrated DNA samples were stored at -80°C until methylation assays were performed. Samples were then thawed overnight at 4°C and then incubated at 56°C for an hour prior to analysis.

Global Methylation

Samples of DNA were analyzed in duplicate for total methylation and 5-hmC methylation using the fluorometric methyl flash hydroxymethylated DNA quantification kit (Epigentek, Farmingdale, NY). Analysis of 5-hmC methylation was included as it has been specifically known to impact gene expression (Branco et al., 2012). Binding solution and multiple wash steps were applied to the samples according to the manufacturer's protocol. Fluorescence of methylation was measured with a Synergy 2 multi-mode plate reader (BioTek, Winooski, VT).

Statistical Analysis

The effects of heifer development on heifer ADG were analyzed by analysis of repeated measures using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). The model included the independent variables of treatment, day, and treatment x day. The effects of heifer development on ADG from AI to pregnancy determination and pubertal status were analyzed using the GLIMMIX procedure in SAS. Birth weight, WW, and ADG were analyzed as a 2 x 2 factorial design (heifer development by sire type) using the GLM procedure in SAS with calf sex and year as replicate included in the model. Total methylation and 5-hmC methylation were analyzed as a 2 x 2 factorial design using the GLIMMIX procedure in SAS; calf sex was included in the model, and DNA concentration was run as a covariate to correct for varying DNA concentrations of the samples.

RESULTS

Heifer Performance

There was no difference between replicates ($P = 0.99$) or treatments ($P = 0.41$) in percentage of mature body weight reached by the start of the breeding season (65.2 ± 0.8% vs 66.1 ± 0.8% for drylot- and range-developed heifers, respectively; Figure 3). There was no effect of treatment ($P = 0.93$), replicate ($P = 0.99$), or treatment x replicate ($P = 0.99$) on the percentage of heifers reaching puberty prior to the beginning of the breeding season (97.3 vs. 93.6% for drylot- and range-developed heifers, respectively; Figure 4). Drylot-developed heifers had reduced ADG ($P < 0.01$; 0.21 ± 0.03 kg/d) compared to range-developed heifers (0.58 ± 0.03 kg/d) from AI to pregnancy determination (Figure 5).

Offspring Birth Weight

Heifer calves (34.3 ± 0.60 kg) and AI-sired calves (32.9 ± 0.50 kg) had lighter BW than bull calves ($P = 0.04$; 34.9 ± 0.45 kg; Figure 6) and NS-sired calves ($P < 0.003$; 35.7 ± 0.40 kg; Figure 7), respectively. Calves from drylot-developed heifers also had lighter BW ($P < 0.004$; 33.3 ± 0.50 kg) compared to calves from range-developed heifers (35.3 ± 0.50 kg; Figure 8). However, no interaction occurred between heifer development and sire type ($P = 0.70$; Figure 9), and there was no difference between replicates ($P = 0.99$).

Offspring Weaning Weight

Heifer calves tended to have lighter WW (201.9 ± 3.40 vs 208.9 ± 3.20 kg) than bull calves ($P = 0.09$; Figure 10), but AI-sired calves had heavier WW (221.8 ± 5.10 vs 189.1 ± 2.70 kg) than NS-sired calves ($P < 0.0001$; Figure 11). Calves from drylot-developed heifers had lighter WW (200.2 ± 3.40 vs 210.6 ± 3.60 kg) than calves from

range-developed heifers (P = 0.04; Figure 12). No interaction between heifer development and sire type was detected (P = 0.99; Figure 13), and there was no difference between replicates (P = 0.95).

Offspring Average Daily Gain

Offspring ADG was not impacted by calf sex (P = 0.44; Figure 14), sire type (P = 0.65; Figure 15), or heifer development (P = 0.30; Figure 16). However, there tended to be an interaction between heifer development and sire type on ADG, with NS-sired calves from drylot-developed heifers (0.78 ± 0.02 kg) tending to exhibit reduced ADG (P = 0.07) compared to NS-sired calves from range-developed heifers (0.83 ± 0.04 kg) while there was no difference in ADG between AI-sired calves from drylot-developed heifers (0.83 ± 0.04 kg) and AI-sired calves from range-developed heifers (0.82 ± 0.08 kg; Figure 17). Natural service-sired drylot calves also tended to exhibit reduced ADG compared to AI-sired calves from drylot-developed heifers but had similar ADG compared to AI-sired calves from range-developed heifers (Figure 17). There was no difference between replicates (P = 0.26).

Offspring Total Methylation

No differences were observed in offspring total methylation by calf sex (P = 0.35; Figure 18), sire type (P = 0.32; Figure 19), or heifer development (P = 0.55; Figure 20). No interaction between heifer development and sire type was observed (P = 0.92; Figure 21).

Offspring 5-hmC Methylation

There was a sex tendency (P = 0.10) for heifer calves to express greater methylation than bull calves (Figure 22), but sire type did not affect offspring 5-hmC

methylation (P = 0.35; Figure 23). Heifer development tended to impact offspring 5-hmC methylation (P = 0.10), with calves from drylot-developed heifers tending to express greater methylation than calves from range-developed heifers (Figure 24). An interaction between heifer development and sire type was observed (P = 0.003), with NS-sired calves from drylot-developed heifers showing the greatest 5-hmC methylation compared to AI-sired drylot, AI-sired range, and NS-sired range calves (Figure 25).

Table 1. Nutrient analysis (DM basis) in dried distillers grains plus solubles (DDGS) and conventional supplement.

Item	**DDGS**	**Conventional Supplement**
CP, %	29.70	31.0
Fat, %	11.60	3.3
TDN, %	84.60	69.6
Calcium, %	0.06	0.37
Phosphorous, %	0.79	1.11
Potassium, %	1.09	1.31
Magnesium, %	0.34	0.45
Copper, mg/kg	6	61
Zinc, mg/kg	99	112
Manganese, mg/kg	18	56

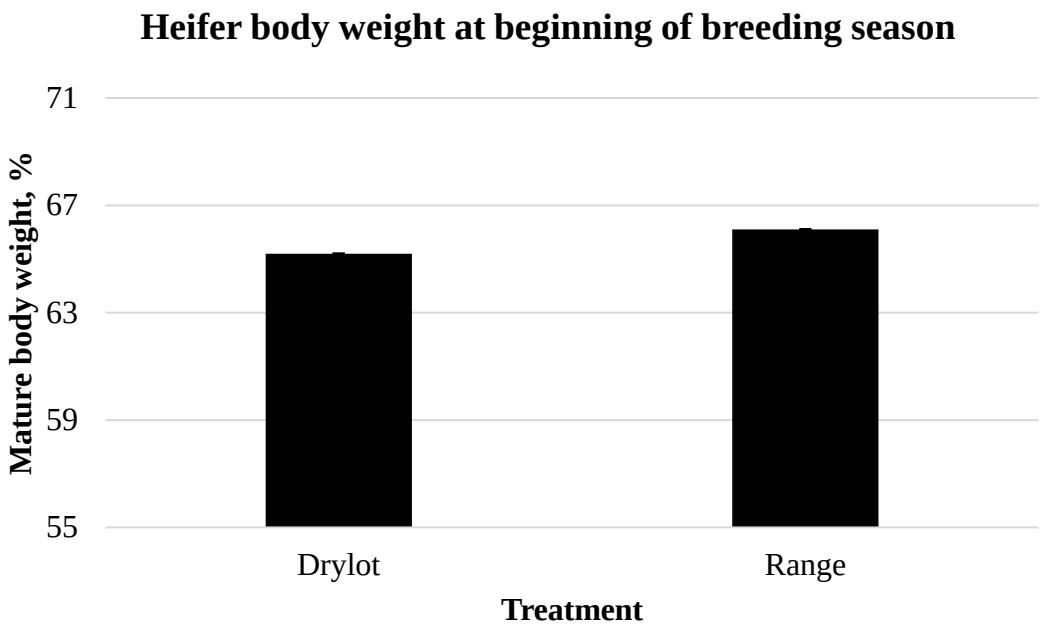

Figure 3. Heifer body weight at the beginning of the breeding season by treatment. No difference was seen between replicates (P = 0.99) or treatments (P = 0.41).

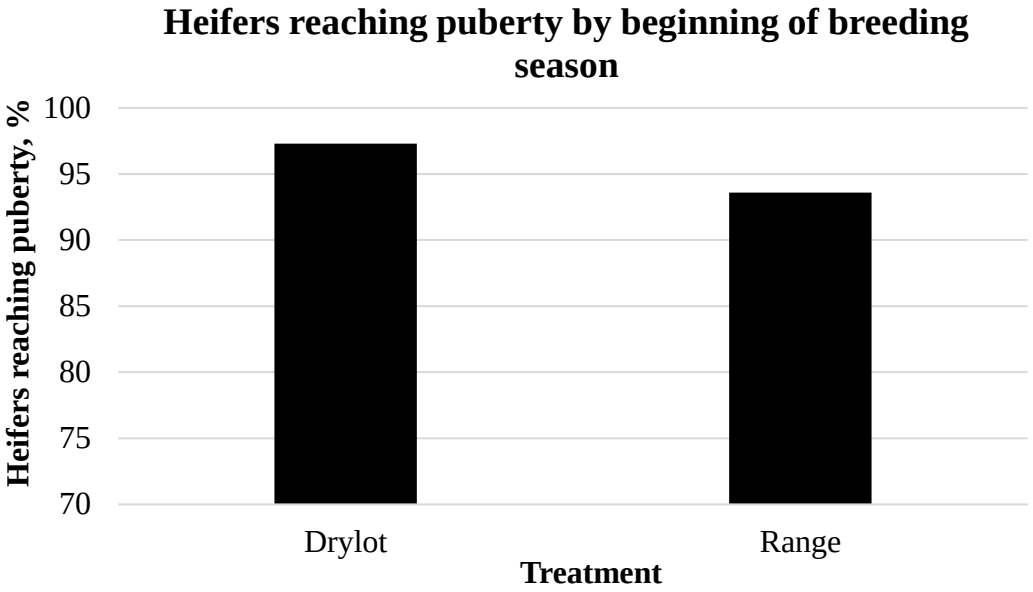

Figure 4. Heifers reaching puberty by the beginning of the breeding season by treatment. Treatment (P = 0.93), replicate (P = 0.99), and treatment x replicate (P = 0.99) did not affect the percentage of heifers reaching puberty prior to the breeding season.

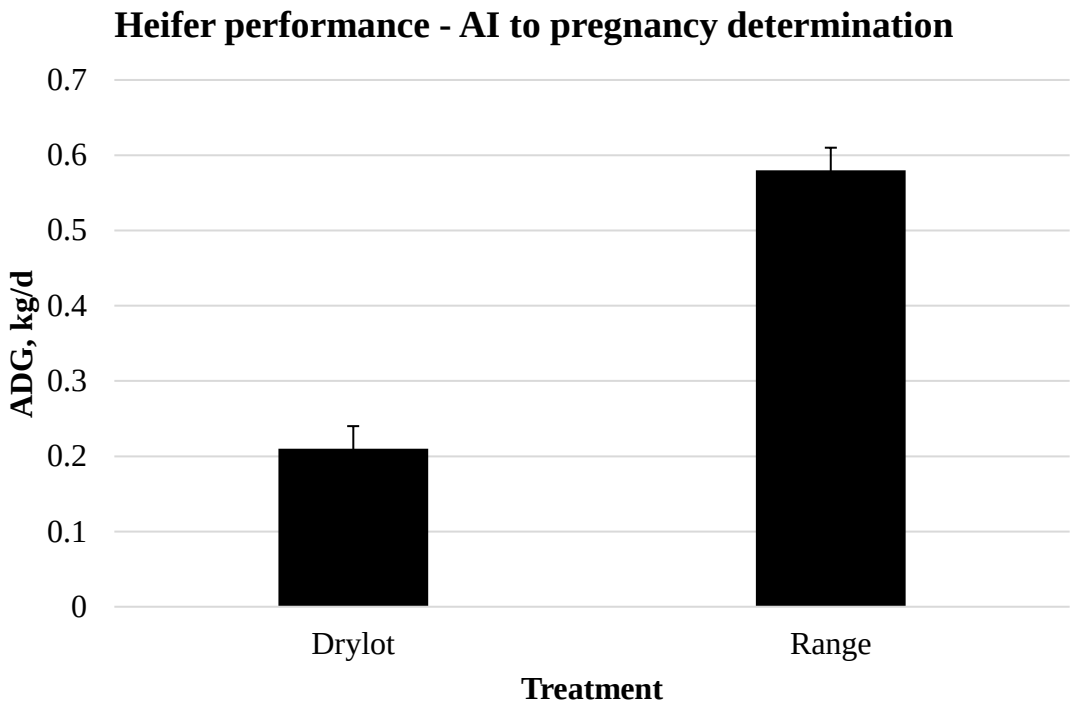

Figure 5. Effect of treatment on heifer ADG from AI to pregnancy determination. Drylot-developed heifers had reduced ADG compared to range-developed heifers ($P < 0.01$).

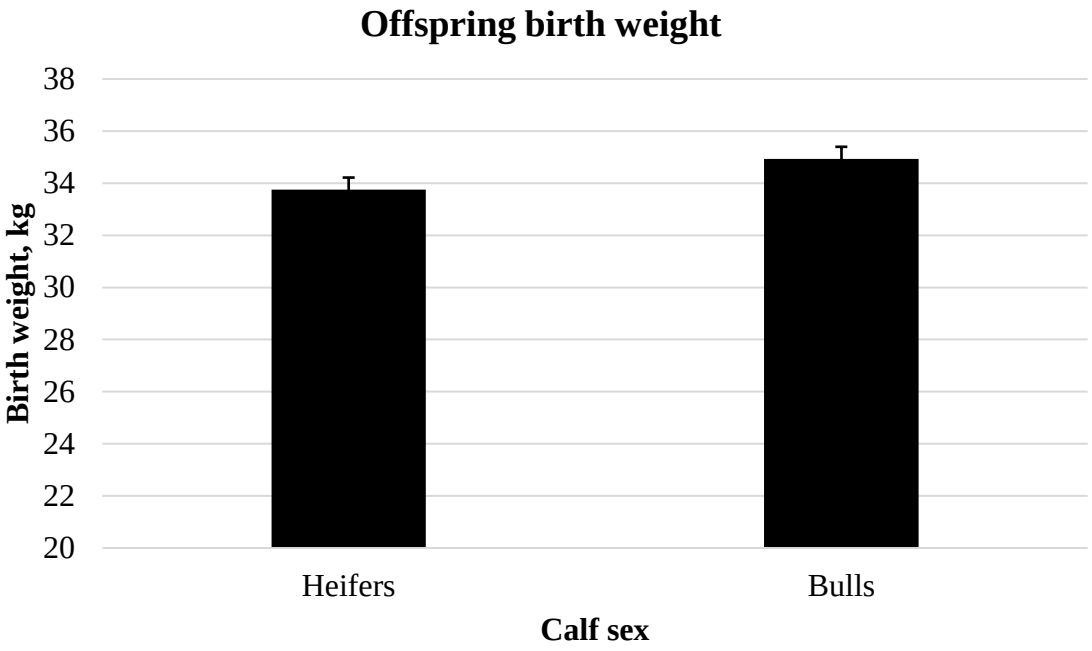

Figure 6. Effect of calf sex on offspring birth weight. Heifer calves had lighter birth weights compared to bull calves (P = 0.04).

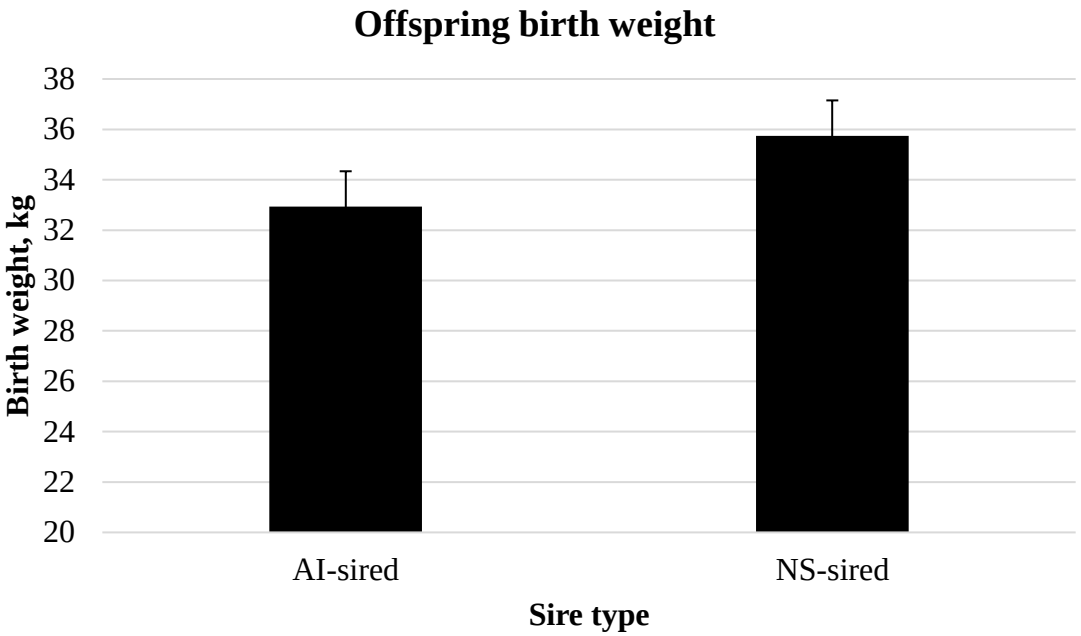

Figure 7. Effect of sire type on offspring birth weight. Natural service-sired (NS-sired) calves had heavier birth weights than AI-sired calves (P = 0.003).

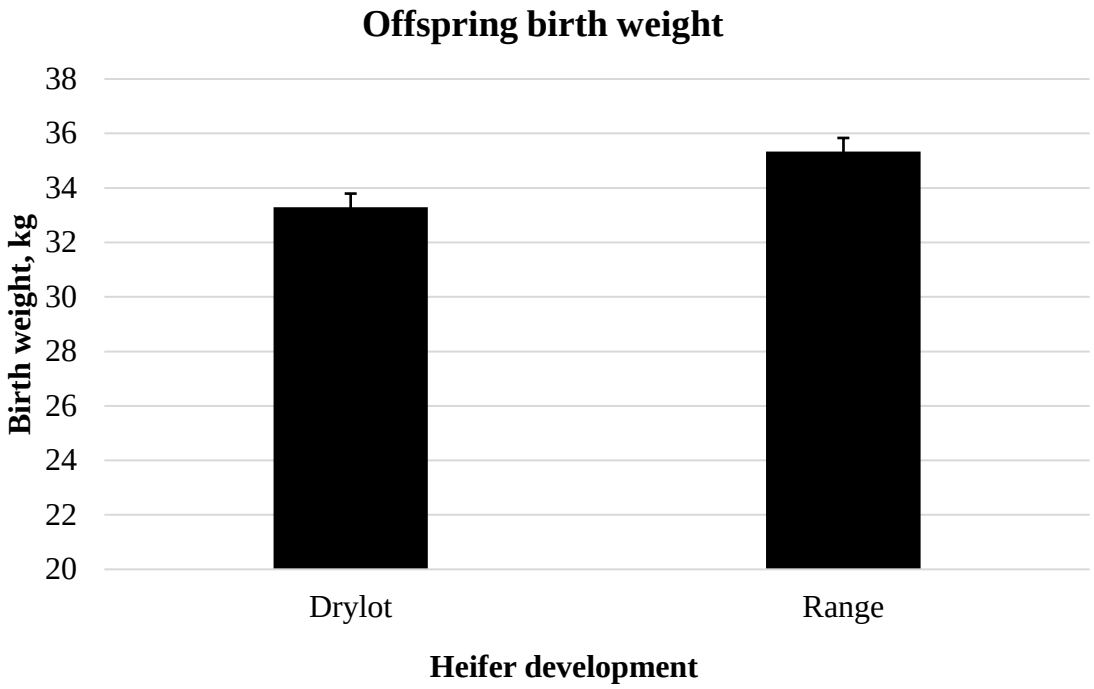

Figure 8. Effect of heifer development on offspring birth weight. Calves from drylot-developed heifers had lighter birth weights compared to calves from range-developed heifers (P = 0.004).

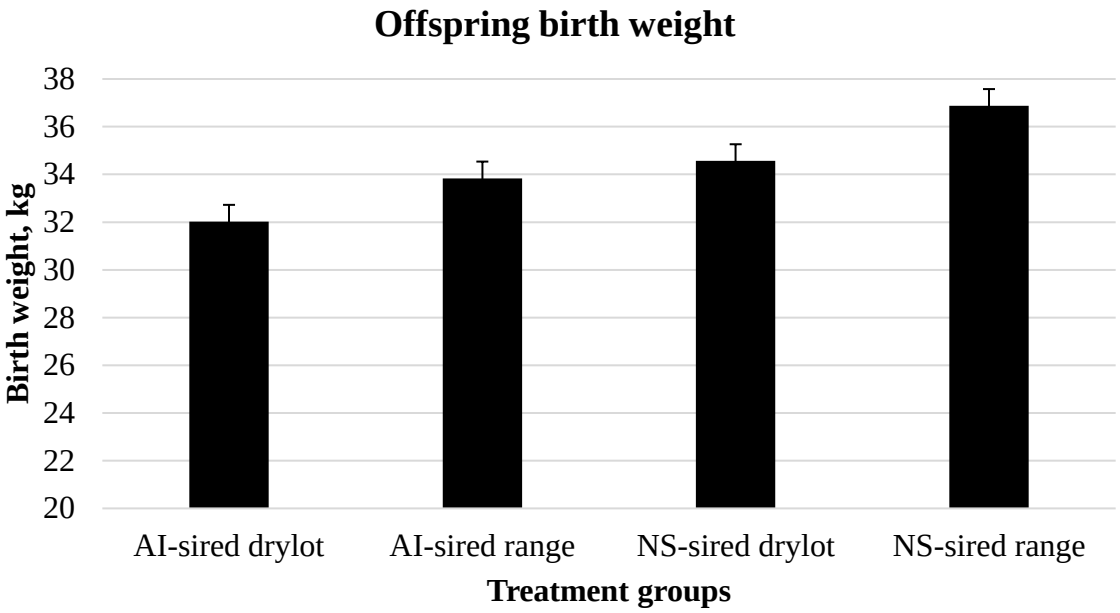

Figure 9. Effect of heifer development and sire type on offspring birth weight, where NS-sired = natural service-sired. No interaction between heifer development and sire type was observed for offspring birth weight (P = 0.70).

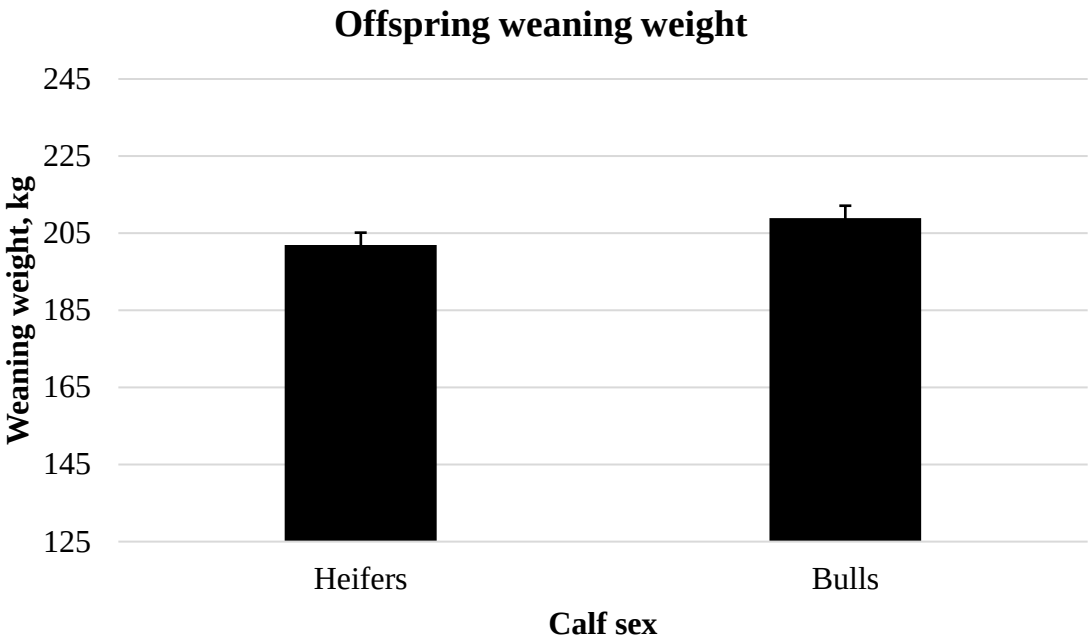

Figure 10. Effect of calf sex on offspring weaning weight. Heifer calves tended to have lighter weaning weights compared to bull calves ($P = 0.09$).

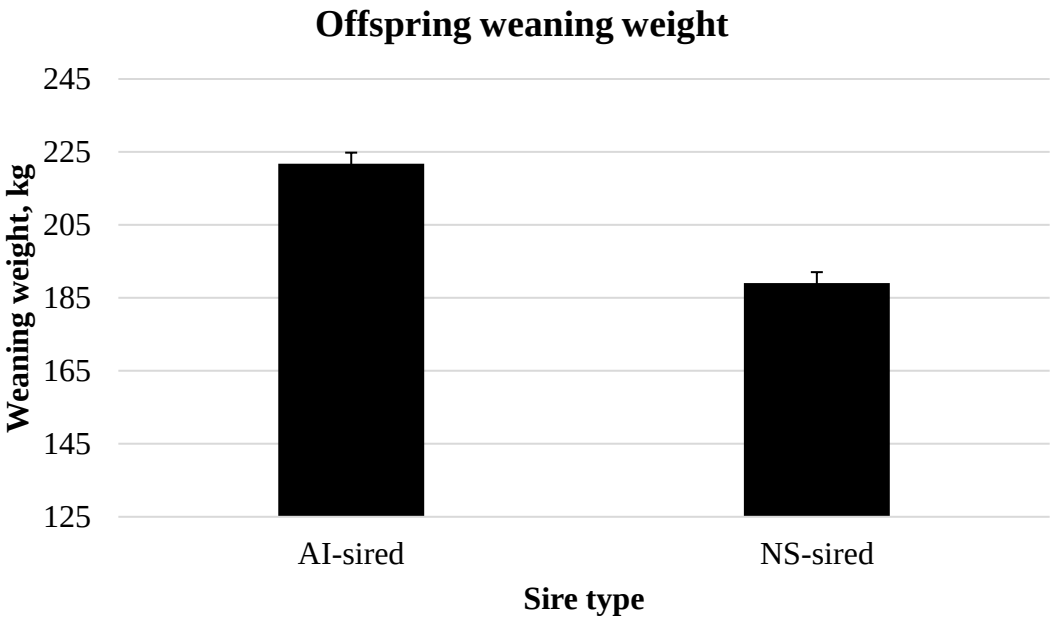

Figure 11. Effect of sire type on offspring weaning weight. Natural service-sired (NS-sired) calves had lighter weaning weights compared to AI-sired calves ($P < 0.0001$).

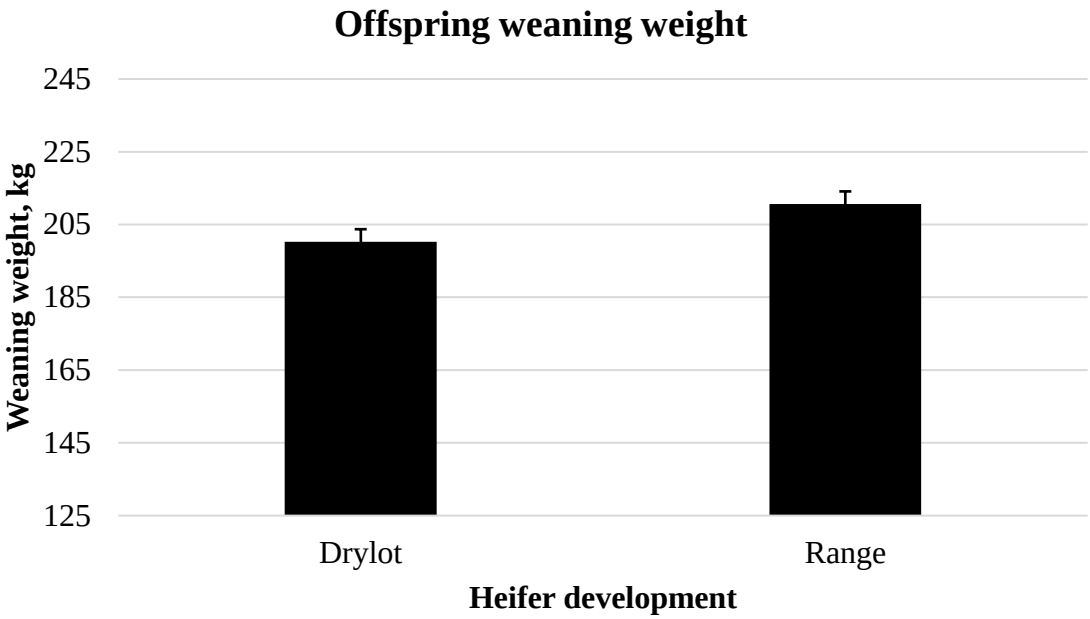

Figure 12. Effect of heifer development on offspring weaning weight. Calves from drylot-developed heifers had lighter weaning weights compared to calves from range-developed heifers (P = 0.04).

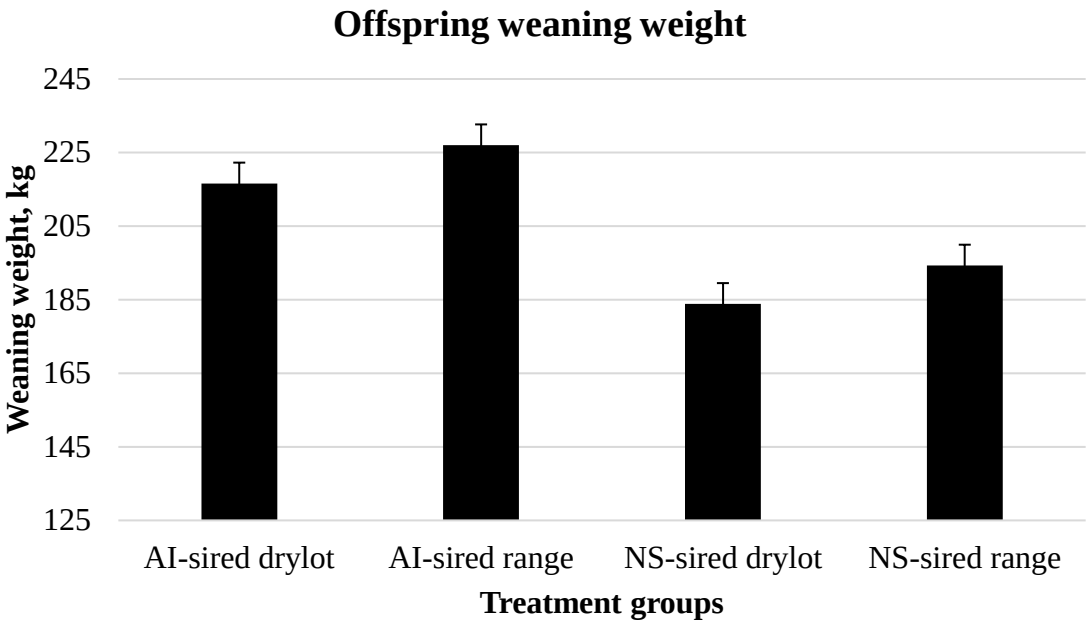

Figure 13. Effect of heifer development and sire type on offspring weaning weight, where NS-sired = natural service-sired. No interaction was observed between heifer development and sire type on offspring weaning weight (P = 0.99).

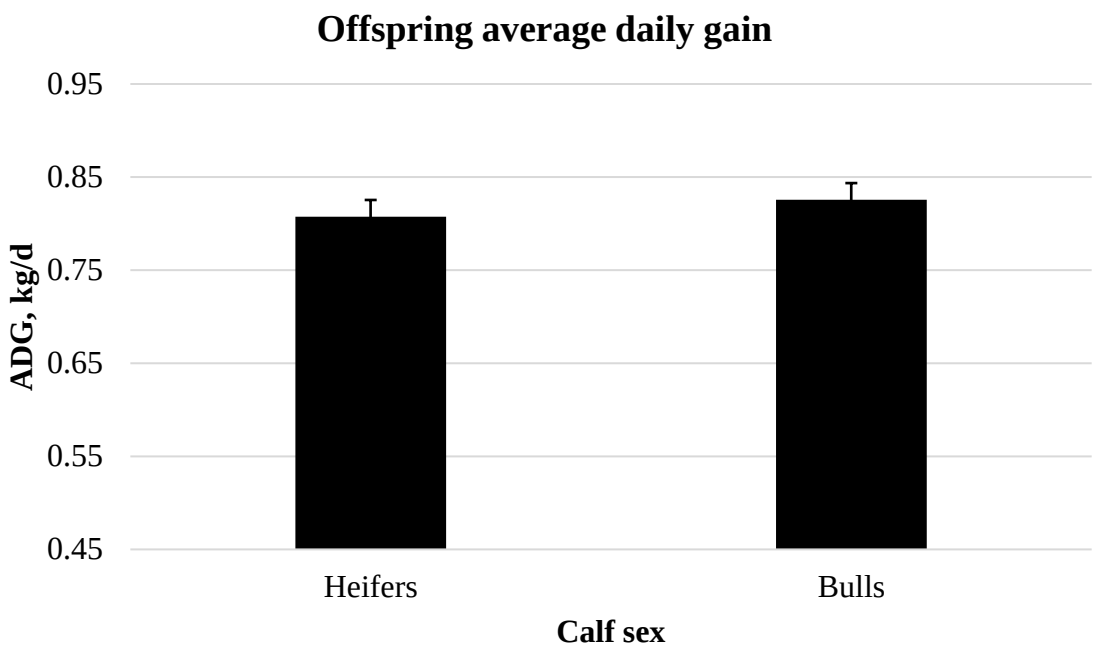

Figure 14. Effect of calf sex on offspring ADG. There was no difference in ADG between heifer and bull calves (P = 0.44).

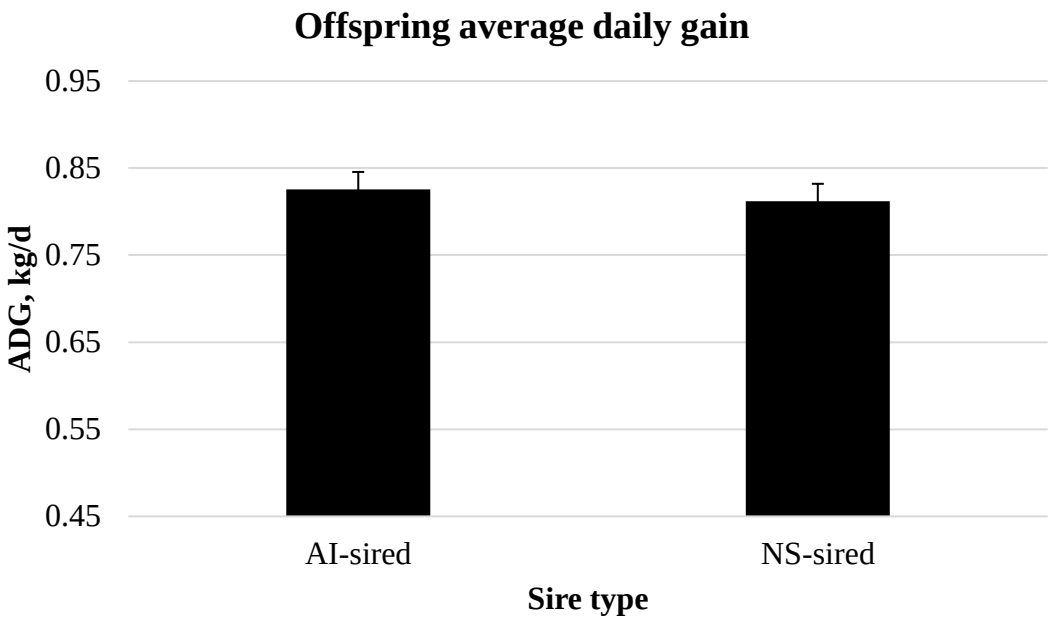

Figure 15. Effect of sire type on offspring ADG. There was no difference in offspring ADG between AI-sired and natural service-sired (NS-sired) calves (P = 0.65).

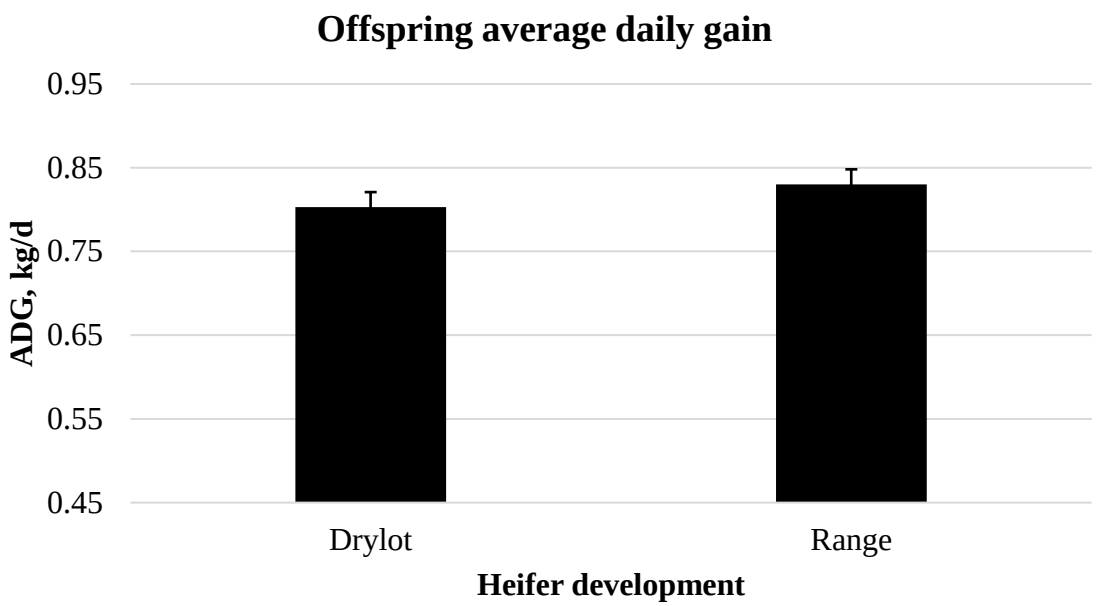

Figure 16. Effect of heifer development on offspring ADG. There was no difference in offspring ADG between calves from drylot- versus range-developed heifers (P = 0.30).

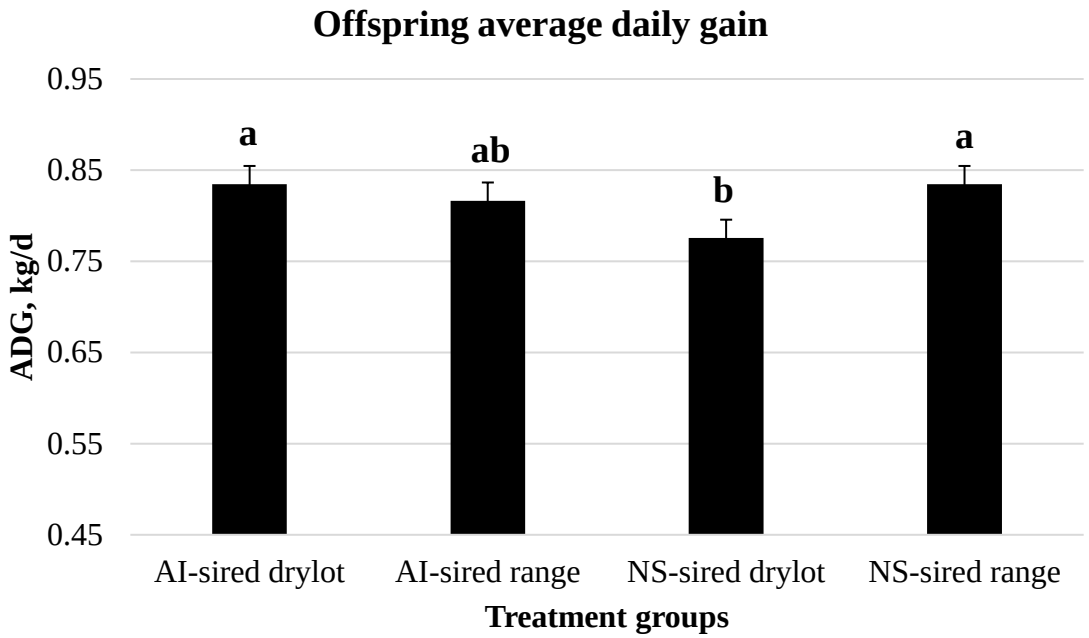

Figure 17. Effect of heifer development and sire type on offspring ADG. Natural service-sired (NS-sired) range calves and AI-sired drylot calves tended to have increased ADG compared to NS-sired drylot calves ($P = 0.07$).
[ab] Superscripts differ ($P < 0.004$).

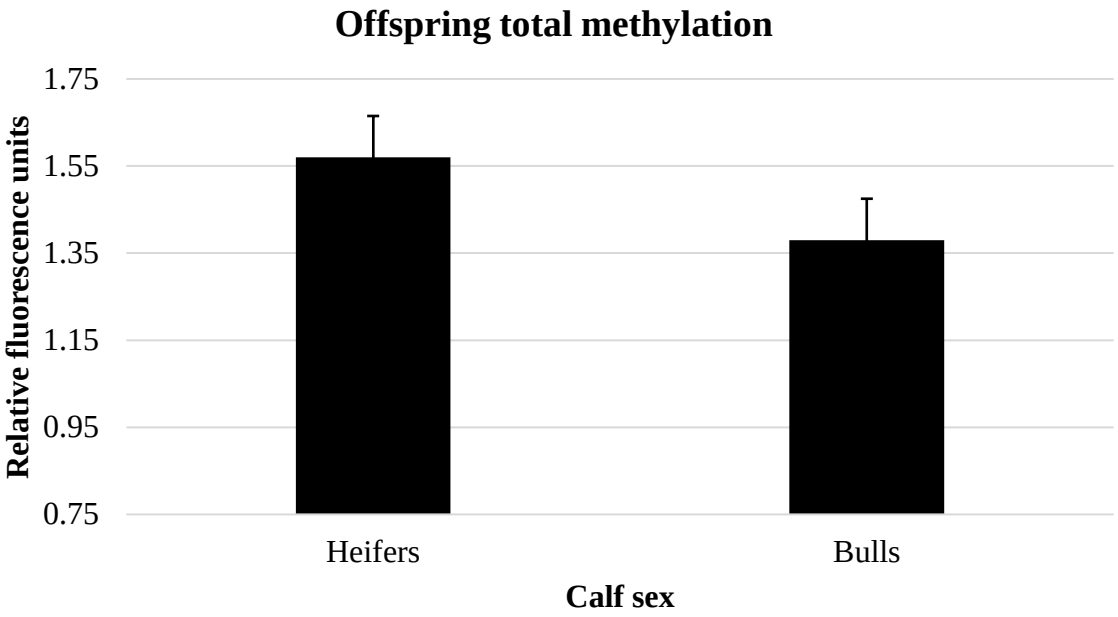

Figure 18. Effect of calf sex on offspring total methylation. There was no difference in offspring total methylation between heifer and bull calves (P = 0.35).

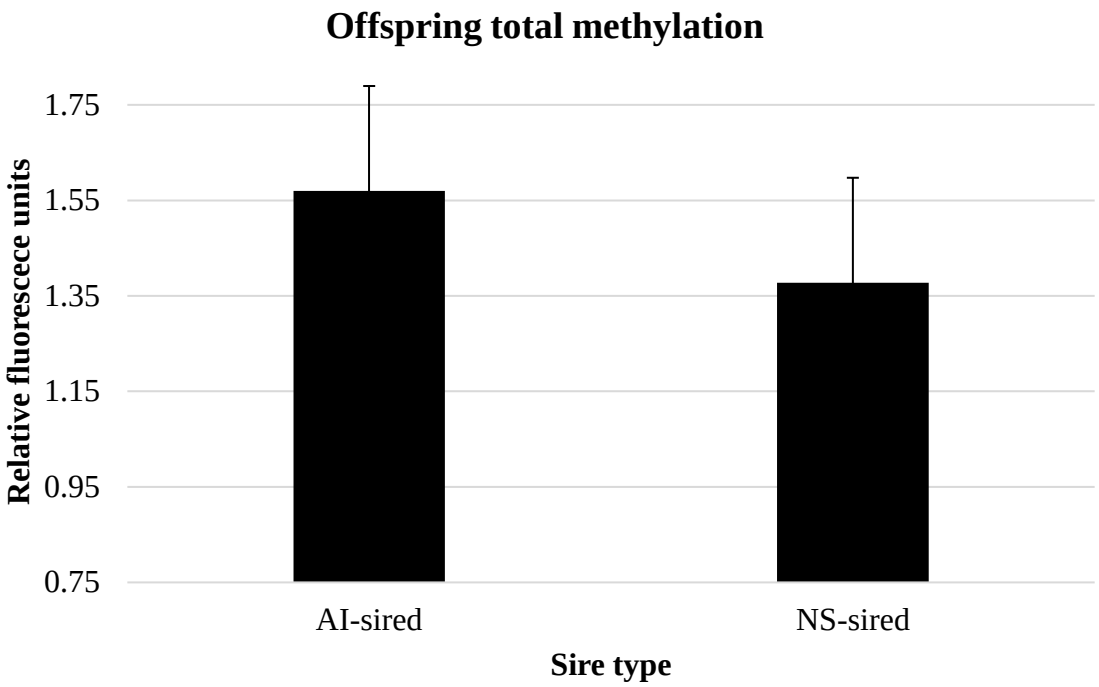

Figure 19. Effect of sire type on offspring total methylation. There was no difference in offspring total methylation between AI-sired and natural service-sired (NS-sired) calves (P = 0.32).

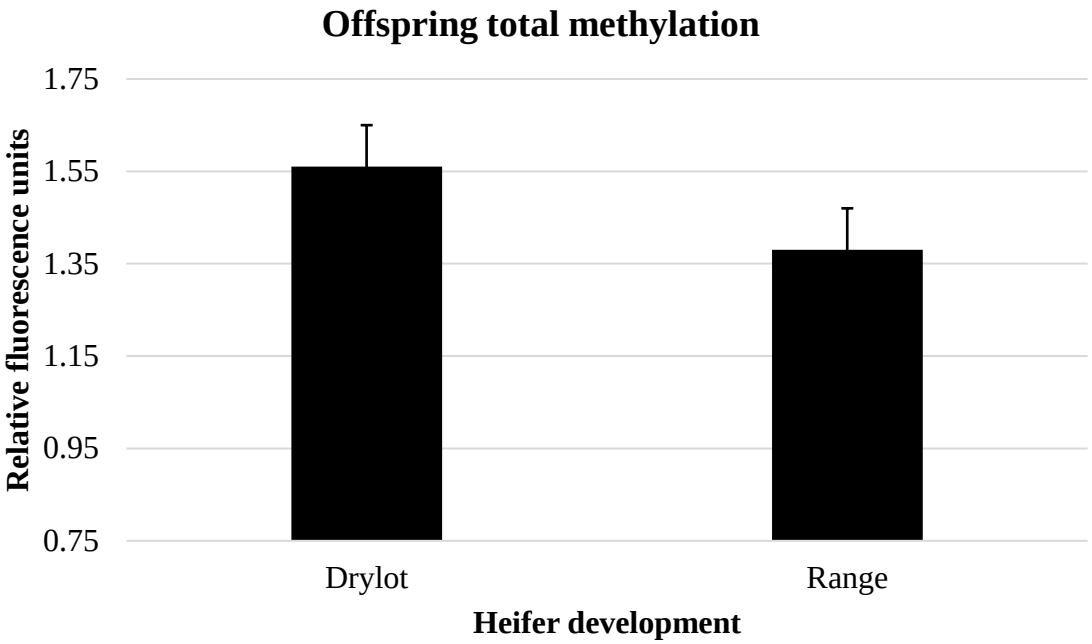

Figure 20. Effect of heifer development on offspring total methylation. There was no difference in offspring total methylation between calves from drylot- versus range-developed heifers (P = 0.55).

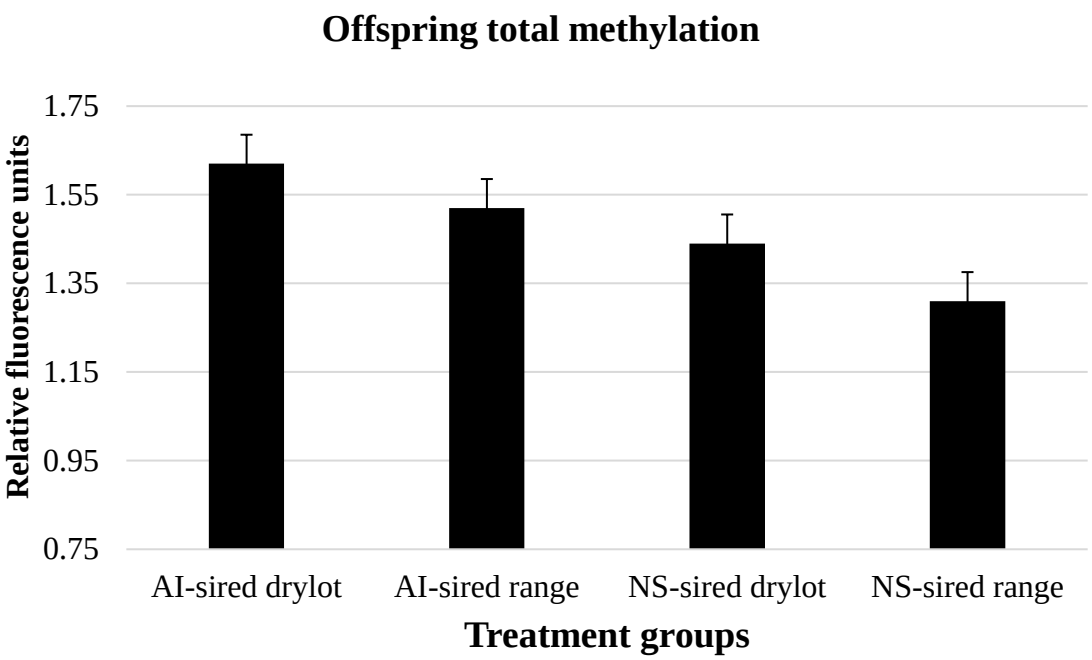

Figure 21. Effect of heifer development and sire type on offspring total methylation, where NS-sired = natural service-sired. There was no interaction between heifer development and sire type on offspring total methylation (P = 0.92).

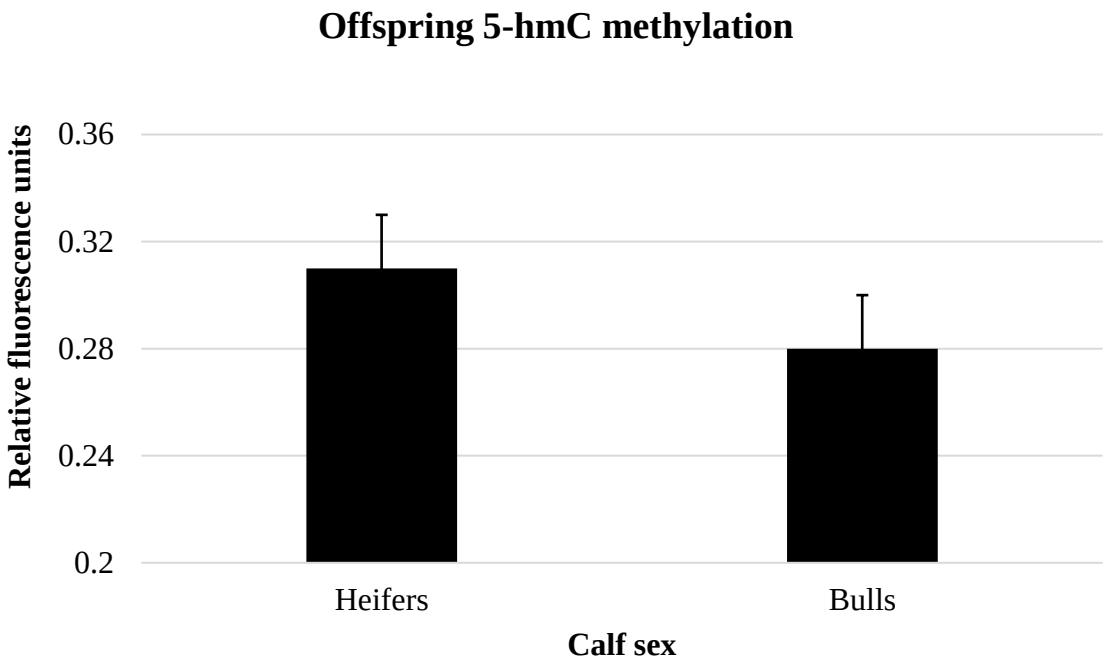

Figure 22. Effect of calf sex on offspring 5-hmC methylation. Heifer calves tended to express greater 5-hmC methylation compared to bull calves (P = 0.10).

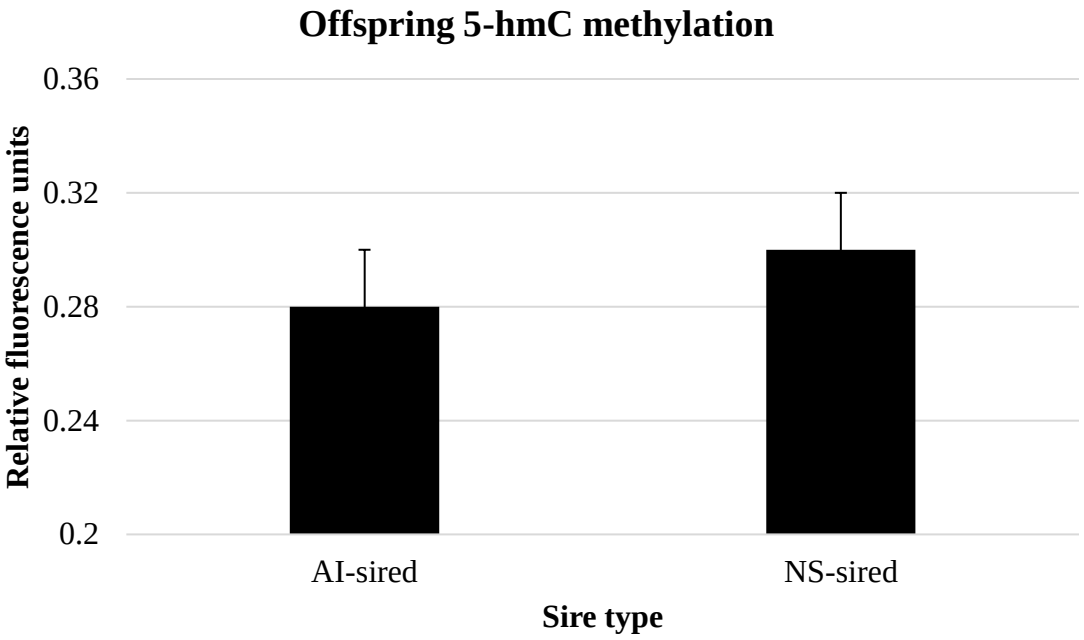

Figure 23. Effect of sire type on offspring 5-hmC methylation. There was no difference in 5-hmC methylation between AI-sired and natural service-sired (NS-sired) calves (P = 0.35).

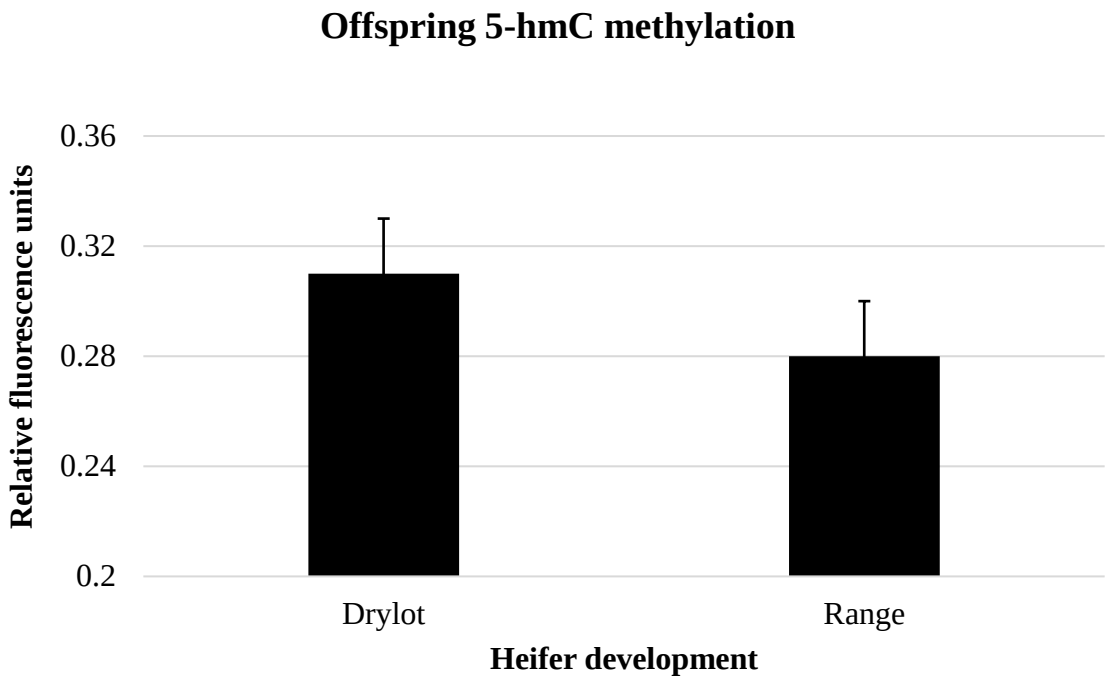

Figure 24. Effect of heifer development on offspring 5-hmC methylation. Calves from drylot-developed heifers tended to express greater 5-hmC methylation compared to calves from range-developed heifers (P = 0.10).

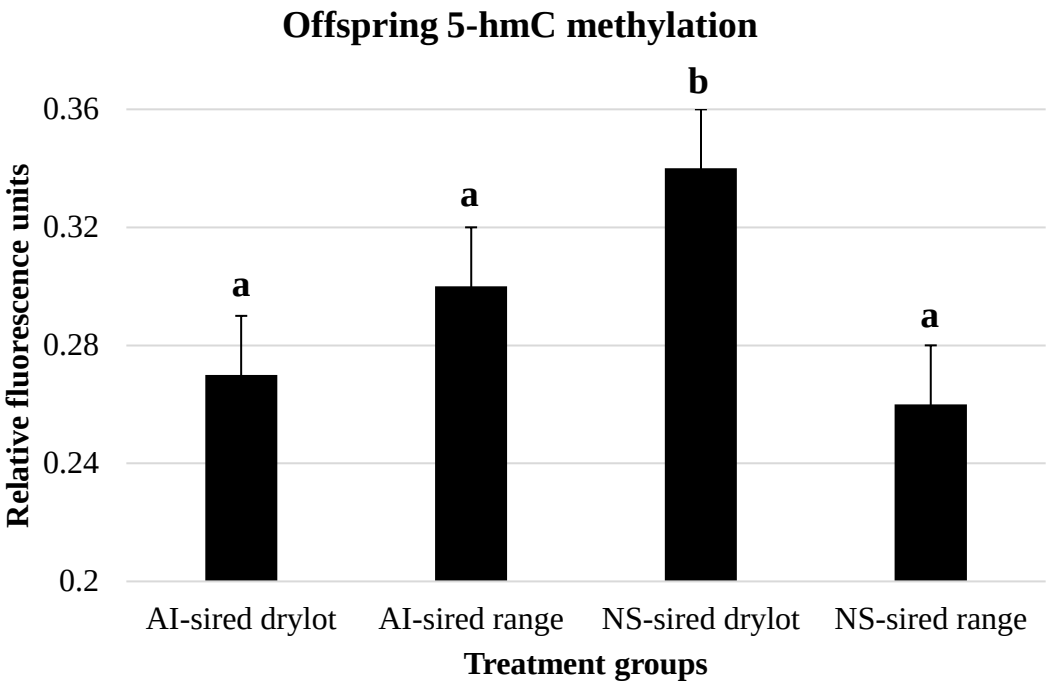

Figure 25. Effect of heifer development and sire type on offspring 5-hmC methylation. Natural service-sired (NS-sired) range, AI-sired range, and AI-sired drylot calves expressed less 5-hmC methylation compared to NS-sired drylot calves ($P = 0.003$).

[ab] Superscripts differ ($P < 0.05$).

DISCUSSION

Heifer calves are commonly reported to have lighter BW and WW compared to their male counterparts (Long et al., 2012), which was confirmed in the current study. Calves sired by AI also had lighter BW but heavier WW compared to NS-sired calves, which is likely due to selectivity in choosing a sire through AI that allows for these growth characteristics (Bourdon and Brinks, 1982). Heavier WW of AI-sired calves may also be a result of calves being born earlier in the calving season and thus given more time to grow until weaning compared to NS-sired calves that were born later in the calving season.

Growth characteristics of the offspring may be impacted by changes in the uterine environment during gestation. Nutritional status of the dam influences the uterine environment, and changes in maternal nutrition may impact conceptus development and postnatal growth of the offspring (Du et al., 2010). Method of heifer development places heifers in a negative energy balance if they are not acclimated to grazing (Perry et al., 2013), which may have implications on the uterine environment, conceptus development, and postnatal offspring development. Heifers develop grazing habits during the pre-weaning period when on pasture with their dams (Provenza and Balph, 1987). Heifers that are moved to a drylot at weaning, however, do not retain these skills and exert more energy in foraging and ingesting nutrients when reacquainted with a grazing environment (Arnold and Maller, 1977; Osuji, 1974). Heifers that grazed between weaning and breeding exhibited better retention of grazing skills and had increased ADG the following summer compared to heifers placed in a drylot (Olson et al., 1992). Heifers in the current study underwent a negative energy balance when moved from a drylot situation to

forage-grazing setting immediately following AI, as evidenced by a previous study when drylot-developed heifers moved to a spring grazing setting lost 1.6 ± 0.17 kg/d during the first week of grazing compared with range-developed heifers that gained 0.88 ± 0.16 kg/d (Perry et al., 2013). Drylot-developed heifers also had reduced AI pregnancy success (49.1%) compared to heifers with prior grazing experience (59.4%). However, overall breeding season conception rates were not different between drylot- and range-developed heifers in the current study, indicating that drylot-developed heifers became acclimated to grazing after an adaptation period (Perry et al., 2013). No difference was detected in mature body weight between drylot- and range-developed heifers at the beginning of the breeding season; therefore, decreased dry matter intake due to drylot-developed heifers re-learning grazing behavior and expending additional energy post-AI resulted in subsequent body weight loss and reduced pregnancy success to AI in drylot-developed heifers. Calves from drylot-developed heifers in the current study also had lighter BW and WW compared to calves from range-developed heifers, suggesting that lack of grazing experience for drylot-developed heifers created nutritional stress during the early stages of embryogenesis that impacted long-term development of the offspring.

Nutritional stress in utero did not appear to impact subsequent offspring ADG in AI-sired calves, as ADG of AI-sired calves from drylot-developed heifers was similar to ADG of AI-sired calves from range-developed heifers. However, ADG of NS-sired calves from drylot-developed heifers was reduced compared to ADG of NS-sired calves from range-developed heifers. Although embryo development was likely not impacted, as drylot-developed heifers would have adapted to a grazing environment by the time of natural service, nutritional stress may have had a negative impact on follicular

development when drylot-developed heifers were first turned out to a grazing setting after AI. Small antral follicular growth is sensitive to nutritional insults (Gutiérrez et al., 1997), and a period of nutritional restriction may have impacted competency of the oocyte, subsequent embryo development, and postnatal performance.

The process of DNA methylation, which represses long-term gene expression in the conceptus, is susceptible to stresses such as changes in maternal nutrition (Gicquel et al., 2008; Holland and Rakyan, 2013) and may be a potential mechanism mediating conceptus and postnatal development in response to maternal nutrition. Drylot-developed heifers in the current study experienced nutrient restriction immediately following AI while re-learning how to graze efficiently; therefore, it is likely that DNA methylation was impacted in the conceptus during this time, as DNA methylation occurs during the beginning stages of blastocyst development (Bird, 2002). In the current study, no differences were observed in total DNA methylation for offspring across treatments; however, tendencies for changes in offspring 5-hmC methylation patterns were observed in concurrence with changes in offspring performance. Calves from drylot-developed heifers had a tendency for increased 5-hmC methylation compared to calves from range-developed heifers and also had lighter BW and WW. Natural service-sired calves from drylot-developed heifers expressed greater 5-hmC methylation compared to calves from all other treatment groups and also tended to have reduced ADG compared to AI-sired calves from drylot-developed heifers and NS-sired calves from range-developed heifers. This data indicates a possible association between reduced offspring performance and increased 5-hmC methylation in this study. As 5-hmC methylation is associated with regulation of gene expression (Branco et al., 2012; Murrell et al., 2013), 5-hmC

methylation in the offspring may have been impacted in utero during the adaptation period when heifers were developing foraging skills and experiencing a negative energy balance. Changes in maternal nutrition during early embryogenesis have the potential to impact all cells within the fetus, as cells during this time are totipotent and have yet to differentiate into specific tissues (Gicquel et al., 2008; Santos and Dean, 2004). Any changes that affect totipotent cells will impact all future development of tissues within the fetus. Therefore, lack of grazing experience for drylot-developed heifers in the current study at the time of AI may be responsible for 1) reduced AI pregnancy success of drylot-developed heifers compared to range-developed heifers and 2) reduced growth traits of the offspring due to changes in 5-hmC methylation and gene expression during early embryogenesis.

In conclusion, results from this study show compelling evidence to further support a correlation between heifer development and sire type on offspring performance and progeny 5-hmC DNA methylation. While expression of total offspring methylation was not different between treatments, an increase in 5-hmC methylation corresponded with lighter BW and WW of calves from drylot-developed heifers with a tendency for reduced ADG in NS-sired calves from drylot-developed heifers. These results indicate that the period of nutrient restriction drylot-developed heifers experience while adapting to grazing at the beginning of the breeding season may have long-lasting impacts on offspring performance and may be explained in part by the mechanism of DNA methylation.

CHAPTER III

EFFECTS OF PRE- AND POST-INSEMINATION MATERNAL PLANE OF NUTRITION ON UTERINE ENVIRONMENT AND EMBRYO DEVELOPMENT

ABSTRACT

Nutritional changes immediately after insemination can result in increased embryonic mortality, but the mechanisms that cause this increased embryonic mortality are not known. Therefore, the objective of this study was to evaluate the impacts of a nutritional change immediately following AI on estrus expression and interval to estrus in heifers, peripheral metabolites, uterine luminal fluid metabolites, and day 6 embryo quality. Sixty Angus-cross heifers (351 ± 47 kg) were allotted into two pre-AI treatments: low or high. Low treatment heifers were limit-fed ground cornstalks and mineralized soybean meal and urea to achieve 64.1% maintenance. High treatment heifers had ad libitum access to ground cornstalks and supplemented mineralized soybean meal, urea, and corn to achieve 139% maintenance. Heifers remained in their respective treatments for 33 to 36 days and were then inseminated upon estrus expression following the PG 6-d CIDR protocol with semen from a single collection of a single beef sire. Following AI, 30 heifers were randomly reassigned within treatment, creating four nutritional treatments: low treatment remaining on low (LL), low treatment moving to high (LH), high treatment remaining on high (HH), and high treatment moving to low (HL). Heifers remained on treatments post-AI for six days and were then flushed for embryo and uterine fluid collection. Blood samples were collected daily from AI to embryo collection. Interval to estrus, and uterine luminal fluid (ULF) concentrations of NEFA, glucose, and protein were analyzed using the GLM procedure of SAS. Estrus expression

and embryo recovery rates were analyzed with the GLIMMIX procedure in SAS while embryo stage and grade were analyzed using the MIXED procedure of SAS. Weekly heifer weights, energy intake, and plasma concentrations of NEFA, glucose, and protein were analyzed through repeated measures using the MIXED procedure in SAS. Heifers in the LL treatment had the lowest overall energy intake (64.1%, 6.60 Mcal/d; $P < 0.0001$) compared to LH heifers (81.8%, 8.44 Mcal/d), HH heifers (139%, 14.03 Mcal/d), and HL heifers (116%, 12.02 Mcal/d). Time impacted amount of maintenance energy consumed, with heifers consuming the greatest level of intake the week prior to treatment switch (104%; $P < 0.0001$). There was also a treatment x time effect, in which HH and LL heifers maintained their level of intake throughout the study, whereas HL heifers' plane of intake declined sharply during the last six days after the treatment switch in contrast to LH heifers, which rapidly increased their plane of intake ($P < 0.0001$). A treatment effect was observed on average heifer weight throughout the study ($P < 0.0001$), with LL heifers exhibiting lighter body weights (332.1 ± 2.1 kg) compared to LH heifers (340.0 ± 2.1 kg), HH heifers (351.0 ± 2.2 kg), and HL heifers (359.5 ± 2.2 kg). Time also impacted heifer weight ($P = 0.002$), with similar weights across treatments from week -4 (trial start) through week -1 (352.2 kg ± 2.9 kg, 347.7 ± 2.9 kg, 348.3 ± 2.9 kg, 349.4 ± 2.9 kg, and 344.5 ± 2.9 kg for trial start weight, week -4, week -3, week -2, and week -1 respectively). The trial start weight and weeks -3 and -2 weights were heavier compared to d 0 (340.3 ± 2.9 kg) and d 6 (337.0 ± 2.9 kg) weights. Weights during weeks -4 and -1 were similar compared to the d 0 weight but heavier compared to the d 6 weight while weights between d 0 and 6 were similar. A treatment x time interaction was observed ($P = 0.02$), with LL heifers losing weight throughout the study (-0.81 ± 0.24 kg/d) while HH

heifers maintained body weight (0 ± 0.26 kg/d). Heifers in the HL treatment maintained weight prior to AI (0.26 ± 0.29 kg/d) but lost weight during the last six days after AI (-2.90 ± 1.42 kg/d), whereas LH heifers lost weight prior to AI (-0.72 ± 0.22 kg/d) but did not exhibit weight gains during the last six days after AI (1.37 ± 0.94 kg/d). No difference was observed between low and high treatments preceding AI for estrus expression ($P = 0.22$). No difference was observed between pre-AI low and high treatments for interval to estrus among heifers expressing estrus ($P = 0.57$). When heifers that did not express estrus were included with estrus heifers, there was no statistical difference between low and high treatments for interval to estrus ($P = 0.20$). Pre-AI treatment did not impact embryo recovery rate ($P = 0.57$). Post-AI treatment approached a tendency to affect embryo recovery rate ($P = 0.12$), with high treatment heifers tending to have greater embryo recovery rates (48.3 ± 9.1%) compared to low treatment heifers (27.4 ± 9.6%). There was no interaction between pre- and post-AI treatments on embryo recovery rate ($P = 0.75$). Embryo stage was impacted by pre-AI treatment ($P = 0.05$), with high treatment heifers preceding AI yielding embryos with a more advanced stage (stage = 2.98 ± 0.4) compared to low treatment heifers (stage = 1.79 ± 0.4). Post-AI treatment also tended to affect embryo stage ($P = 0.07$), with low treatment heifers tending to yield embryos that were at a less advanced stage (stage = 1.83 ± 0.5) compared to high treatment heifers (stage = 2.93 ± 0.3). However, there was no interaction between pre- and post-AI treatments on embryo stage ($P = 0.42$). Pre-AI plane of nutrition approached a tendency to impact embryo grade ($P = 0.17$), with low treatment heifers tending to have embryos with a poorer grade (grade = 3.21 ± 0.4) compared to high treatment heifers (grade = 2.40 ± 0.4). Post-AI nutrition tended to affect embryo grade (*P*

= 0.08), with low treatment heifers yielding embryos with a poorer grade (grade = 3.33 ± 0.5) compared to high treatment heifers (grade = 2.29 ± 0.5). However, there was no interaction between pre- and post-AI plane of nutrition on embryo grade (P = 0.37). Heifers in the LL (0.59 ± 0.04 mEq/L) and HL treatments (0.61 ± 0.04 mEq/L) had greater plasma NEFA concentrations (P < 0.0001) compared to LH heifers (0.37 ± 0.04 mEq/L) and HH heifers (0.34 ± 0.04 mEq/L). Time also impacted NEFA concentrations (P < 0.0001), with the greatest NEFA concentrations occurring on d 3 after AI (0.68 ± 0.03 mEq/L), compared to d 0 (AI; 0.49 ± 0.03 mEq/L) and d 6 after AI (0.26 ± 0.03 mEq/L). There was also a treatment x time interaction (P < 0.0001), with LL and HL heifers exhibiting a sharp increase in NEFA concentrations from d 0 to d 3 and then declining again to d 6, whereas LH heifers showed a continual decline in NEFA concentrations from d 0 through d 6. Treatment did not impact plasma glucose concentrations (P = 0.49); however, time affected glucose concentrations (P = 0.002), with the greatest concentrations occurring on d 0 (0.75 ± 0.02 mg/dL) and declining on d 3 (0.71 ± 0.02 mg/dL) and d 6 (0.66 ± 0.02 mg/dL). There was no interaction between treatment and time (P = 0.81). Treatment impacted plasma protein concentrations (P = 0.003), with LL heifers (0.61 ± 0.007 mcg/mL) having greater plasma protein concentrations than LH heifers (0.58 ± 0.007 mcg/mL) and HH heifers (0.57 ± 0.007 mcg/mL) but similar concentrations compared to HL heifers (0.59 ± 0.007 mcg/mL). Heifers in the HL and LH treatments had similar plasma protein concentrations, whereas LH heifers also had similar protein concentrations compared to HH heifers. Time did not impact plasma protein concentrations (P = 0.22), and there was no interaction between treatment and time on plasma protein concentrations (P = 0.84). Uterine luminal fluid

NEFA concentrations were not impacted by pre-AI treatment (P = 0.95) or post-AI treatment (P = 0.74). There was also no interaction between pre- and post-AI treatment (P = 0.76). However, estrus expression tended to impact ULF NEFA concentrations (P = 0.10), with heifers that expressed estrus tending to have greater concentrations of ULF NEFA (0.02 ± 0.002 mEq/L) compared to heifers which did not show estrus (0.006 ± 0.006 mEq/L). Concentrations of ULF glucose were not impacted by pre-AI treatment (P = 0.41), post-AI treatment (P = 0.70), or pre- and post-AI treatment interaction (P = 0.27). Furthermore, ULF glucose concentrations were not impacted by estrus expression (P = 0.61). Uterine luminal fluid concentrations of protein were not impacted by pre-AI treatment (P = 0.55) nor post-AI treatment (P = 1.0). Furthermore, there was no interaction between pre- and post-AI treatments (P = 0.76); however, estrus expression approached a tendency to impact ULF protein concentrations (P = 0.13), with heifers expressing estrus tending to have greater ULF protein concentrations (0.56 ± 0.14 mcg/mL) compared to heifers not expressing estrus (0.33 ± 0.06 mcg/mL). In summary, results from this study indicate that the early stages of embryo development are sensitive to maternal plane of nutrition; therefore, proper nutritional management is critical around the time of AI to ensure heifers are not placed in a negative energy balance nor adversely impacting embryo survival and early conceptus development.

INTRODUCTION

Early embryonic failure accounts for 44% of reproductive failure from a single service to AI in the beef industry (Diskin and Sreenan, 1980), with the majority of embryonic mortality occurring between eight and 16 d post insemination (Diskin et al., 2011). Management practices can influence embryonic fatality; therefore, managing plane of nutrition is crucial to optimizing pregnancy success to AI in heifers.

Short-term nutritional insults immediately following insemination have been reported to impede embryogenesis and decrease embryo quality (Kruse et al., 2017). Differing planes of nutrition prior to and following insemination have also been reported to impact embryo survival. Dunne et al. (1999) reported that heifers fed 200% of maintenance requirements prior to insemination and switched to 80% following AI had significantly reduced embryo survival compared to heifers remaining at 80% or 200% throughout the trial or fed 80% and then increased to 200% after insemination. Although the mechanism for embryonic mortality is not yet determined, maternal nutrient restriction creates an unfavorable environment for embryogenesis and results in increased embryonic failure. Changes in concentrations of peripheral maternal metabolites may mediate changes in both the follicular fluid and the endometrium (Bossis et al., 1999), thereby impacting follicular development, oocyte competency, and uterine secretions necessary for embryonic survival. Therefore, the objective of this study was to determine how pre- and post-insemination planes of nutrition in heifers impact interval to estrus, estrus expression, maternal metabolite circulation, uterine environment, and embryo development to day 6 of gestation.

MATERIALS AND METHODS

Experimental Design

All treatments were approved by the South Dakota State University Institutional Animal Care and Use Committee. Angus-crossbred heifers (n = 60), approximately 12 mo of age, from the SDSU Cottonwood Range and Livestock Field Station were used to evaluate the impacts of nutrient manipulation on embryo development and uterine environment. This research was conducted during the spring of 2017 at the SDSU Beef Breeding Unit located in Brookings, South Dakota. Heifers were randomly assigned to one of two treatments and were blocked within treatment by initial body weight (351 ± 47 kg). Treatments were designated as heifers fed 139% maintenance (high treatment) and heifers fed 64.1% maintenance (low treatment). Diets were formulated using the Beef Cattle Nutrient Requirements Model (NRC, 2016). Heifers were evenly distributed across body weight blocks and treatments based on initial body weight (low treatment: low block [n = 10], medium block [n = 10], high block [n = 10]; high treatment: low block [n = 10]; medium block [n = 10], high block [n = 10]). High treatment heifers were provided *ad libitum* access to ground cornstalks (Table 2) in concrete bunks (1.06 m of bunk space/hd). Ground cornstalks were weighed on a daily basis and offered to heifers, and ground cornstalk orts were collected weekly, weighed, and subsampled for nutrient analysis. High treatment daily intake was calculated per body weight block as a percent of body weight based on pen average weekly consumption (Table 3). High treatment heifers were individually supplemented with corn and mineralized soybean meal and urea (Table 2) daily at 1400 h to meet 139% (14.03 Mcal/d) of maintenance requirements for low, medium, and high body weight blocks. Corn and soybean meal supplement was provided based on body weight block (Table 4). Low treatment heifers were offered

ground cornstalks individually (Table 2) twice daily at 0700 h and 1500 h with a feeding time of 2 h. Ground cornstalk refusals were collected after each feeding and weighed. Orts were collected after each feeding from d 33 to d 41 and subsampled for nutrient analysis. Orts quality was similar to feed offered; thus, nutrient quality of ground cornstalks consumed by heifers was not adjusted for orts quality. Low treatment daily intake was calculated per body weight block as an average of individual daily consumption (Table 3). Low treatment heifers were individually supplemented with mineralized soybean meal and urea daily (Table 4) at 1500 h to meet 64.1% (6.60 Mcal/d) of maintenance requirements for low, medium and high body weight blocks. Heifers were acclimated to individual feeding 1 wk prior to the start of the experiment.

Initial heifer body weight was determined from an average of weights taken on two consecutive days prior to the start of the experiment. Additional body weights were collected weekly prior to afternoon supplementation and feeding, at the time of AI, and during conceptus flushing. Initial body condition scores (BCS) were determined by two trained technicians, based both on visual observation and palpation of the ribs and vertebra (1 = thin and 9 = obese) prior to the start of the experiment and again at the end of the experiment prior to embryo collection.

Synchronization and Estrus Detection

Heifers remained in their respective treatments for 33 to 36 days prior to artificial insemination (AI). Heifers were inseminated in four replicates with an equal number of heifers from each treatment in each replicate to accommodate the number of heifers in which uterine flushes could be performed per day to evaluate embryo recovery and embryo characteristics. Heifers were synchronized for breeding using the prostaglandin

(PG) 6-d controlled internal drug release (CIDR) protocol, which consisted of the administration of $PGF_{2\alpha}$ (PG; 25 mg as 2 mL of Lutalyse HighCon i.m.; Zoetis, Florham Park, NJ) on d -9, insertion of an Eazi-Breed CIDR insert (1.38 g progesterone; Zoetis, Florham Park, NJ) and administration of GnRH (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) on d -6, and CIDR removal and administration of $PGF_{2\alpha}$ (PG; 25 mg as 2 mL of Lutalyse HighCon i.m.; Zoetis, Florham Park, NJ) on d 0. Estrus was monitored visually following CIDR removal from 530 h to 2100 h in conjunction with EstroTect patches (Western Point, Inc., Apple Valley, MN). Heifers that had greater than half of the patch scratched off were classified as exhibiting standing estrus. Heifers were bred 8 – 12 h after their first standing activity by one of three trained AI technicians with semen from a single collection of a single beef sire. Breeding by AI technicians was distributed evenly among treatments. If estrus was not observed after 91 h, the heifer's follicular dynamics were assessed by transrectal ultrasonography using an Aloka 500V ultrasound with a 7.5 MHz linear probe (Aloka, Wallingford, CT). Follicles > 6 mm in diameter were recorded, and GnRH (100 µg as 2 mL of Factrel i.m.; Pfizer Animal Health, Madison, NJ) was administered to induce ovulation at the time of insemination. Two heifers (1 LL, 1 HL) did not have a large follicle and were removed from plasma and uterine luminal fluid analyses but remained in the animal performance analyses.

Treatment Change

Following AI, 30 heifers were randomly reassigned within treatment, with 15 heifers from the low treatment moved to the high treatment diet and 15 heifers from the high treatment moved to the low treatment diet. Thirty heifers remained in their original treatments post-AI, creating four nutritional treatments: low treatment remaining on low

(LL), low treatment moving to high (LH), high treatment remaining on high (HH), and high treatment moving to low (HL). Heifers were evenly redistributed among body weight blocks to ensure that low and high treatment diets each had 30 heifers and 10 heifers remained in each body weight block. Heifers remained on treatments post-AI for six days until embryo recoveries were performed.

Embryo Collection and Evaluation

Six days after AI, all animals were subjected to transcervical uterine catheterization. The side of ovulation was confirmed by the presence of a CL using transrectal ultrasonography (7.5-MHz linear array transducer, IBEX, EVO, Loveland, CO), a catheter was placed in the uterine horn ipsilateral to the CL, and the horn was flushed with flush media (100 mL 10X Dulbecco's phosphate buffered solution, 50 mL 0.269% $CaCl_2$, and 850 mL double-distilled H_2O). Heifers were initially flushed with 15 mL of flush media to maintain a constant volume. Recovered media was filtered, collected, and snap frozen for further analysis. Flush media was assessed under a microscope to determine if an embryo was present. If no conceptus was recovered, the uterine horn was then flushed an additional two or three times to increase the likelihood of embryo recovery. Recovered embryos (n = 12) were assigned a quality grade (scale 1 to 5; 1 = excellent, 2 = good, 3 = fair, 4 = poor, 5 = dead or degenerative) and evaluated for stage of development (scale 1 to 9; 1 = unfertilized, 2 = 2- to 12-cell, 3 = early morula, 4 = morula, 5 = early blastocyst, 6 = blastocyst, 7 = expanded blastocyst, 8 = hatched blastocyst, 9 = expanding hatched blastocyst) according to International Embryo Transfer Society standards (IETS, 2009) by an embryologist blind to treatment. Three heifers (2 LL, 1 LH) were not successfully flushed and were removed from all analyses.

Sampling and Analysis

Blood samples were collected daily by jugular venipuncture into 10 mL Vacutainer tubes containing EDTA (Fischer Scientific, Pittsburgh, PA) from the day of AI through d 6. Blood was immediately placed on ice and shipped to the laboratory. Plasma was harvested following centrifugation (1200 x g for 30 minutes at 4°C) and stored at -20°C. Collected blood was analyzed for determination of plasma concentrations of non-esterified fatty acids (NEFA), glucose, and protein. Uterine luminal fluid (ULF) was stored at -80°C until analyses were conducted for NEFA, glucose, and protein concentrations.

Plasma and ULF concentrations of NEFA were quantified in duplicate with an enzymatic colorimetric assay kit (NEFA-C Wako Chemicals, Richmond, VA) with intra- and inter-assay coefficients of variation of 8.2% and 5.4% for plasma and 23% and 18% for ULF, respectively. Working standards (0, 0.50, 1.00, and 1.97 mEq/L) were prepared by serial dilution using Milli-Q water and standard provided in NEFA-C kit. Enzymatic color reactive reagents A and B were prepared per instructions provided with assay kit. In a 96-well, flat-bottomed, microtiter plate; sample, standard, Milli-Q water (blank; 5 µL) and internal lab control (2.5, 5, and 10 µL) were incubated with 200 µL Color Reagent A at 37°C for five minutes. Following addition of 100 µL of Color Reagent B, sample plates were again incubated at 37°C for five minutes. Absorbance was measured at 550 nm on the SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA).

Plasma and ULF concentrations of glucose were determined in duplicate with a Glucose Liquicolor Kit (Stanbio Laboratory, Boerne, TX) with intra- and inter-assay coefficients of variation of 9.2% and 16% for plasma and 30% and 24% for ULF,

respectively. Assay materials were warmed to room temperature, at which time reagent (1 mL) provided with assay kit was pipetted into 96 12 x 75 glass tubes and incubated at 37°C for five minutes. Working standards (0, 100, 200, 300, and 400%) were prepared by pipetting 0, 5, 10, 15, and 20 µL respectively of provided standard into appropriate tubes. Sample was added at 5 µL, mixed thoroughly, and incubated at 37°C for five minutes. Sample and standard (300 µL) were then transferred from each tube onto a 96-well plate. Absorbance was measured at 500 nm on the SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA).

Plasma and ULF concentrations of protein were assessed using the Braford protein colorimetric assay with an intra-assay coefficient of variation of 4.6% for plasma and 5.3% for ULF. Working standards (0, 25, 125, 250, 500, 750, 1,000, 1,500, and 2,000 µg/mL) were prepared by serial dilution using Easy Buffer B (IMV Technologies USA, Maple Grove, MN) and bovine serum albumin from the Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Plasma samples underwent a 1:100 volumetric dilution using Easy Buffer B and were pipetted into a 96-well plate with standard (10 µL). Coomassie Plus Reagent (Thermo Scientific, Rockford, IL) was brought to room temperature and added to samples and standard (300 µL). Sample plates were shaken in the SpectraMax Plus 384 microplate reader for 30 seconds and incubated at room temperature for 10 min. Absorbance was then measured at 595 nm.

Nutrient Analysis

All feed ingredients and orts were analyzed by Dairyland Laboratories (Arcadia, WI). Cornstalks were subsampled daily and composited into three samples for analysis (Table 2). Soybean meal was subsampled prior to the study for appropriate supplement

formation and was analyzed. Soybean meal and urea were mixed proportionally in 113.4 kg batches and subsampled per batch prior to the addition of minerals. Subsamples were pooled and analyzed for crude protein, ADF, aNDF, aNDFom, lignin, AD-ICP, ND-ICP, fat, ash, TDN, and NEl (Table 2). Phosphorous, trace mineral salt, vitamins A and E, and calcium were included in the supplement to balance for mineral deficiencies according to the Beef Cattle Nutrient Requirements Model (NRC, 2016).

Statistical Analysis

Interval to estrus and estrus expression were analyzed using the GLM procedure of SAS with pre-AI treatment included in the model. Interval to estrus included separate models analyzing heifers that expressed estrus versus all heifers within the study (animals that did not exhibit estrus were included at an interval of 91 h). Embryo recovery was analyzed using the GLIMMIX procedure of SAS with pre- and post-AI treatments included as independent variables. Embryo stage and grade were analyzed using the MIXED procedure of SAS with pre- and post-AI treatments included as independent variables. Weekly heifer weights, energy intake, and plasma concentrations of NEFA, glucose, and protein were analyzed through repeated measures using the MIXED procedure of SAS, with the indicated best fit model of compound symmetry as the covariance structure and treatment, time, and treatment x time included as independent variables. Concentrations of ULF NEFA, glucose, and protein were analyzed using the GLM procedure of SAS. Treatment, presence of an embryo, and estrus expression were included in the model as independent variables.

RESULTS

Energy Intake

Heifers in the LL treatment had the lowest overall energy intake (64.1%, 6.60 Mcal/d; $P < 0.0001$; Figure 26) compared to LH heifers (81.8%, 8.44 Mcal/d), HH heifers (139%, 14.03 Mcal/d), and HL heifers (116%, 12.02 Mcal/d) throughout the entire feeding period. Time impacted amount of maintenance energy consumed ($P < 0.0001$; Figure 27), with heifers consuming less during week -4 (96.1%) in comparison to weeks -2 and -1 prior to AI and at treatment switch (103.9% and 104.3% respectively). There was also a treatment x time effect, in which HH and LL heifers maintained their level of intake throughout the study, whereas HL heifers' plane of intake declined sharply during the last six days after the treatment switch in contrast to LH heifers, which rapidly increased their plane of intake ($P < 0.0001$; Figure 28).

Animal Performance

A treatment effect was observed on heifer weight at embryo collection ($P < 0.0001$), with LL heifers exhibiting lighter body weights (332.1 ± 2.1 kg; Figure 29) compared to LH heifers (340.0 ± 2.1 kg), HH heifers (351.0 ± 2.2 kg), and HL heifers (359.5 ± 2.2 kg). Time also impacted heifer weight ($P = 0.002$; Figure 30), with similar weights across treatments from the beginning of the trial through week -1 (352.2 kg ± 2.9 kg, 347.7 ± 2.9 kg, 348.3 ± 2.9 kg, 349.4 ± 2.9 kg, and 344.5 ± 2.9 kg for trial start weight, week -4, week -3, week -2, and week -1, respectively). The trial start weight and weeks -3 and -2 weights were heavier compared to d 0 (340.3 ± 2.9 kg) and d 6 (337.0 ± 2.9 kg) weights. Weights during weeks -4 and -1 were similar compared to the d 0 weight but heavier compared to the d 6 weight while weights between d 0 and 6 were similar. A treatment x time interaction was observed ($P = 0.02$; Figure 31), with LL heifers losing

weight throughout the study (-0.81 ± 0.24 kg/d) while HH heifers maintained body weight (0 ± 0.26 kg/d). Heifers in the HL treatment maintained weight prior to AI (0.26 ± 0.29 kg/d) but lost weight during the last six days after AI (-2.90 ± 1.42 kg/d), whereas LH heifers lost weight prior to AI (-0.72 ± 0.22 kg/d) but did not exhibit significant weight gains during the last six days after AI (1.37 ± 0.94 kg/d).

Estrus Expression and Interval to Estrus

No difference was observed between low and high treatments preceding AI for estrus expression (P = 0.22; Figure 32). No difference was observed between low and high treatments preceding AI for interval to estrus among heifers expressing estrus (P = 0.57; Figure 33). When heifers that did not express estrus were included in the model, there was no difference between low and high treatments for interval to estrus (P = 0.20; Figure 34).

Embryo Recovery, Stage, and Grade

Plane of nutrition between low and high treatments prior to AI did not impact embryo recovery rate (P = 0.57; Figure 35). Plane of nutrition between low and high treatments following AI approached a tendency to affect embryo recovery rate (P = 0.12; Figure 36), with high treatment heifers tending to have greater embryo recovery rates (48.3 ± 9.1%) compared to low treatment heifers (27.4 ± 9.6%). There was no interaction between pre- and post-AI treatments on embryo recovery rate (P = 0.75; Figure 37).

Embryo stage was impacted by pre-AI plane of nutrition (P = 0.05; Figure 38), with high treatment heifers preceding AI yielding embryos with a more advanced stage (stage = 2.98 ± 0.4) compared to low treatment heifers (stage = 1.79 ± 0.4). Post-AI plane of nutrition also tended to affect embryo stage (P = 0.07; Figure 39), with low treatment heifers following AI tending to yield embryos that were at a less advanced stage (stage =

1.83 ± 0.5) compared to high treatment heifers (stage = 2.93 ± 0.3). However, there was no interaction between pre- and post-AI treatments on embryo stage (P = 0.42; Figure 40).

Pre-AI plane of nutrition approached a tendency to impact embryo grade (P = 0.17; Figure 41), with low treatment heifers preceding AI tending to have embryos with a poorer grade (grade = 3.21 ± 0.4) compared to high treatment heifers (grade = 2.40 ± 0.4). Post-AI plane of nutrition tended to affect embryo grade (P = 0.08; Figure 42), with low treatment heifers yielding embryos with a poorer grade (grade = 3.33 ± 0.5) compared to high treatment heifers (grade = 2. 29 ± 0.5). However, there was no interaction between pre- and post-AI plane of nutrition on embryo grade (P = 0.37; Figure 43).

Plasma: Non-Esterified Fatty Acids

Heifers in the LL (0.59 ± 0.04 mEq/L) and HL treatments (0.61 ± 0.04 mEq/L) had greater NEFA concentrations (P < 0.0001; Figure 44) compared to LH heifers (0.37 ± 0.04 mEq/L) and HH heifers (0.34 ± 0.04 mEq/L). Time also impacted NEFA concentrations (P < 0.0001; Figure 45), with the greatest NEFA concentrations occurring on d 3 after AI (0.68 ± 0.03 mEq/L) and least concentrations on d 6 after AI (0.26 ± 0.03 mEq/L), compared with immediate concentrations on d 0 (AI; 0.49 ± 0.03 mEq/L). There was also a treatment x time interaction (P < 0.0001; Figure 46), with LL and HL heifers exhibiting a sharp increase in NEFA concentrations from d 0 to d 3 and then declining again to d 6. Plasma NEFA concentrations increased moderately from d 0 to d 3 for HH heifers before declining to d 6, whereas LH heifers showed a continual decline in NEFA concentrations from d 0 through d 6.

Plasma: Glucose

Treatment did not impact plasma glucose concentrations (P = 0.49; Figure 47); however, time affected glucose concentrations (P = 0.002; Figure 48), with similar plasma glucose concentrations occurring on d 0 (0.75 ± 0.02 mg/dL) and d 3 after AI (0.71 ± 0.02 mg/dL) before declining on d 6 (0.66 ± 0.02 mg/dL). There was no interaction between treatment and time (P = 0.81; Figure 49).

Plasma: Protein

Treatment impacted plasma protein concentrations (P = 0.003; Figure 50), with LL heifers (0.61 ± 0.007 mcg/mL) having greater plasma protein concentrations than LH heifers (0.58 ± 0.007 mcg/mL) and HH heifers (0.57 ± 0.007 mcg/mL) but similar concentrations compared to HL heifers (0.59 ± 0.007 mcg/mL). Heifers in the HL and LH treatments had similar plasma protein concentrations, whereas LH heifers also had similar protein concentrations compared to HH heifers (Figure 50). Time did not impact plasma protein concentrations (P = 0.22; Figure 51), and there was no interaction between treatment and time on plasma protein concentrations (P = 0.84; Figure 52).

Uterine Luminal Fluid: Non-Esterified Fatty Acids

Uterine luminal fluid NEFA concentrations were not impacted by pre-AI treatment (P = 0.95; Figure 53) or post-AI treatment (P = 0.74; Figure 54). There was also no interaction between pre- and post-AI treatment (P = 0.76; Figure 55). However, estrus expression tended to impact ULF NEFA concentrations (P = 0.10; Figure 56), with heifers that expressed estrus tending to have greater concentrations of ULF NEFA (0.02 ± 0.002 mEq/L) compared to heifers which did not show estrus (0.006 ± 0.006 mEq/L).

Uterine Luminal Fluid: Glucose

Concentrations of ULF glucose were not impacted by pre-AI treatment ($P = 0.41$; Figure 57), post-AI treatment ($P = 0.70$; Figure 58), or pre- and post-AI treatment interaction ($P = 0.27$; Figure 59). Furthermore, ULF glucose concentrations were not impacted by estrus expression ($P = 0.61$; Figure 60).

Uterine Luminal Fluid: Protein

Uterine luminal fluid concentrations of protein were not impacted by pre-AI treatment (P = 0.55; Figure 61) nor post-AI treatment ($P = 1.0$; Figure 62). Furthermore, there was no interaction between pre- and post-AI treatments ($P = 0.76$; Figure 63); however, estrus expression approached a tendency to impact ULF protein concentrations ($P = 0.13$; Figure 64), with heifers expressing estrus tending to have greater ULF protein concentrations (0.56 ± 0.14 mcg/mL) compared to heifers not expressing estrus (0.33 ± 0.06 mcg/mL).

Table 2. Nutrient analysis (DM basis) of cornstalks and soybean meal (SBM) supplement

Item	Cornstalks	SBM Supplement
CP,%	5.11	65.47
ADF	51.78	5.33
aNDF	77.81	7.43
aNDFom	75.41	7.03
Lignin (Sulfuric Acid)	5.95	0.37
Lignin	7.89	5.24
AD-ICP	0.81	0.34
ND-ICP	0.82	10.48
Fat, %	1.08	1.88
Ash	7.19	7.10
TDN, %	51.59	79.55

Table 3. Ground cornstalks consumed by body weight block within treatment on an as-fed-basis in pounds, calculated on a daily basis per week.

	LL[1]				LH		
Week	**L**	**M**	**H**	**Week**	**L**	**M**	**H**
-4	6.1	6.8	7.4	**-4**	6.2	6.9	7.6
-3	7.2	8.2	8.2	**-3**	7.5	8.4	9.0
-2	7.5	8.2	8.6	**-2**	7.5	8.3	9.3
-1	7.3	8.1	8.7	**-1**	7.1	8.2	9.0
d 0	7.1	7.8	8.5	**d 0**	6.9	7.6	8.3
d 6	6.5	7.4	7.6	**d 6**	11.1	11.2	11.6

	HH[2]				HL		
Week	**L**	**M**	**H**	**Week**	**L**	**M**	**H**
-4	9.8	10.5	11.2	**-4**	9.8	10.5	11.2
-3	9.2	9.8	10.4	**-3**	9.2	9.8	10.4
-2	9.7	10.3	10.9	**-2**	9.7	10.3	10.9
-1	11.1	11.7	11.9	**-1**	11.1	11.7	11.9
d 0	11.8	12.6	13.5	**d 0**	11.8	12.6	13.5
d 6	10.3	10.7	11.4	**d 6**	5.6	5.7	5.8

[1] Low treatment intake calculated as an average of individual consumption.

[2] High treatment calculated as a percent of body weight from pen average weekly consumption.

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

L - Low body weight block

M - Medium body weight block

H - High body weight block

Table 4. Dietary ingredients (kg) supplemented for each body weight block.

Item	**Low**[1]			**High**[1]		
(kg)	*Low*[2]	*Medium*[2]	*High*[2]	*Low*[2]	*Medium*[2]	*High*[2]
Corn	0	0	0	1.542	1.588	1.588
SBM	0.222	0.231	0.236	0.367	0.381	0.39
Urea	0.014	0.014	0.014	0.023	0.023	0.023

[1] Nutritional treatment

[2] Body weight block

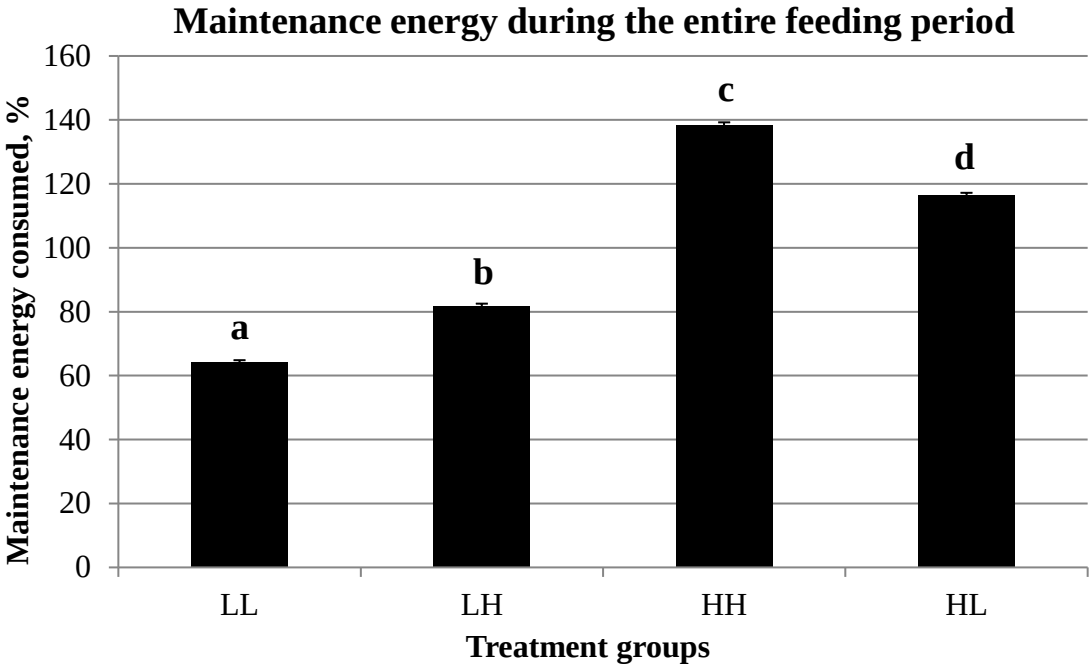

Figure 26. Effect of treatment on maintenance energy consumed. Energy intake for LL heifers was the least compared to HH heifers which had the greatest ($P < 0.0001$).

[abcd] Superscripts differ ($P < 0.0001$).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

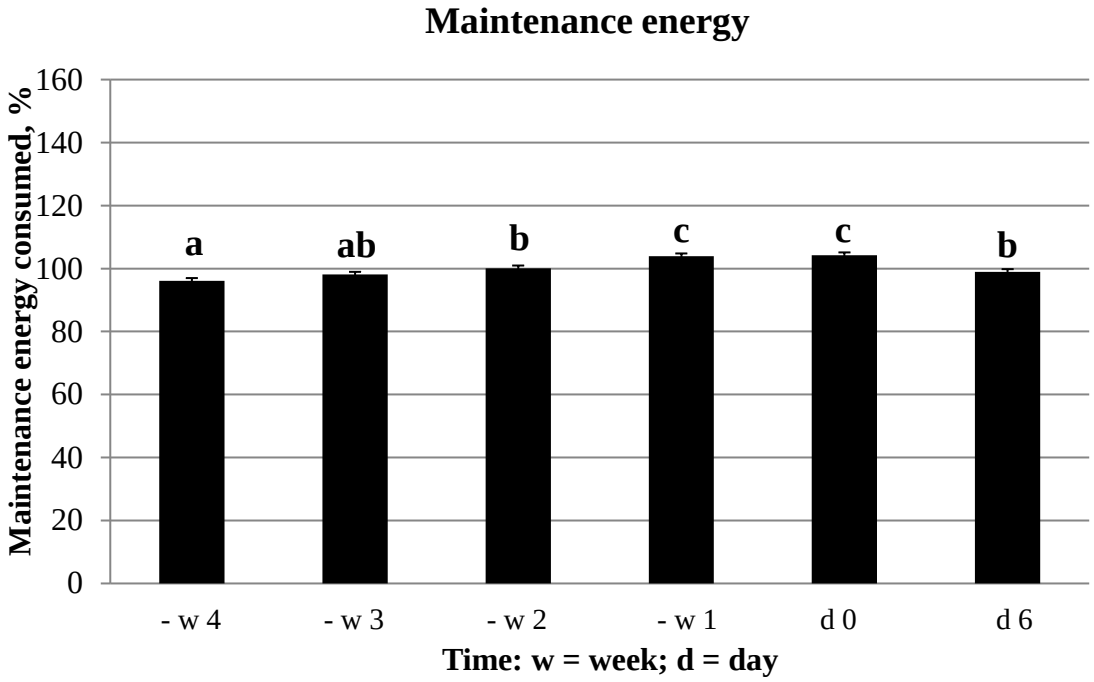

Figure 27. Effect of time on maintenance energy consumed, where d 0 = AI and d 6 = embryo collection. Heifer intake increased weekly before dropping off from d 0 through d 6 ($P < 0.0001$).

[abc] Superscripts differ ($P < 0.03$).

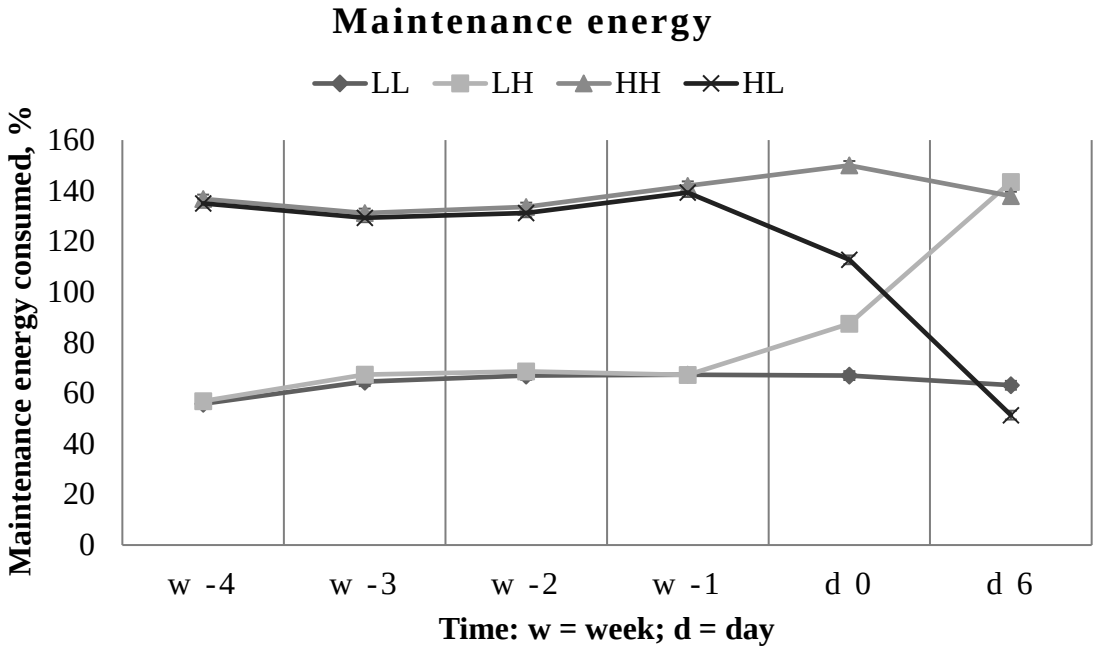

Figure 28. Effect of treatment x time interaction on maintenance energy consumed, where d 0 = AI and d 6 = embryo collection. While LL and HH heifers maintained relatively constant energy intakes, HL heifers' energy intake declined rapidly from d 0 to d 6 while LH heifers' energy intake increased sharply ($P < 0.0001$).

LL - Low-low treatment, heifers on low treatment before and after AI.
LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.
HH - High-high treatment, heifers on high treatment before and after AI.
HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

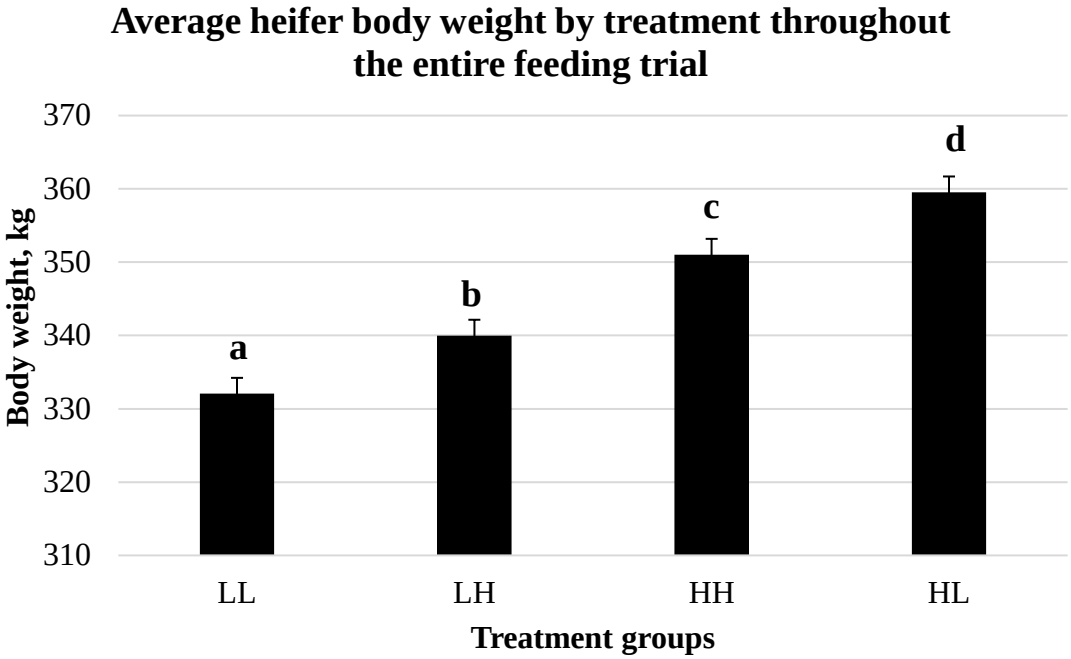

Figure 29. Effect of treatment on average heifer body weight. Body weights for HL heifers were the greatest, while LL heifers weighed the least ($P < 0.0001$).

[abcd] Superscripts differ ($P < 0.008$).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

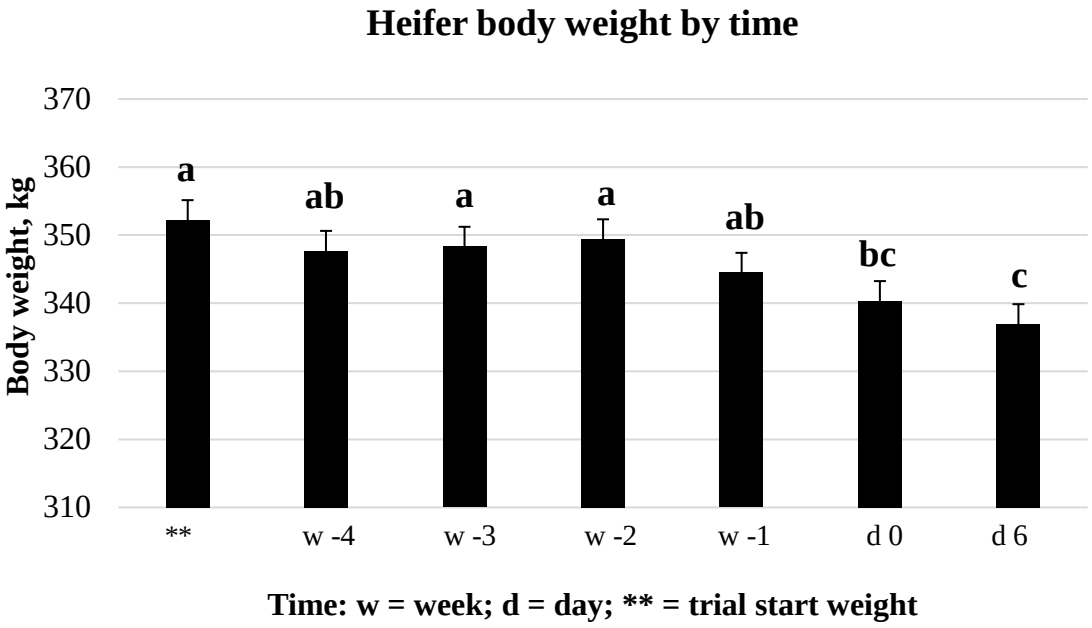

Figure 30. Effect of time on average heifer body weight, where d 0 = AI and d 6 = embryo collection. Heifer body weights across treatments were similar at the beginning of the trial through week -1, but the trial start weight and weeks -3 and -2 weights were heavier compared to d 0 and d 6 weights. Weights during weeks -4 and -1 were similar to the d 0 weight but heavier compared to the d 6 weight. Weights between d 0 and 6 were similar ($P = 0.002$).

[abc] Superscripts differ ($P < 0.05$).

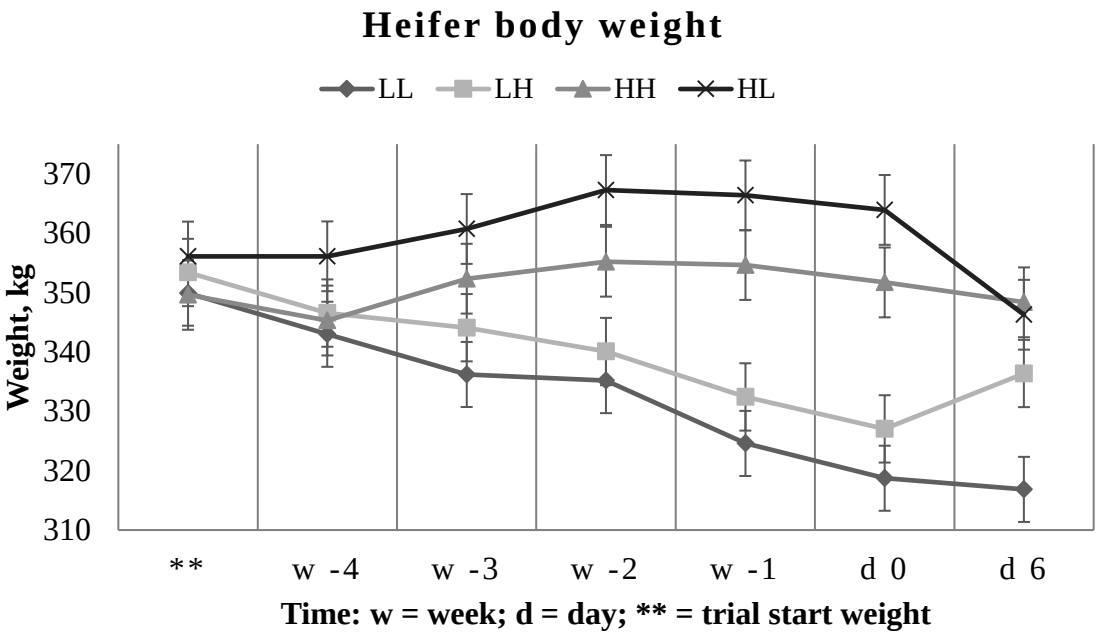

Figure 31. Effect of treatment x time interaction on heifer body weight, where d 0 = AI and d 6 = embryo collection. Body weights declined over time for LL heifers, whereas HH heifers maintained weight. Heifers in the HL treatment gained weight but then lost weight during the treatment switch from d 0 to d 6. Heifers in the LH treatment lost weight until the treatment switch but there was no significant weight gain from d 0 to d 6 (P = 0.02).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

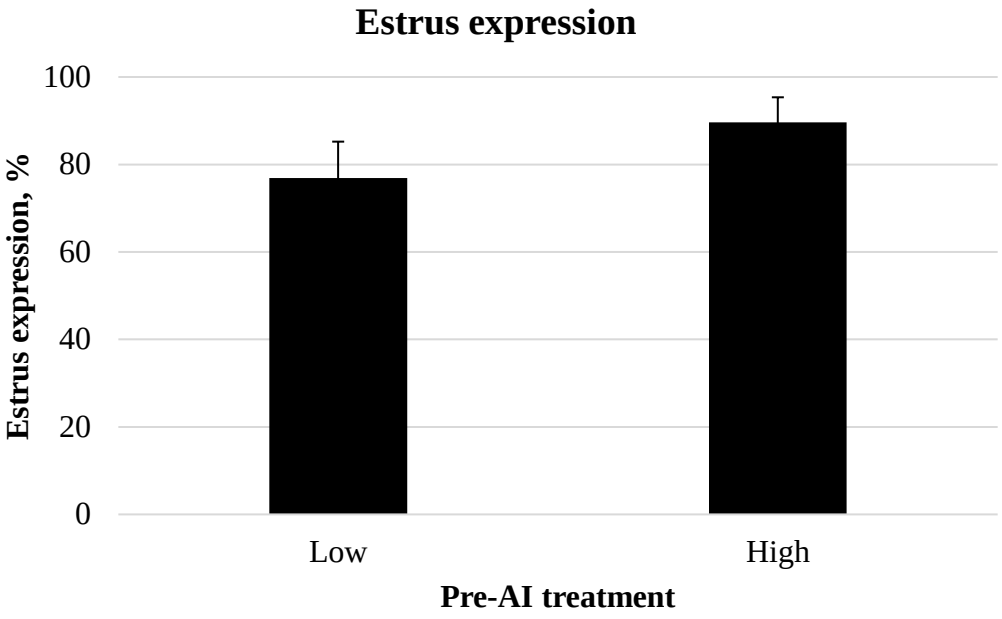

Figure 32. Effect of treatment on estrus expression. No difference was observed between low and high treatments preceding AI for estrus expression (P = 0.22).

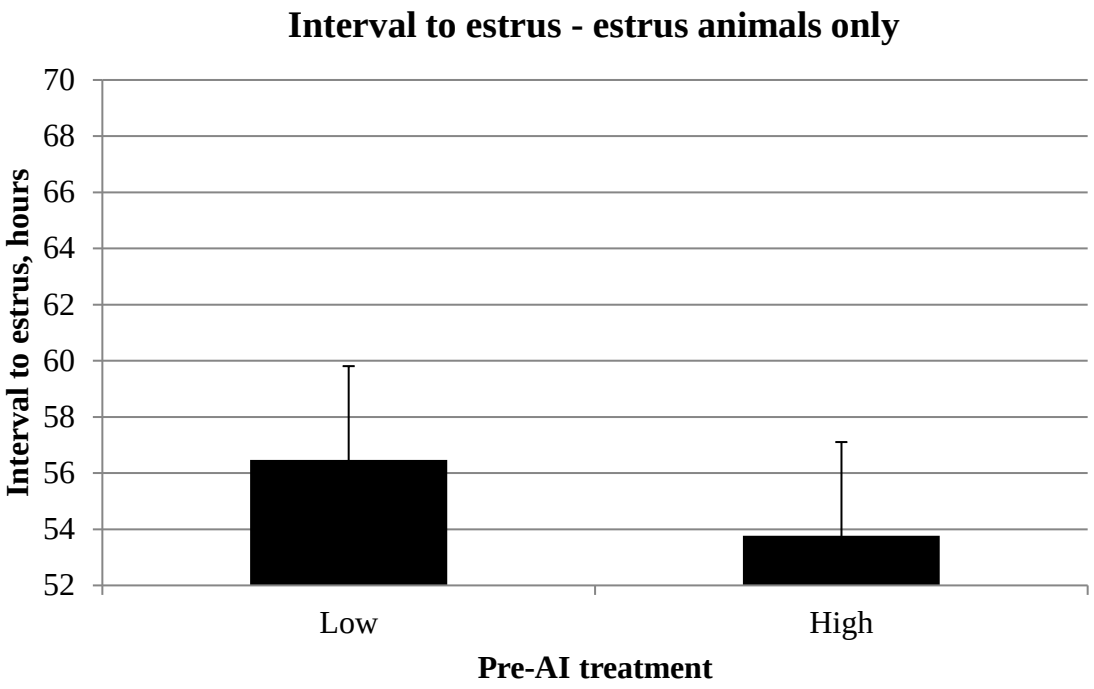

Figure 33. Effect of treatment on interval to estrus. No difference was observed for interval to estrus between low and high treatment animals preceding AI (P = 0.57).

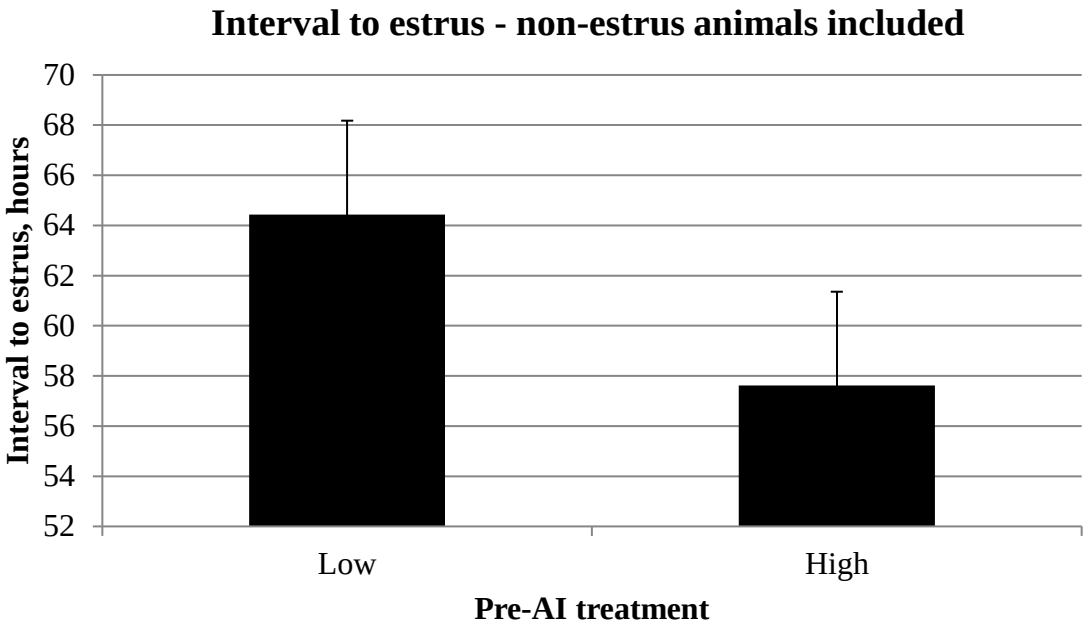

Figure 34. Effect of treatment on interval to estrus. No difference occurred between low and high treatment animals for interval to estrus preceding AI (P = 0.20).

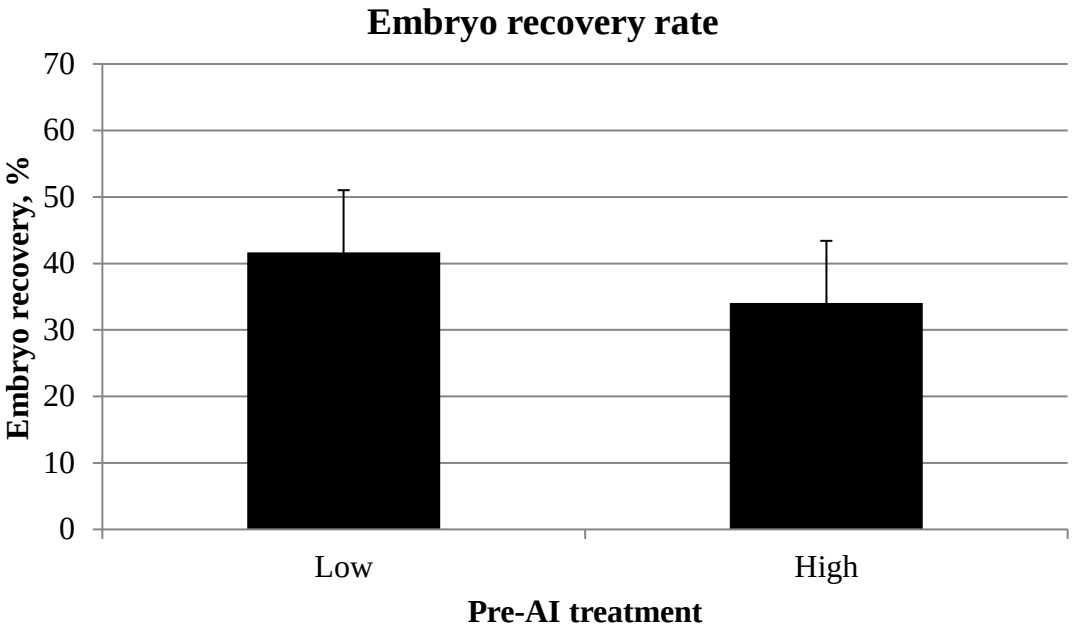

Figure 35. Effect of pre-AI plane of nutrition on embryo recovery rate on d 6. Plane of nutrition prior to AI did not impact embryo recovery rate (P = 0.57).

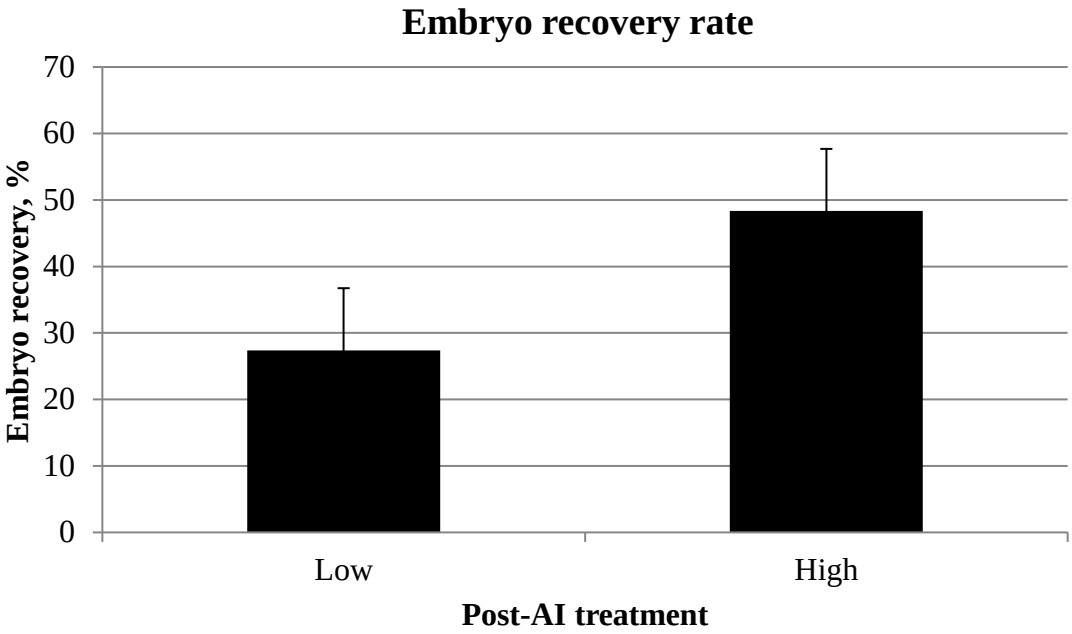

Figure 36. Effect of post-AI plane of nutrition on embryo recovery rate on d 6. Plane of nutrition following AI approached a tendency to affect embryo recovery rate, with high nutrition after AI yielding a greater recovery rate (P = 0.12).

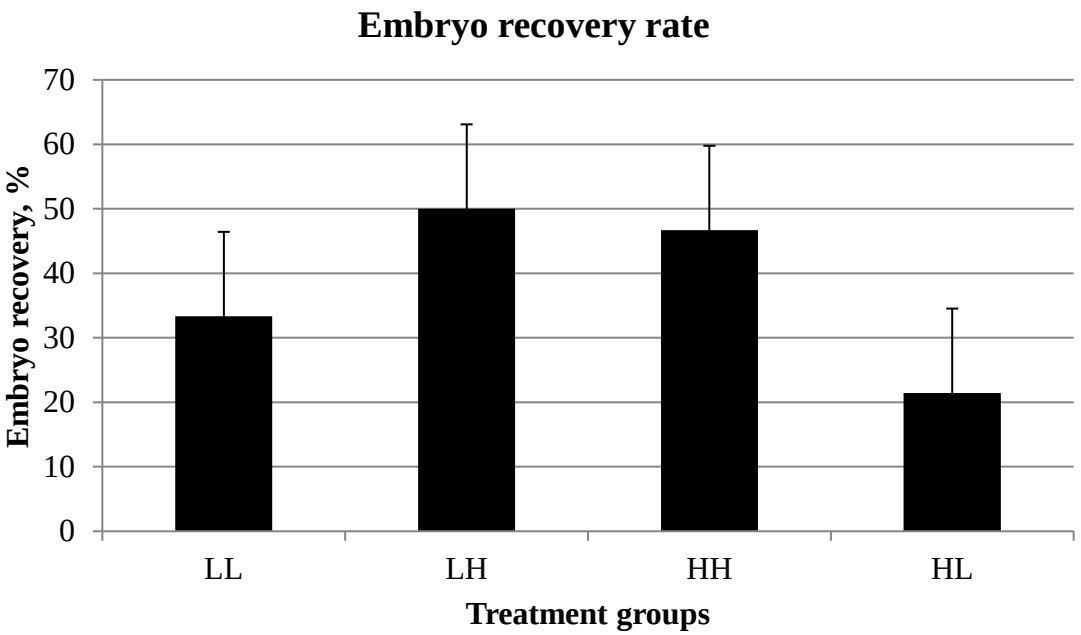

Figure 37. Effect of pre- and post-AI plane of nutrition on embryo recovery rate on d 6. There was no interaction between pre- and post-AI treatments on embryo recovery rate ($P = 0.75$).

LL - Low-low treatment, heifers on low treatment before and after AI.
LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.
HH - High-high treatment, heifers on high treatment before and after AI.
HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

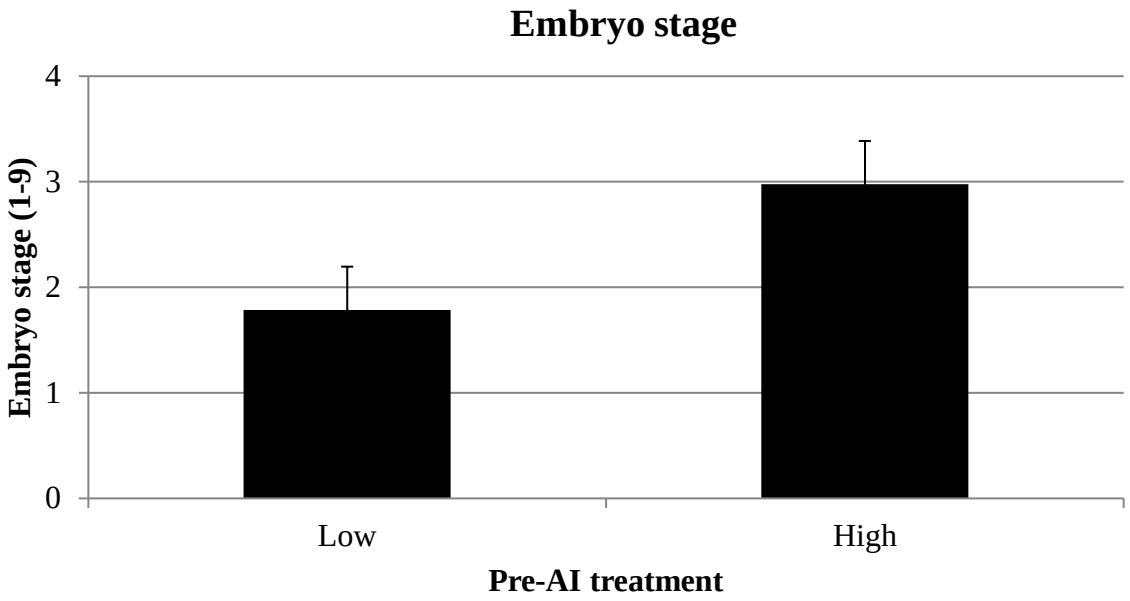

Figure 38. Effect of pre-AI plane of nutrition on embryo stage (scale 1 to 9: 1 = unfertilized; 9 = expanding hatched blastocyst) on d 6. Embryos recovered from heifers on the high plane of nutrition prior to AI were at a more advanced stage than embryos recovered from heifers on the low treatment (P = 0.05).

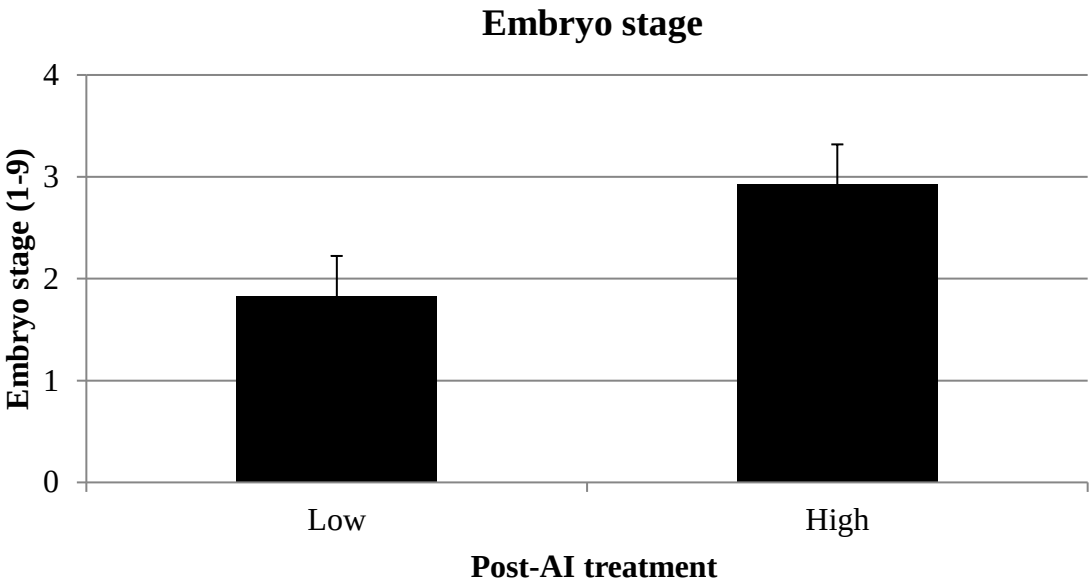

Figure 39. Effect of post-AI plane of nutrition on embryo stage (scale 1 to 9: 1 = unfertilized; 9 = expanding hatched blastocyst) on d 6. Embryos recovered from heifers on the low plane of nutrition following AI tended to be at a less advanced stage compared to embryos from heifers on the high plane of nutrition (P = 0.07).

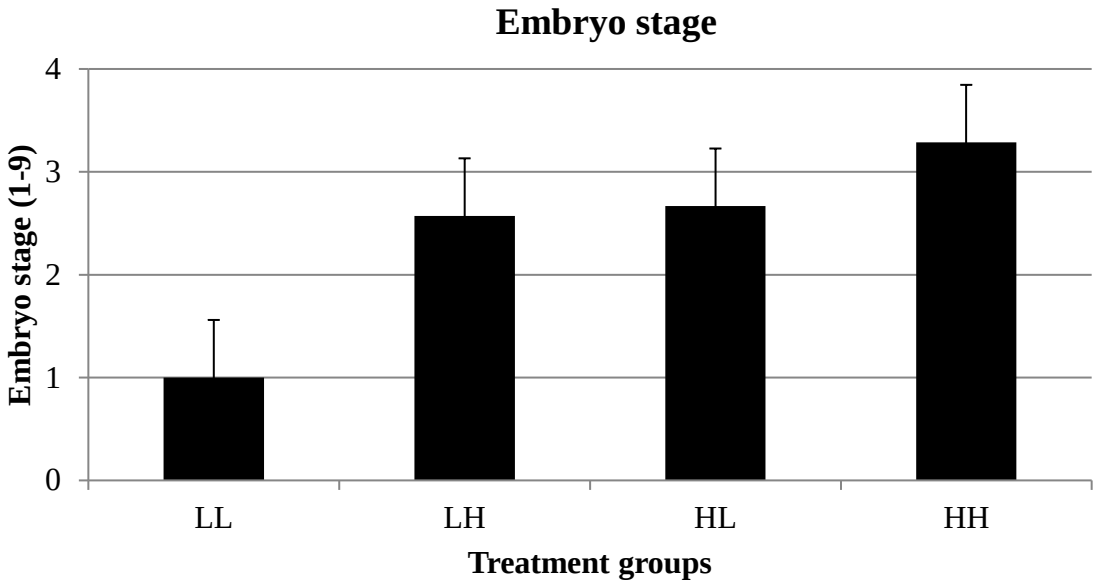

Figure 40. Effect of pre- and post-AI plane of nutrition interaction on embryo stage (scale 1 to 9: 1 = unfertilized; 9 = expanding hatched blastocyst) on d 6. No interaction was observed between pre- and post-AI treatments on embryo stage ($P = 0.42$).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

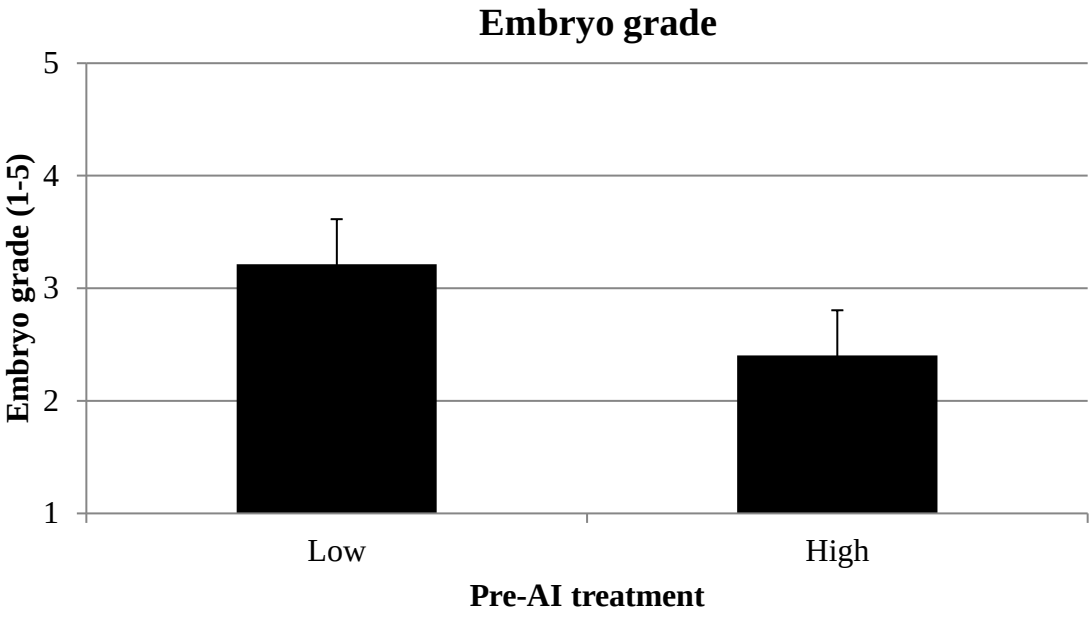

Figure 41. Effect of pre-AI plane of nutrition on embryo grade (scale 1 to 5: 1 = excellent; 5 = dead or degenerative) on d 6. Embryos recovered from heifers on a low plane of nutrition preceding AI approached a tendency to have a poorer grade compared to embryos recovered from high treatment heifers ($P = 0.17$).

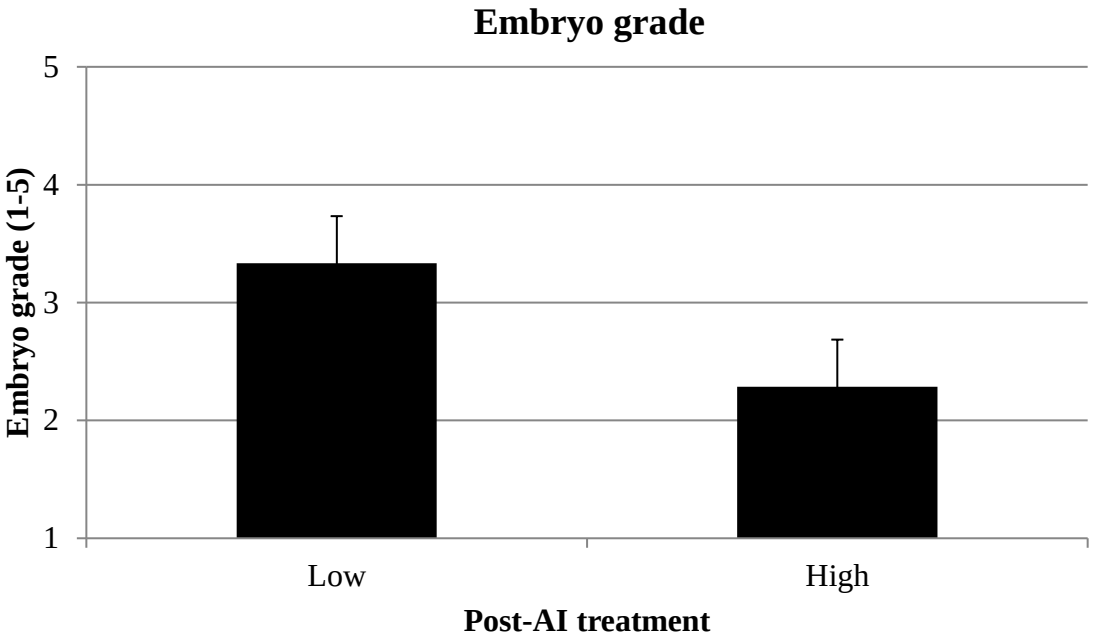

Figure 42. Effect of post-AI plane of nutrition on embryo grade (scale 1 to 5: 1 = excellent; 5 = dead or degenerative) on d 6. Embryos from low treatment heifers tended to have a poorer grade compared to embryos from high treatment heifers (P = 0.08).

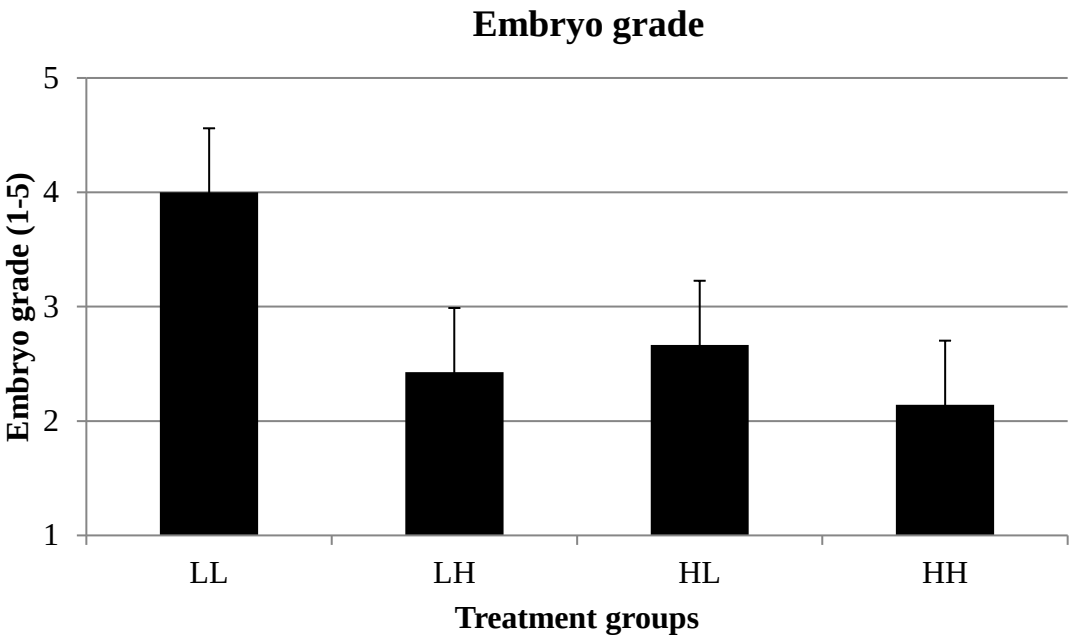

Figure 43. Effect of pre- and post-AI plane of nutrition interaction on embryo grade (scale 1 to 5: 1 = excellent; 5 = dead or degenerative) on d 6. No interaction between pre- and post-AI treatments was observed on embryo grade (P = 0.37).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

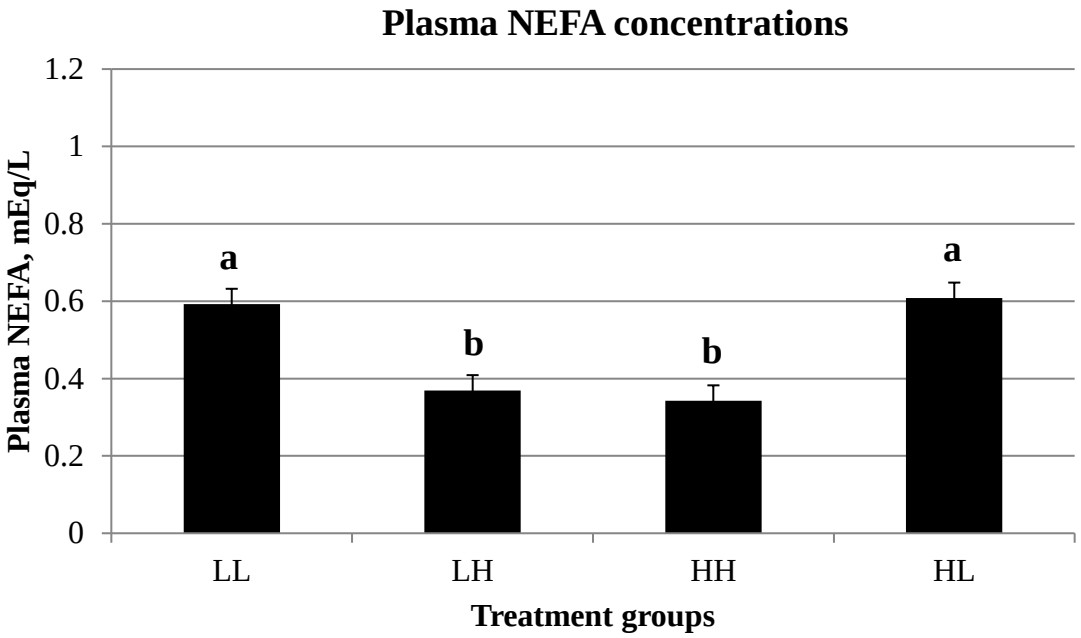

Figure 44. Effect of treatment on plasma NEFA concentrations. Heifers in the HL and LL treatments had the greatest NEFA concentrations ($P < 0.0001$).

[ab] Superscripts differ ($P < 0.0001$).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

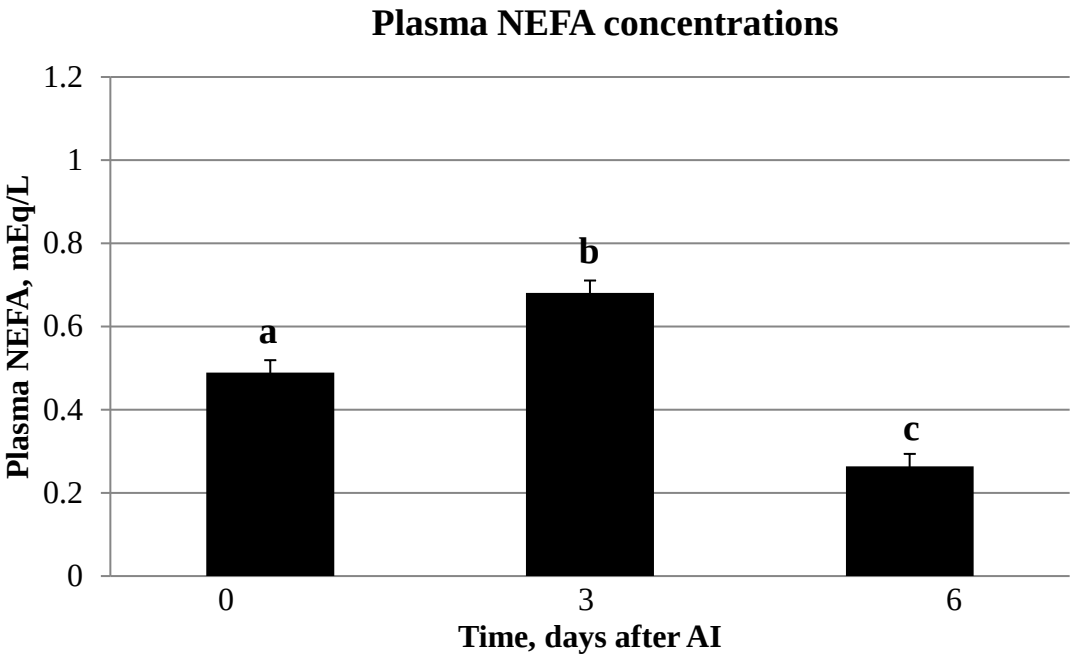

Figure 45. Effect of time on plasma NEFA concentrations, where d 0 = AI and d 6 = embryo collection. Plasma NEFA concentrations were different by time; d 3 had the greatest NEFA concentrations followed by d 0, with d 6 having the least concentrations ($P < 0.0001$).

[abc] Superscripts differ ($P < 0.0001$).

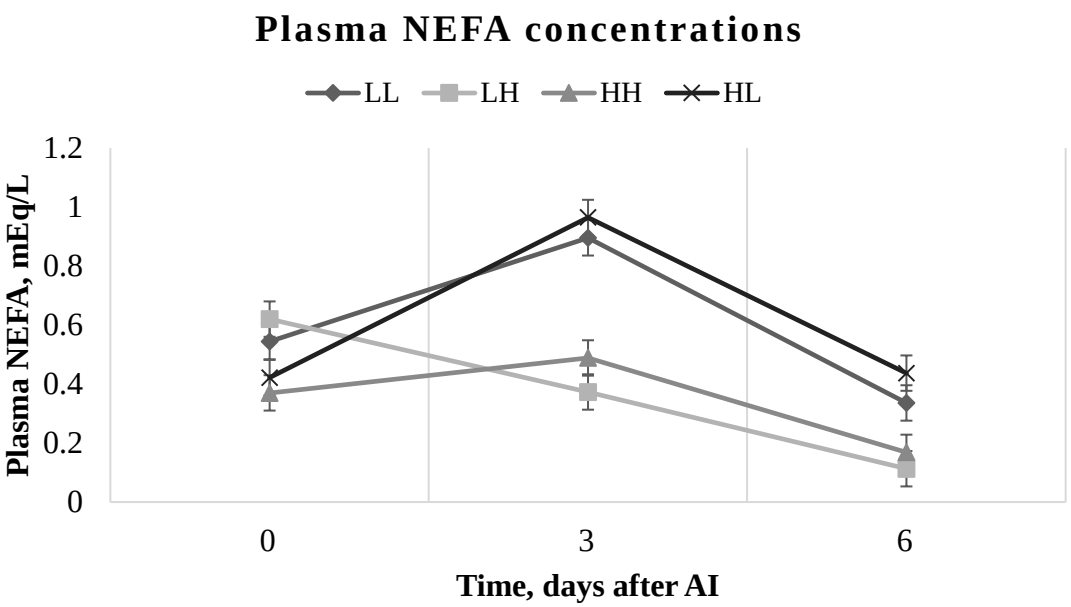

Figure 46. Effect of treatment x time interaction on plasma NEFA concentrations, where d 0 = AI and d 6 = embryo collection. Heifers in the LL and HL treatments had the sharpest increase and decline in NEFA concentrations. Plasma NEFA concentrations increased moderately from d 0 to d 3 for HH heifers before declining to d 6, whereas LH heifers had a steady decline in NEFA concentrations ($P < 0.0001$).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

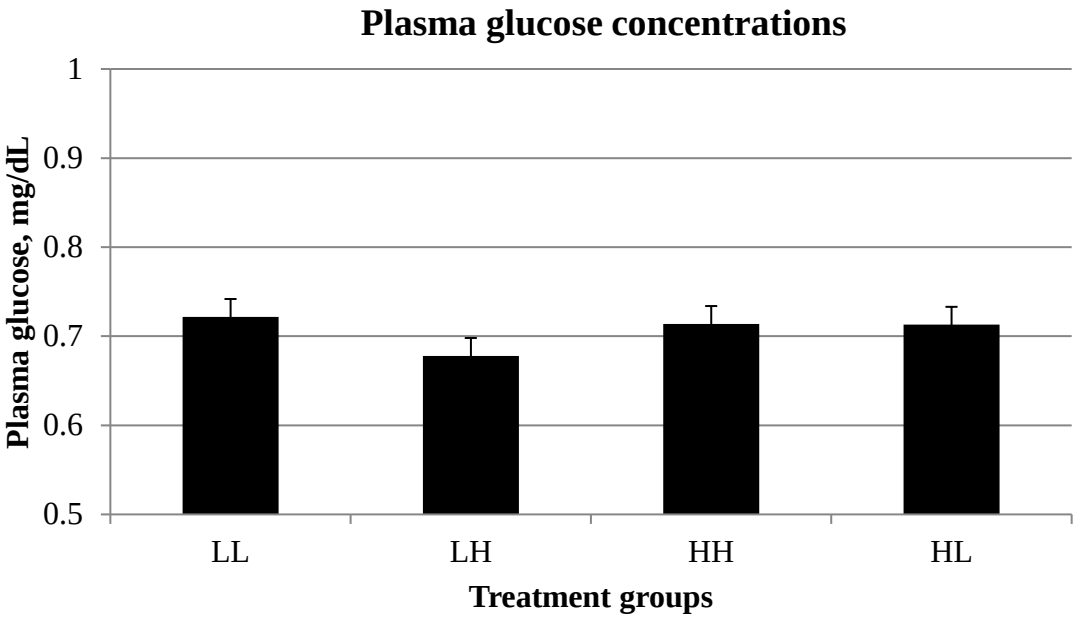

Figure 47. Effect of treatment on plasma glucose concentrations. Plasma glucose concentrations were not affected by treatment ($P = 0.49$).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

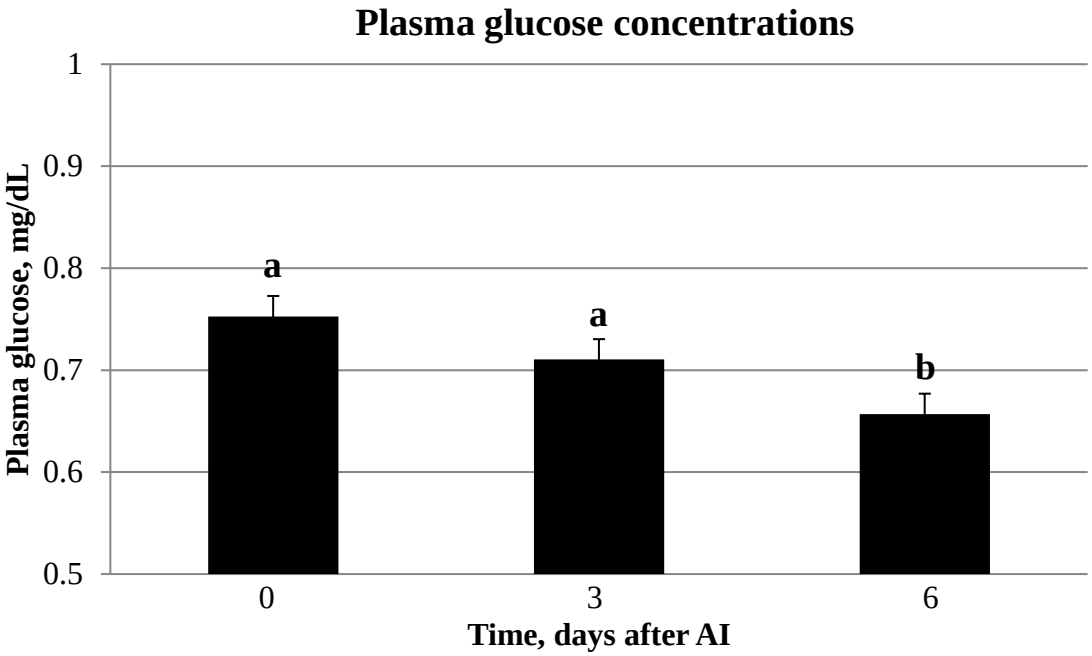

Figure 48. Effect of time on plasma glucose concentrations, where d 0 = AI and d 6 = embryo collection. Plasma glucose concentrations were similar from d 0 to d 3 before declining to d 6 after AI ($P = 0.002$).

[ab] Superscripts differ ($P < 0.05$).

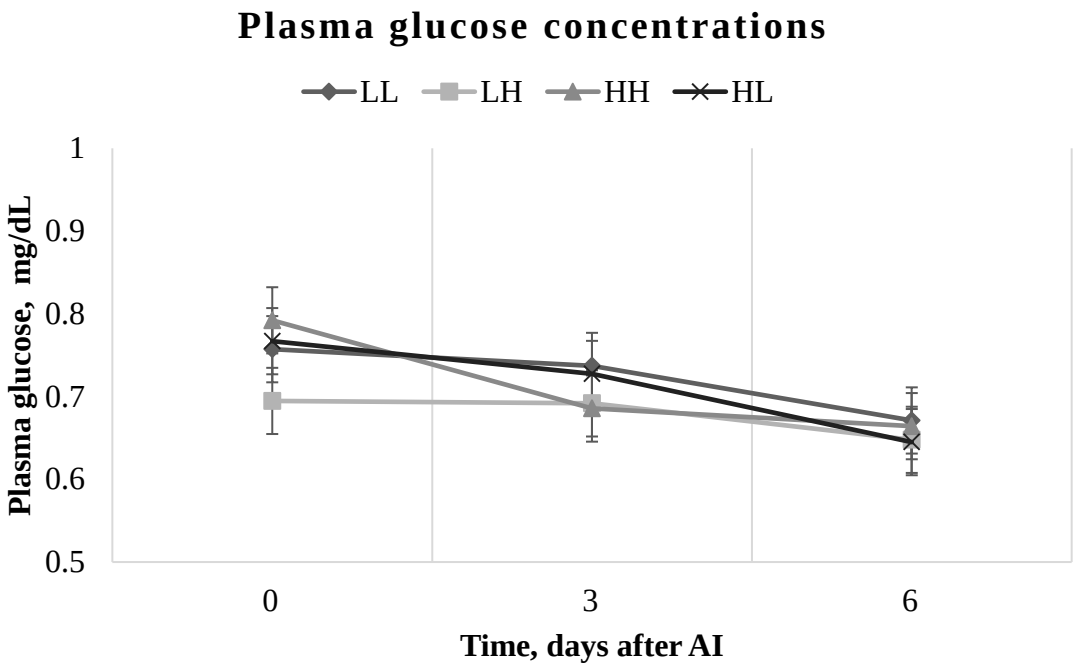

Figure 49. Effect of treatment x time interaction on plasma glucose concentrations, where d 0 = AI and d 6 = embryo collection. No interaction between treatment and time was observed on plasma glucose concentrations (P = 0.81).

LL - Low-low treatment, heifers on low treatment before and after AI.
LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.
HH - High-high treatment, heifers on high treatment before and after AI.
HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

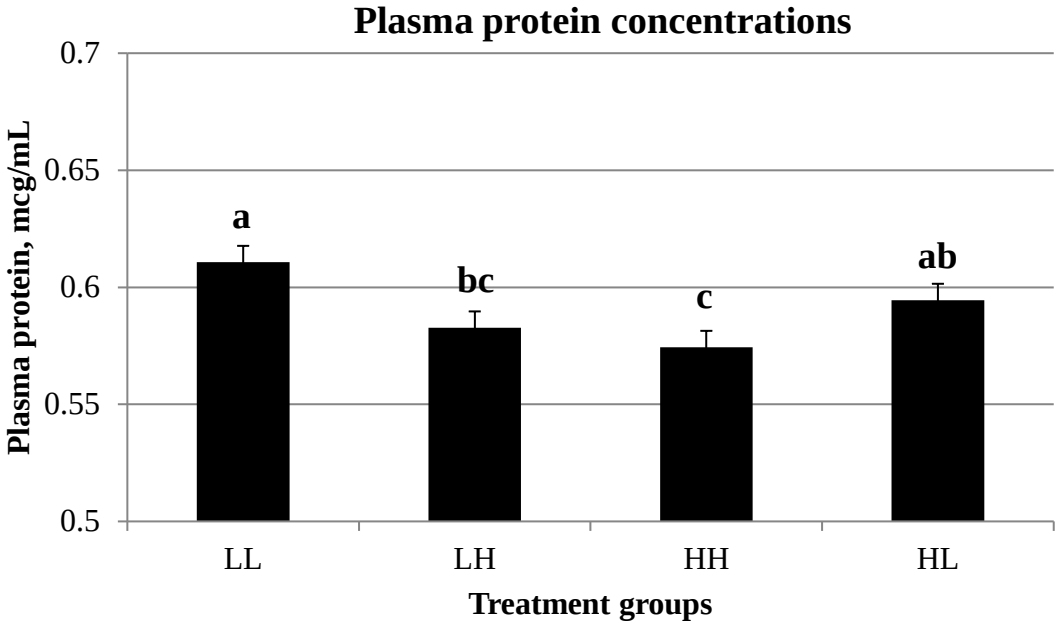

Figure 50. Effect of treatment on plasma protein concentrations. Heifers in the LL treatment had greater plasma protein concentrations compared to LH and HH treatments but similar concentrations compared to HL heifers. Heifers in the HL and LH treatments had similar plasma protein concentrations while LH heifers also had similar concentrations compared to HH heifers ($P = 0.003$).

[abc] Superscripts differ ($P < 0.05$).

LL - Low-low treatment, heifers on low treatment before and after AI.

LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.

HH - High-high treatment, heifers on high treatment before and after AI.

HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

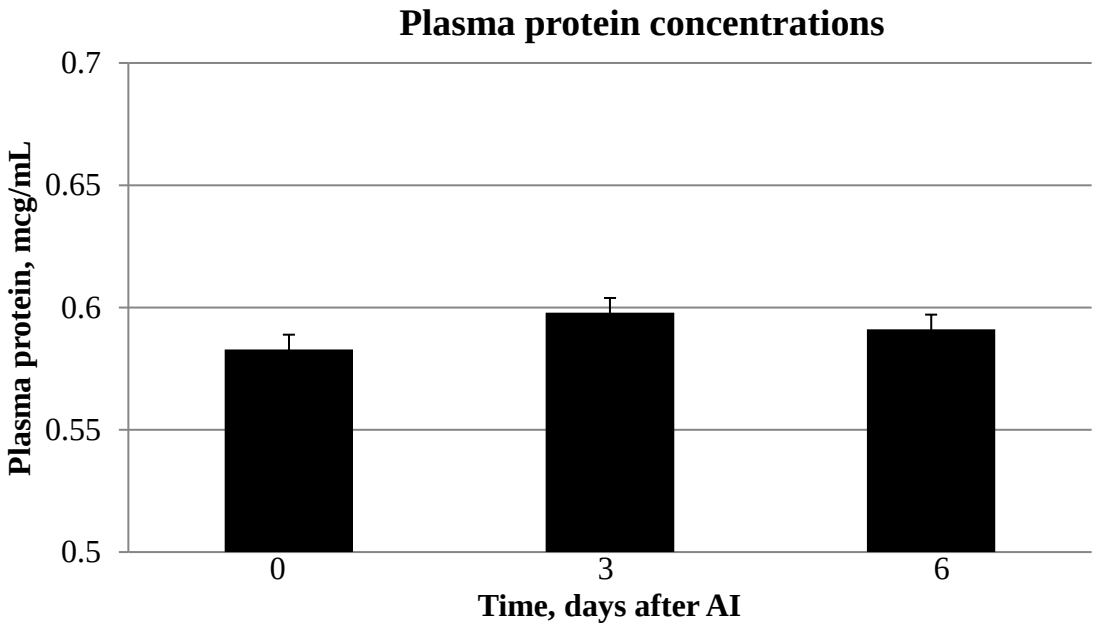

Figure 51. Effect of time on plasma protein concentrations, where d 0 = AI and d 6 = embryo collection. Plasma protein concentrations were not impacted by time (P = 0.22).

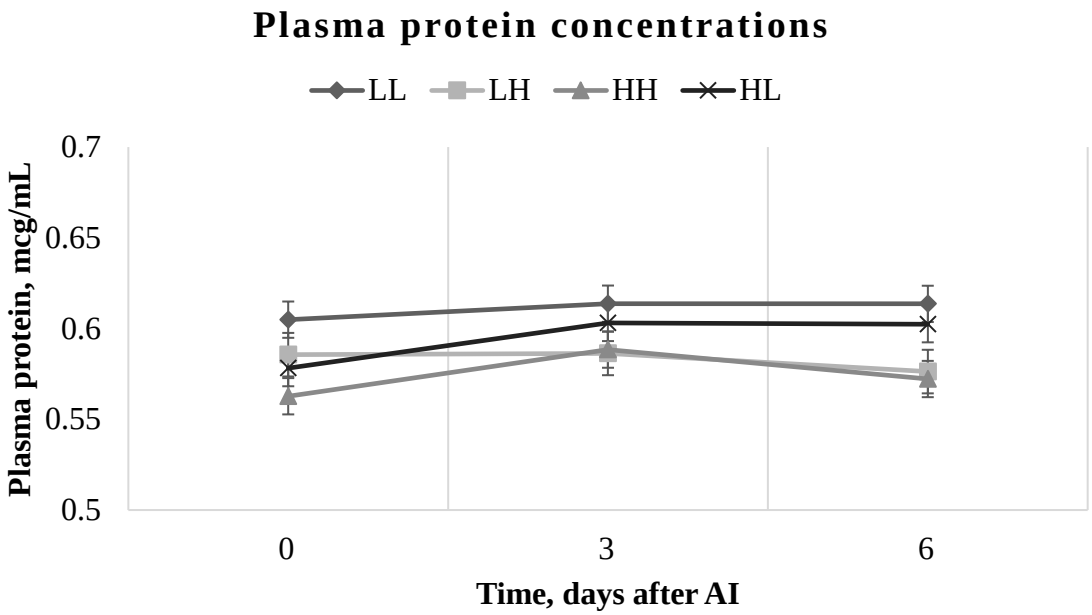

Figure 52. Effect of treatment x time interaction on plasma protein concentrations, where d 0 = AI and d 6 = embryo collection. No interaction was observed between treatment and time on plasma protein concentrations (*P* = 0.84).

LL - Low-low treatment, heifers on low treatment before and after AI.
LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.
HH - High-high treatment, heifers on high treatment before and after AI.
HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

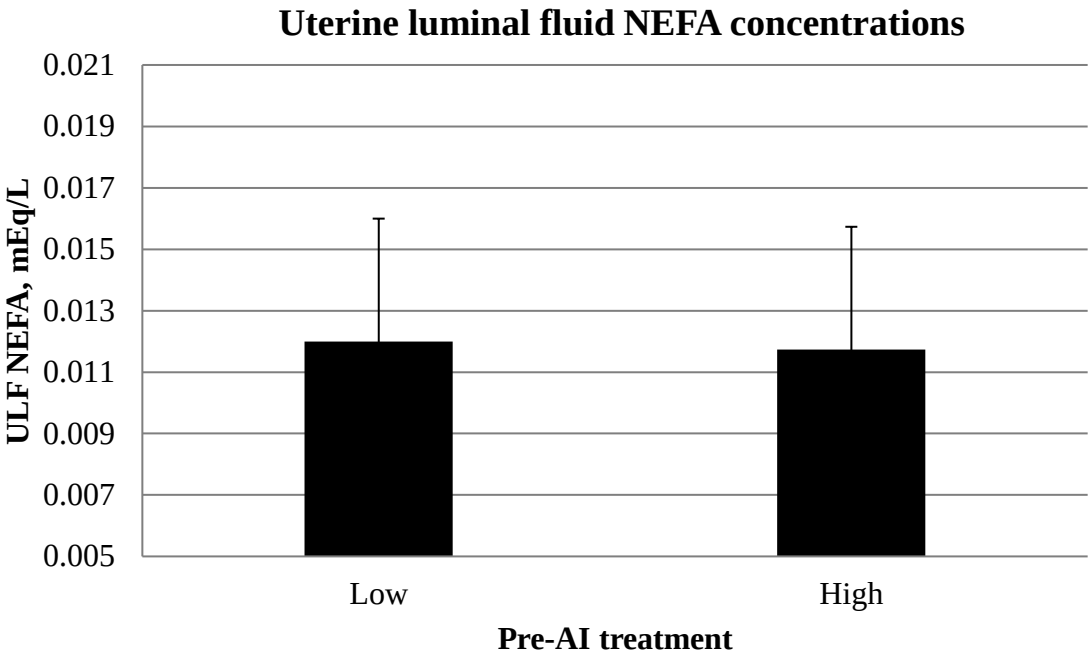

Figure 53. Effect of pre-AI plane of nutrition on ULF NEFA concentrations. Plane of nutrition prior to AI did not impact ULF NEFA concentrations (*P* = 0.95).

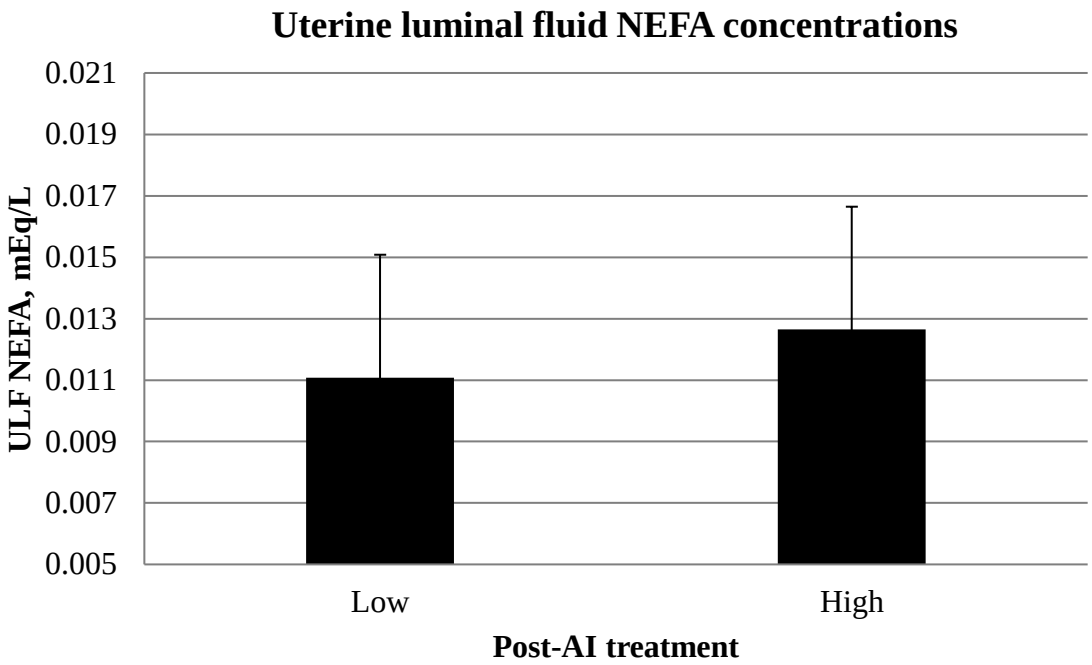

Figure 54. Effect of post-AI plane of nutrition on ULF NEFA concentrations. Plane of nutrition following AI did not impact ULF NEFA concentrations (P = 0.74).

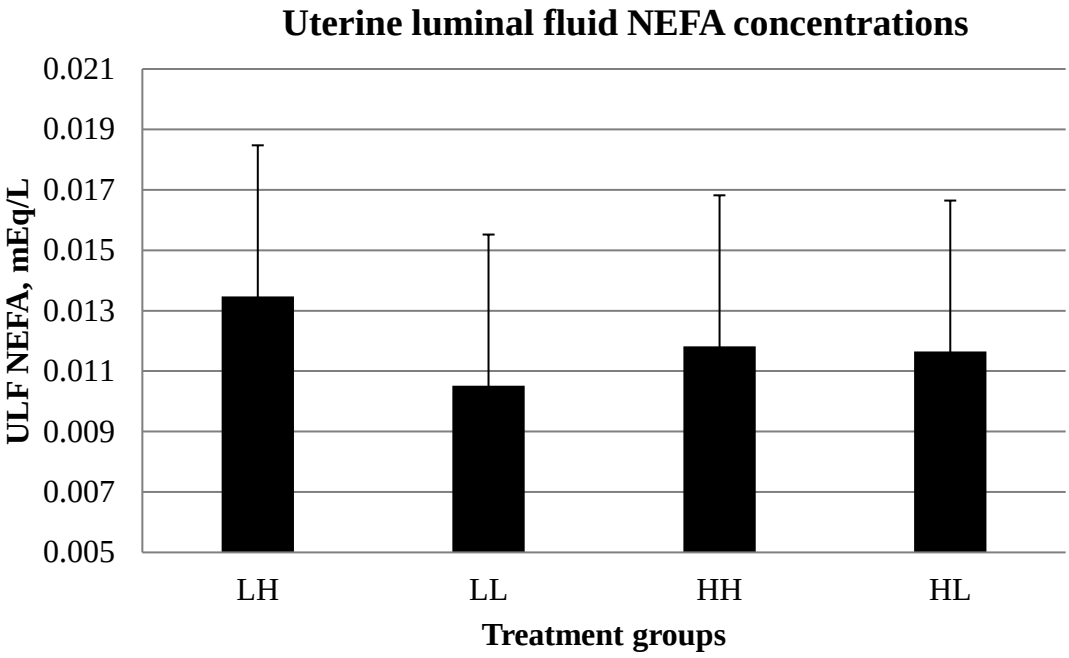

Figure 55. Effect of pre- and post-AI plane of nutrition interaction on ULF NEFA concentrations. No interaction between pre- and post-AI plane of nutrition was observed on ULF NEFA concentrations (P = 0.76).

LL - Low-low treatment, heifers on low treatment before and after AI.
LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.
HH - High-high treatment, heifers on high treatment before and after AI.
HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

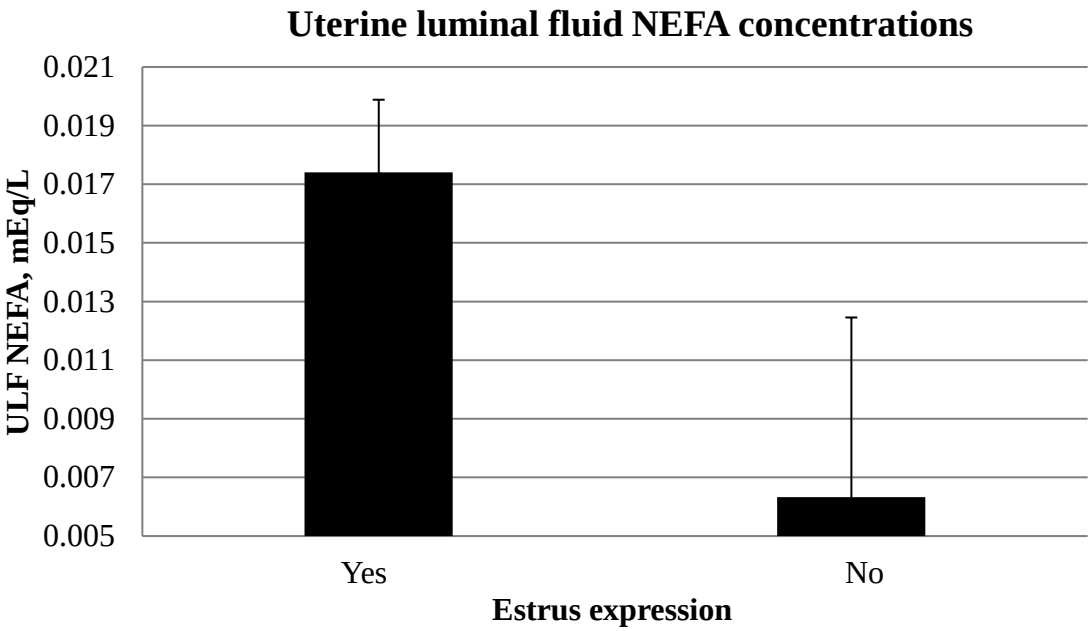

Figure 56. Effect of estrus expression on ULF NEFA concentrations. Heifers that expressed estrus tended to have greater ULF NEFA concentrations compared to heifers that didn't express estrus (P = 0.10).

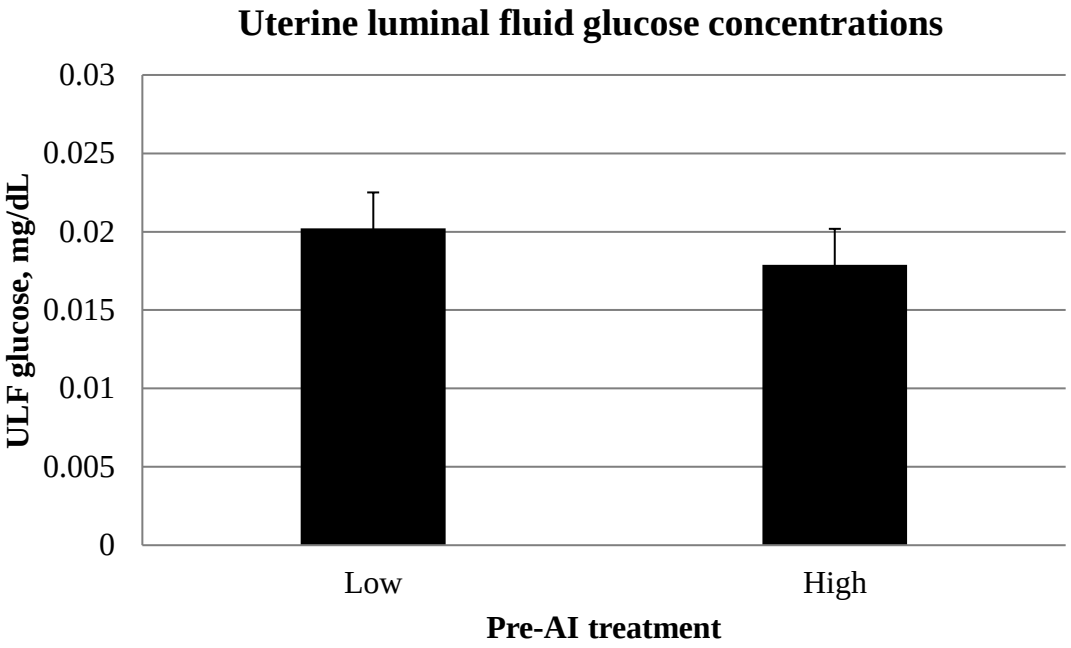

Figure 57. Effect of pre-AI plane of nutrition on ULF glucose concentrations. Plane of nutrition preceding AI did not impact ULF glucose concentrations (*P* = 0.41).

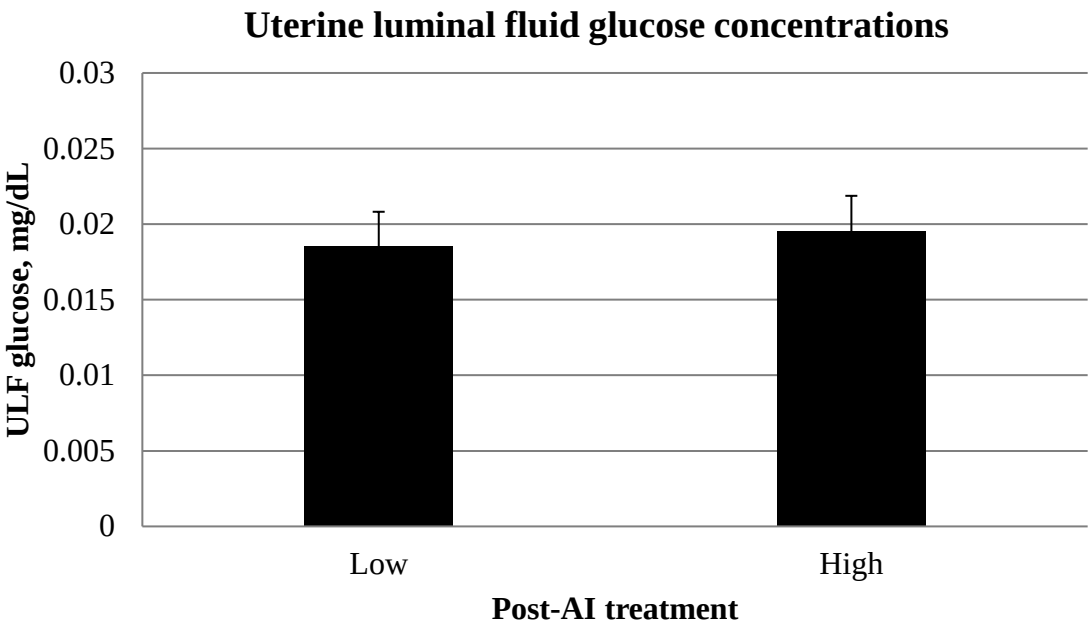

Figure 58. Effect of post-AI plane of nutrition on ULF glucose concentrations. Plane of nutrition following AI did not impact ULF glucose concentrations (P = 0.70).

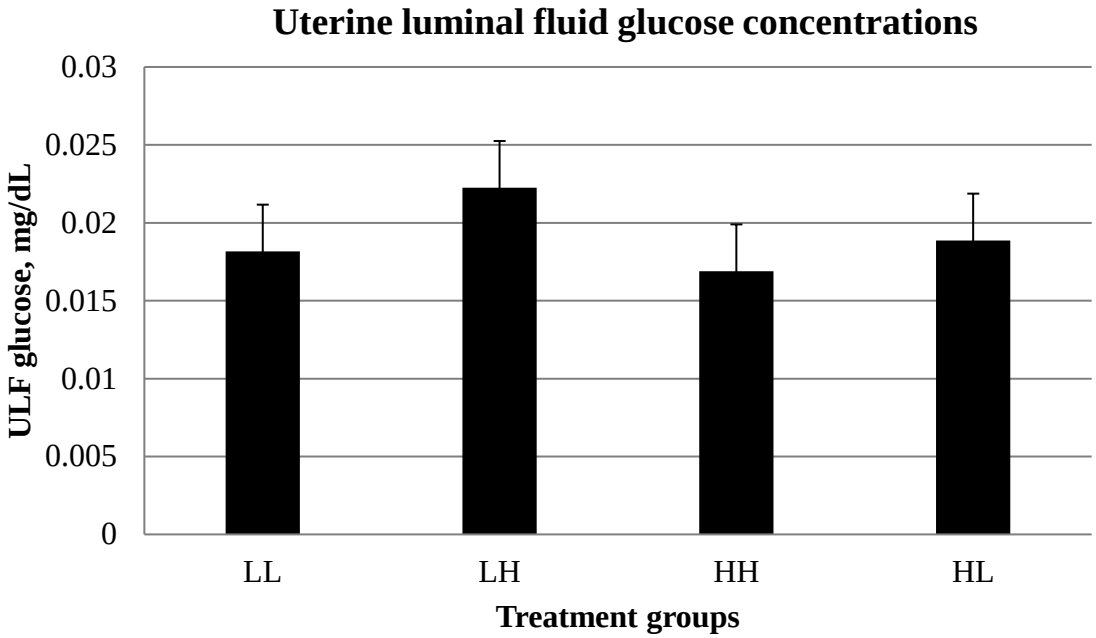

Figure 59. Effect of pre- and post-AI plane of nutrition interaction on ULF glucose concentrations. No interaction was observed between pre- and post-AI plane of nutrition on ULF glucose concentrations ($P = 0.27$).

LL - Low-low treatment, heifers on low treatment before and after AI.
LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.
HH - High-high treatment, heifers on high treatment before and after AI.
HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

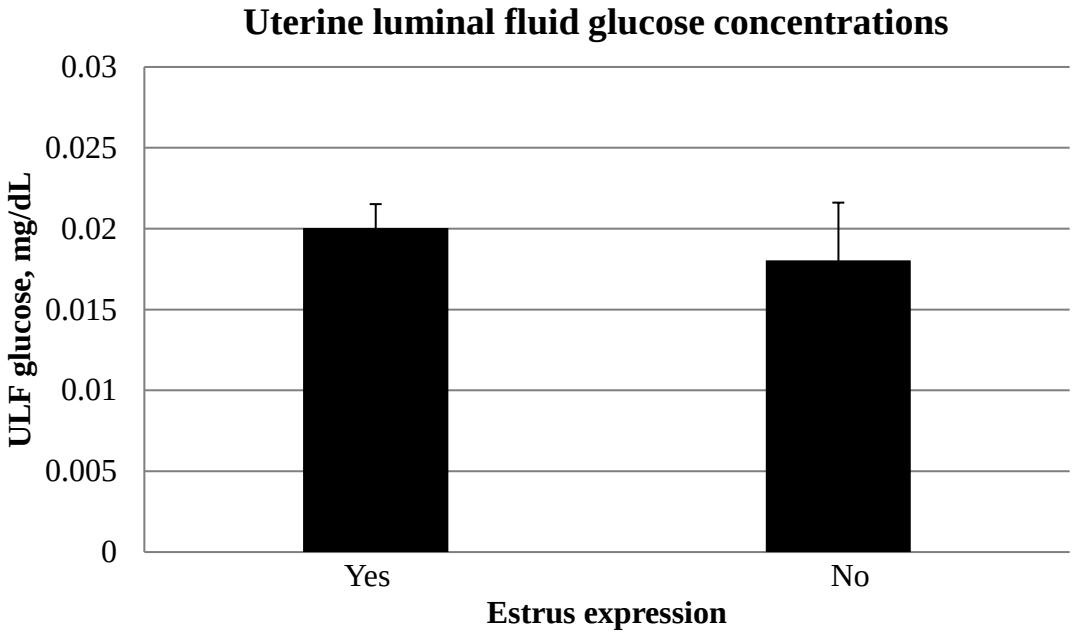

Figure 60. Effect of estrus expression on ULF glucose concentrations. Concentrations of ULF glucose were not impacted by estrus expression (P = 0.61).

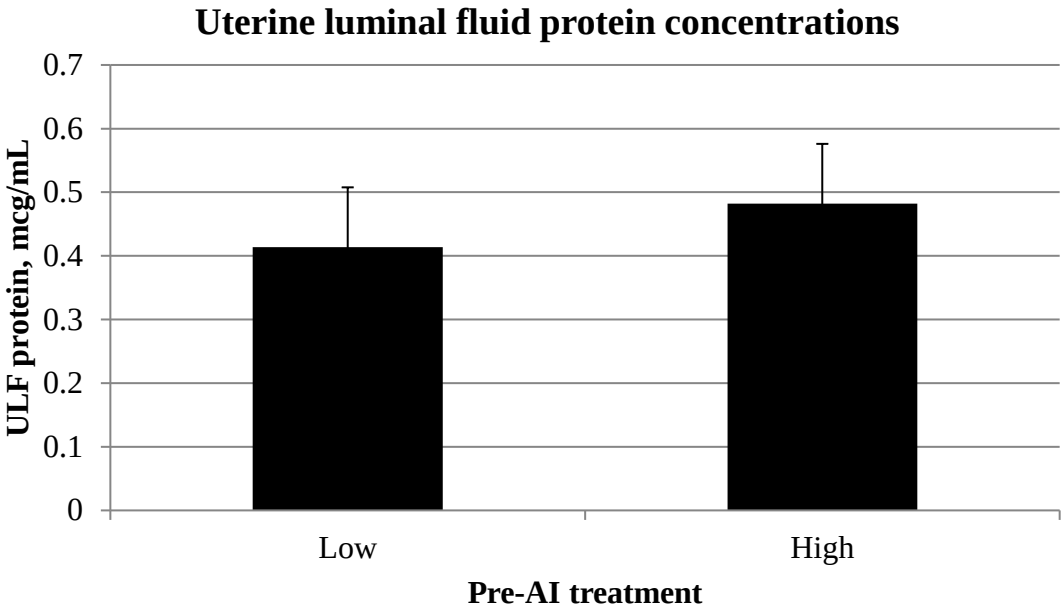

Figure 61. Effect of pre-AI plane of nutrition on ULF protein concentrations. Plane of nutrition prior to AI did not impact ULF protein concentrations (P = 0.55).

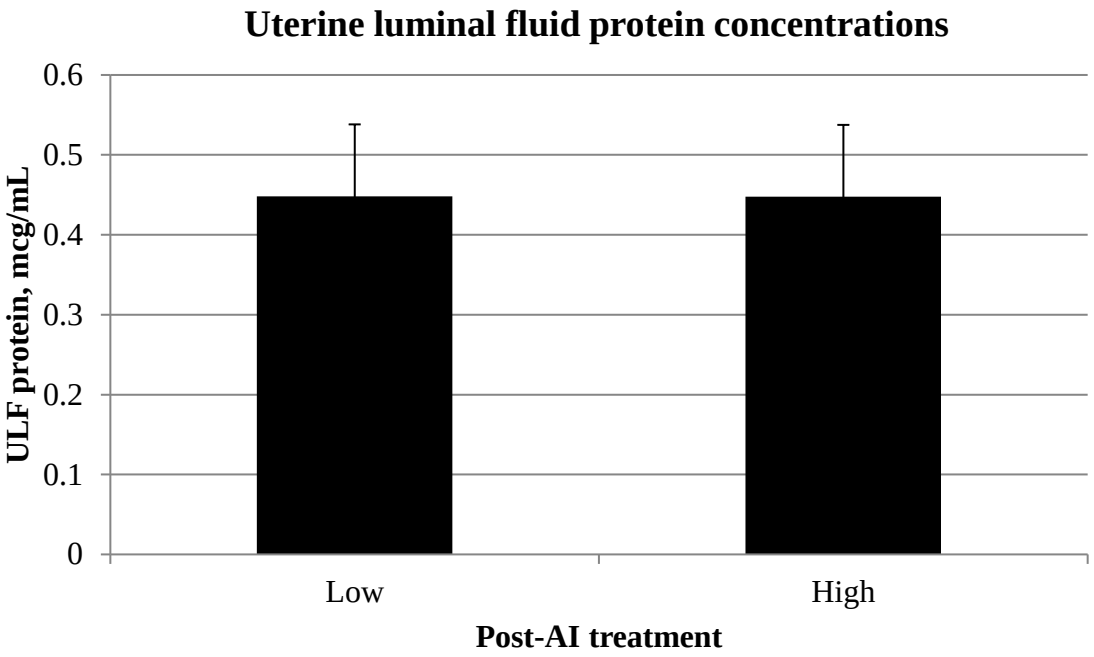

Figure 62. Effect of post-AI plane of nutrition on ULF protein concentrations. Plane of nutrition following AI did not affect ULF protein concentrations (P = 1.0).

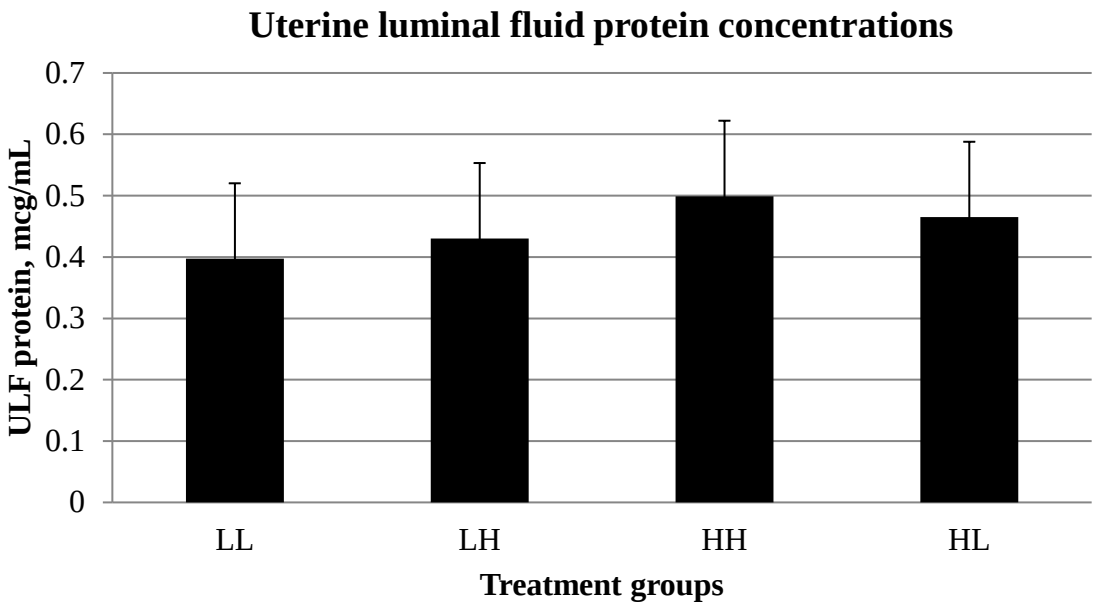

Figure 63. Effect of pre- and post-AI plane of nutrition interaction on ULF protein concentrations. No interaction between pre- and post-AI plane of nutrition was observed on ULF protein concentrations ($P = 0.76$).

LL - Low-low treatment, heifers on low treatment before and after AI.
LH - Low-high treatment, heifers on low treatment before AI and high treatment after AI.
HH - High-high treatment, heifers on high treatment before and after AI.
HL - High-low treatment, heifers on high treatment before AI and low treatment after AI.

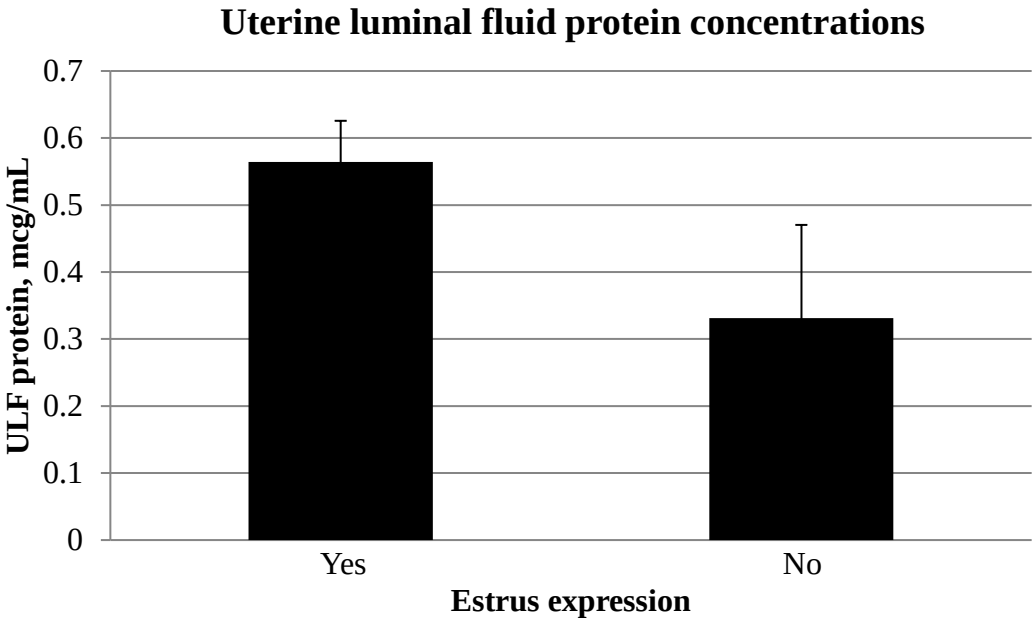

Figure 64. Effect of estrus expression on ULF protein concentrations. Heifers that expressed estrus approached a tendency to have greater ULF protein concentrations compared to heifers that did not express estrus (P = 0.13).

DISCUSSION

Heifer body weight fluctuations by treatment were not surprising, as diets were formulated in the current study to either place heifers in a negative energy balance or maintain weight. The patterns of weight gain and loss for LH and HL treatments preceding and following AI were similar compared to what was reported by Dunne et al. (1990).

In the current study, interval to estrus was not different, but low treatment heifers approached a tendency to exhibit a longer interval to estrus compared to high treatment heifers. This lack of difference is likely due to the limited number of animals used in the current study. However, previous research has reported that estrus response may be impacted by nutrient restriction. In heifers undergoing acute short-term nutrient restriction (40% maintenance for 2 weeks), estrus response in restricted heifers was 39% compared to 95% in control heifers (Mackey et al., 1999). Nutrient restriction was longer but not as acute in the current study; however, the current study approached a tendency for fewer low treatment heifers to express estrus compared to high treatment heifers and may be further validated by a larger number of animals in future studies. In addition, both long-term chronic nutrient restriction and acute short-term nutrient restriction reduced dominant follicular growth and had the potential to inhibit dominant follicles from ovulating (Mackey et al., 1999; Rhodes et al., 1996). Mackey et al. (1999) also observed increased plasma concentrations of pre-emergence FSH in nutrient-restricted heifers, indicating that the dominant follicle was not as adequate in suppressing FSH in nutrient-restricted heifers, likely due to reduced estradiol providing less negative feedback. Reduced LH pulse frequency in nutrient-restricted heifers also reduced follicular growth

rate, suggesting that dominant follicles required more time to reach ovulatory size and to produce enough estradiol to signal estrus and a surge in GnRH and LH prior to ovulation (Mackey et al., 1999). Therefore, reduced estradiol concentrations and follicular growth rate over an extended period of time may delay onset of estrus in nutrient-restricted animals; however, the mechanism behind delayed onset of estrus in response to nutrient restriction has not been determined. While longer interval to estrus has been demonstrated in goats due to nutrient restriction (Mani et al., 1992), minimal research has been reported in this area for beef cattle. Declines in estradiol concentrations associated with reduced dominant follicle size are commonly reported in animals approaching anestrus due to nutrient restriction but are not typically observed in initial cycles of restricted animals (Rhodes et al., 1996), but miniscule changes in estradiol concentrations may be adequate to delay onset of estrus.

In the current study post-insemination nutrition approached a tendency to impact embryo recovery rate, whereas pre-insemination nutrition did not affect embryo recovery rate. Similarly, effect of post-insemination nutrition on embryo survival was reported by Dunne et al. (1999), with heifers fed at 200% maintenance and then to 80% maintenance after insemination having reduced embryo survival by 30 percentage points, compared to no difference in embryo survival among LL, LH, and HH treatment heifers.

Maternal nutrient restriction has also reduced embryo quality, as reported by Kruse et al. (2017), who observed embryos were at a less advanced stage and poorer grade from heifers undergoing nutrient restriction following insemination. The current study supports this finding, as embryo stage and grade were diminished not only by post-AI nutrient restriction, but also in response to pre-AI nutrient restriction.

Circulating concentrations of metabolites indicate associated changes in dietary intake, and in cases of nutrient restriction, peripheral NEFA concentrations are typically elevated in response to nutrient restriction as a result of increased lipolysis for energy (Bossis et al., 1999; Leroy et al., 2005; Van Hoeck et al., 2011). Nutrient-restricted heifers (LL and HL) in the current study expressed greater NEFA concentrations compared to high treatment heifers (LH and HH) and support the inverse relationship between dietary intake and peripheral NEFA concentrations. The impact of time on NEFA concentration in HL heifers may be explained by a period of adaption for heifers to adjust to limit feeding and account for rumen turnover in comparison to former *ad libitum* access to feed. Heifers in the LL and HH treatments also exhibited an increase in NEFA concentrations on d 3 after insemination, which may be due to reduced intake when heifers came into heat prior to AI.

Glucose is an additional metabolite critical to the development of the fetus. As neither the conceptus nor endometrium is capable of producing glucose, thus the embryo is dependent on maternal circulation of glucose (Gao et al., 2009b). Microbial fermentation in the rumen utilizes the majority of glucose within the ruminant for volatile fatty acid (VFA) production; therefore, gluconeogenesis occurs through VFAs such as propionate (Fahey and Berger, 1988). Glucose is tightly regulated within the body, and although differences in plasma glucose concentrations were observed for cows initiating anestrus, plasma glucose concentrations between nutrient restricted and control cows were not notably different until after 10 weeks of nutrient restriction (Richards et al., 1989). As the duration of the current study was only 6 weeks, it is not surprising that no differences were observed in plasma glucose concentrations across treatments. A

collective decrease in plasma glucose concentrations over six days may have been in response to heifers coming into heat and reducing dietary intake, as observed with plasma NEFA concentrations.

Protein can be utilized as an energy substrate in circumstances of nutrient deprivation (Tamminga et al., 1997), as was observed in the current study. Heifers in the LL treatment had increased plasma protein concentrations compared to LH and HH treatments, whereas HL heifers had similar plasma protein concentrations compared to both LL and LH heifers. As HL heifers underwent nutrient restriction, it is expected that HL plasma protein concentrations would be elevated compared to HH heifers.

Embryonic survival is dependent on maternally-derived nutrients, which are supplied to the conceptus via the uterine histotroph, which is composed of enzymes, growth factors, cytokines, lymphokines, hormones, amino acids, proteins, electrolytes, and glucose (Gao et al., 2009a). Estradiol has been reported to influence expression of uterine proteins (Bartol et al., 1981) and may be a potential factor why a tendency for increased ULF concentrations of protein were observed in the current study in response to estrus expression. Although ULF NEFA and protein concentrations tended to increase in response to estrus, concentrations of NEFA, glucose, and protein were not different among treatments on d 6 in response to pre- or post-AI nutrition, suggesting that these metabolites may not be mediating the impacts of maternal nutrition on embryo survival nor conceptus development.

Nutrient restriction may also have repercussions on small antral follicular development in cattle. After the current study, heifers were placed on full feed for 30 days prior to the breeding season. Treatments that underwent nutrient restriction prior to

and/or after AI during the study had a greater percentage of open heifers after the breeding season compared to heifers that did not undergo nutrient restriction (see Appendix), indicating that small antral follicular growth may be sensitive to nutritional insults and may impact future conception rates in cattle (Gutiérrez et al., 1997).

In summary, results from this study indicate that plane of nutrition both prior to and following AI impact embryo survival and early conceptus development. While plasma metabolite concentrations were reflective of nutrient restriction, this trend was not observed in ULF metabolite concentrations, indicating that at d 6 of gestation the uterus does not represent peripheral metabolite circulations. However, placing heifers in a negative energy balance prior to and immediately after AI reduced embryo recovery rate, stage, and grade. This decease may not be mediated through NEFA, glucose, or protein concentrations. Therefore, as the early stages of embryo development are sensitive to maternal plane of nutrition, proper nutritional management is necessary around the time of AI to ensure heifers are not placed in a negative energy balance.

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APPENDIX

Impacts on conceptions rates are evident in beef cattle even after nutrient restriction is terminated. After the current study, heifers were managed as a single group with access to spring pasture and were placed on full feed (*ad libitum* sorghum silage) for 30 days prior to the breeding season. Heifers were synchronized with a fixed-time 7-day CO-Synch + controlled internal drug release (CIDR) protocol and inseminated with semen from a single collection from a single beef sire. Heifers were then moved to pasture for the remainder of the breeding season and were exposed to a clean-up bull. Pregnancy success to AI and overall breeding success were determined 81 days later via transrectal ultrasonography. Heifers assigned to treatments undergoing nutrient restriction prior to and/or after AI from the current study (LL, LH, and HL) had a greater percentage of open heifers after the breeding season (40%, 46.7%, and 40% respectively) compared to heifers that did not undergo nutrient restriction (HH: 20%; Table 4). Small antral follicular growth is sensitive to nutritional insults and has been reported to impact conception rates in cattle (Gutiérrez et al., 1997). As observed by pregnancy rates from heifers in the current study, even short-term nutrient restriction for 6 days (HL) yields similar pregnancy success compared to heifers restricted nutrition for 39 to 42 days (LL heifers), indicating that negative impacts on follicular development may have the potential to impact future pregnancy success.

Table 5. Breeding season pregnancy success across treatments.

	AI, %	Hd/Trmt	***Bull-bred, %***	Hd/Trmt	***Open, %***	Hd/Trmt
LL	33.3	5/15	26.7	4/15	40	6/15
LH	46.7	7/15	6.7	1/15	46.7	7/15
HH	40	6/15	40	6/15	20	3/15
HL	20	3/15	40	6/15	40	6/15