The Effect of Supplemental Protein on the Uterine Environment during Heifer Development

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"I've never lost a game, I just ran out of time"
-Michael Jordan

ABSTRACT

Replacement heifer development is one of the most critical components of beef production. Nutrition affects development and reproductive success of replacement heifers. Our hypothesis was that different levels of protein supplementation would affect ULF of beef heifers, without altering the rate of development. Commercial Angus heifers (*n*=60) were blocked by BW into 4 weight classes. Within each weight class, heifers were randomly assigned to one of three treatment groups of supplemental protein (10% [**CON**], 20% [**P20**], and 40% [**P40**]) in a randomized complete block design with repeated measures. All heifers were allowed ad libitum access to mixed native grass hay. Bodyweight, BCS, and blood samples were collected every 2 weeks. Uterine fluid samples were collected monthly to evaluate components of ULF. Relative uterine pH was calculated by subtracting saline pH from the uterine flush pH. Separate mixed model ANOVAs via PROC GLIMMIX (SAS 9.4, Cary, NC) were used to determine if protein supplementation, time, and their interactions influenced BW, BCS, or ULF. Bodyweight of CON heifers was significantly lower ($P<0.05$) than P20 or P40 groups from sample 4-11. There was an interaction between treatment and time for relative uterine pH ($P=0.002$). At sample 2, the P40 group exhibited less change in pH compared to the CON group (0.33±0.1 vs. 0.74±0.1; $P<0.05$). At sample 3, the P40 (0.23±0.1) group uterine environment was basic compared to the P20 (-0.22±0.1) or CON (-0.13±0.1) groups ($P<0.05$). CON supplemented heifers tended to have more basic raw uterine pH (7.15±0.1) compared to P20 (7.02±0.09) or P40 (6.96±0.09) heifers ($P=0.05$) at detection of puberty. Heifers in weight class 1 had a more acidic uterine environment at detected puberty (6.89±0.1) than weight classes 2, 3, or 4 (6.96±0.1, 7.18±0.1, 7.11±0.1; $P<0.01$).

CON heifers had more Orn (0.12) than P20 (0.07) or P40 heifers (0.03; P=0.03). P40 heifers had more Trp (0.18) than CON (0.25) or P20 (0.19; P=0.03). In conclusion, protein supplementation increased heifer growth and altered the pH and AA content of uterine fluid but did not influence development from weaning to first breeding. All treatments were successful in developing replacement heifers with regards to industry standards.

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CHAPTER ONE
INTRODUCTION

Proper nutritional management of replacement heifers is paramount in maximizing reproductive success. The overarching objective of heifer development is to maximize the percentage of heifers that conceive early in the first breeding season and maintain a pregnancy. Replacement heifers should weigh 50 to 65% of their mature BW (Patterson et al., 1992; Funston et al., 2012) to maximize the number of cyclic females entering the breeding season, since puberty is primarily dictated by age and weight (Wiltbank et al., 1969; Nelsen et al., 1982; Oeydipe et al., 1982). Heifers which conceive earlier in their first breeding season have longer recovery time before the next breeding season resulting in improved overall longevity in the herd (Day and Nagueira, 2013). During development, a positive correlation between body condition score and fertility has been observed, provided that heifers do not become over conditioned (Utter et al., 1993). Heifers supplemented with additional protein experience elevated growth and fertility rates compared to counterparts developed without protein supplementation (Short and Bellows, 1971). Conversely, overfeeding protein can delay the onset of puberty, reduce pregnancy rates at first service, and cause an abnormally low uterine pH which may negatively impact fertility (Ferrell, 1982; Elrod and Butler, 1993; Lalman et al., 1993; Kane et al., 2004).

By identifying the ideal uterine environment in pre-breeding heifers, maximal productivity via increased fertility and reproductive efficiency can be obtained. Developing management practices that lead to an ideal uterine environment will allow producers to identify and more efficiently raise successful replacement females. Establishment and maintenance of pregnancy is dependent on proper interaction of the uterine environment and embryo to support development until hemotropic exchange can be established (Forde et al., 2014). Deficiencies in

key molecules within the uterine environment are a major contributor to embryonic mortality (Bridges et al., 2013). The largest percentage of embryonic mortality occurs prior to day 14 post-insemination (Dunne et al., 2000). The uterine environment is comprised of secretions and transported molecules that can be quantified and described as the maternal contribution to uterine luminal fluid (ULF; Forde et al., 2014). During the preimplantation period, the embryo is free floating within the uterus and entirely dependent on ULF for nutrients (Bazer 1975).

Several recent studies have analyzed amino acid (AA) concentrations within ULF throughout the estrous cycle (Hugentobler et al., 2007), during pre-attachment (Groebner et al., 2011; Forde et al., 2014), however there is a lack of knowledge regarding the constituents of ULF prior to the onset of puberty. Amino acid concentrations have also been reported at maternal recognition of pregnancy and during early gestation in ULF (Crouse et al., 2019). Despite the potential relationship between maternal nutrition and reproductive efficiency, few studies have analyzed the impact of maternal nutrition on AA concentrations in ULF. Crouse and colleagues (2019) reported that animals receiving a restricted diet had altered, and oftentimes reduced, utero-placental AA concentrations from day 16 to 50 of pregnancy, a time point of development in which the embryo is still largely dependent on ULF. Therefore, our hypothesis was that different amounts of supplemental CP would impact the AA concentrations within the ULF of developing heifers without altering reproductive development. The objectives of the current study were quantifying the AA concentrations and pH in ULF while meeting developmental goals similar to industry standards.

CHAPTER TWO
LITERATURE REVIEW

ABSTRACT

With nearly 6 million replacement heifers entering the US beef herd annually (N.A.S.S., 2019), development of these females is one of the most critical components of beef production. These females must incorporate both genetic progress and the needs of the producer to remain successful within the breeding herd. The importance of early conception in the first breeding season largely affects lifetime production of replacement heifers. Day and Nogueira (2013) determined that heifers conceiving early in the breeding season weaned heavier calves and were more likely to become pregnant as 2-year-old cows. One of the primary reproductive targets for a beef cow herd should be ensuring that heifers calve prior to 2 years of age to ensure long term productivity (Diskin and Kenny, 2014).These benchmarks for long-term success indicates the need for high fertility among replacement heifers at the start of the breeding season, and the importance of promoting sexual maturity at a younger age.

Several studies have demonstrated an improvement in conception rates in subsequent estrus events compared to that of their first cycle (Byerley et al., 1987; Roberts et al., 2019). Recent data from a 9 year study reported that heifers exhibiting at least two cycles prior to breeding are more likely to conceive in the first 21 days of the breeding season (Roberts et al., 2019, further emphasizing the importance of reducing the age at puberty, to allow the best chance for heifers to achieve multiple cycles prior to breeding. Additionally, heifers bred at their third estrus attained 21% higher conception rates than those bred at first estrus (Byerley et al., 1987). These analyses reaffirmed that puberty and cyclicity were not only associated with age, but also their rate of physical maturation. It has been well documented that puberty in beef cattle is primarily dictated by age and weight (Wiltbank et al., 1969; Oeydipe et al., 1982; Nelsen et al., 1985), and traditional development of replacement heifers has targeted raising females to weigh

60% of their mature BW before the start of the breeding season (Patterson et al., 1992). Methods of lowering this target weight without negatively impacting reproductive function have been explored as a method of reducing feed costs and utilizing compensatory gain (Funston et al., 2012; Endecott et al., 2013). By allowing heifers an additional 15-day breeding season (45 vs. 60), pregnancy rates were not compromised in heifers developed to lighter BW, however further studies are necessary before commercial implementation or recommendation.

Replacement heifers weighing 600 lbs., fed to gain 1.5 lbs./day require approximately 9.5 lbs. total digestible nutrients (TDN) and 1.32 lbs. of Crude Protein (CP) each day (Funston et al., 2012). The benefits of supplemental protein to replacement heifers have been documented (Martin et al., 2009; Funston et al., 2012; Endecott et al., 2013). Meeting protein requirements is one helpful way producers can ensure that heifers are receiving adequate amounts of energy during critical periods of growth and development, to avoid compromising reproductive success. Most heifer development programs require supplemental feeding to meet nutritional requirements for adequate growth and development (Funston et al., 2012). A positive correlation between BCS and increased reproductive success has been observed in heifers entering the first breeding season up to a BCS of 6 (Utter et al., 1993) and a dramatic decrease in fertility when BCS is < 5 (Selk et al., 1987). Meeting protein and energy requirements are essential to maintaining a normal Luteinizing Hormone (LH) pulse frequency following the cessation of the negative estradiol feedback loop (Day et al., 1986a) that occurs leading up to a heifers first ovulation. Heifers supplemented with additional protein experience elevated growth and fertility rates compared to counterparts developed without protein supplementation (Short and Bellows, 1971). However, it is important to avoid exceeding protein requirements when supplementing developing heifers as this can result in heavier weights at puberty compared to contemporaries

(Ferrell, 1982; Lalman et al., 1993), reduce pregnancy rates at first service (Kane et al., 2004), and cause an abnormally low uterine pH which may negatively impact fertility (Elrod and Butler, 1993). While protein serves an important role during development, total energy in the diet has also been demonstrated as a limiting factor for growing animals, and heifers that receive inadequate levels of energy during development can have severe repercussions as well. Females developed on low energy diets not only grow slower, but can experience delayed puberty compared to contemporaries, via a failure of pulsatory activity of LH which can negatively alter follicular recruitment and inhibit fertile ovulation (Day et al., 1986a).

In addition to impacting physical development, nutritional planes have been repeatedly demonstrated to impact the uterine environment of beef cattle (Elrod and Butler, 1993; Crouse et al., 2019). High levels of dietary CP have also been shown to illicit an acidic pH change within the uterus, negatively impacting conception rates in dairy heifers (Elrod and Butler, 1993). The resident fluid within the uterine environment of beef cattle is comprised of secretions and transported molecules that can be quantified and described as the maternal contribution to uterine luminal fluid (ULF; Forde et al., 2014). Constituents of uterine luminal fluid (ULF) enter the uterus through facilitated transport across the uterine endometrium or are secreted from uterine glandular epithelium (Bridges et al., 2013). The concept of altering the uterine environment through changes in the diet and more specifically the amount of dietary protein provided has been continually investigated in an effort to improve reproductive efficiency for several decades in both beef and dairy cattle during early pregnancy and across the estrous cycle (Jordan et al., 1983; Elrod and Butler, 1993; Crouse et al., 2019). Increasing CP in the diet from 12% to 23% increased Mg, K, P but decreased Zn in ULF (Jordan et al., 1983).

Amino acids have also been demonstrated to be altered by dietary protein intake in systemic circulation (Geppert et al., 2017a, b), as well as the uterine environment (Crouse et al., 2019), further indicating the role of dietary protein and plane of nutrition in contributing amino acids to the uterine environment for utilization by the developing embryo. Hugentobler et al., (2007) determined that the amino acids His, Phe, Ser, Tyr, Met, Val, Ile, Leu, Gly, Tau, and Thr underwent a temporal change in concentration over the course of the estrous cycle. The current review will present recent studies related to the impact of supplementing replacement heifers, and how these management strategies affect the development and reproductive success of these females.

HEIFER DEVELOPMENT

Post-Weaning

Replacement heifer development must support physical and sexual maturation of weaned calves leading up to their first breeding season. Proper development of replacement heifers requires significant input costs of producers that choose to develop their own. Thus, it is very important to formulate supplements and total rations to meet nutrient requirements without over-feeding replacement heifers (NRC, 2016). Creep feeding females designated as replacement animals is not typically recommended prior to weaning to avoid over conditioning which can deposit fat in the udder, inhibiting lifetime milk production and decrease calf weaning weights (Hixon et al., 1982). Recent research has shown that differences in development often occur based on both feed-type and nutritional inputs provided during the post-weaning period (Marston et al., 1994). Rate of growth for replacement females during the entire post-weaning period plays a larger role on reproductive success than that of growth rate immediately prior to breeding (Roberts et al., 2009). Although obvious developmental differences exist between breeds and types of cattle (Diskin and Kenny, 2014), replacement heifers must gain 0.45 - 0.68 kg / day

post-weaning in order to enter the breeding season at an adequate size (Funston et al., 2012). Plane of nutrition has been clearly demonstrated to impact age at puberty (Day and Nogueira, 2013). Development systems range from intensive systems with high feed inputs to more extensive systems that rely more heavily on compensatory gain leading up to the first breeding season (Endecott et al., 2013); there is renewed interest in evaluating these systems in more detail with long-term consideration is currently occurring (Roberts et al., 2009; Day and Nogueira, 2013; Roberts et al., 2019). Classically, it was reported that heifer development systems do not impact pregnancy rates as long as that animals reached an appropriate pre-breeding BW (reviewed by Patterson et al., 1992), however recent reports (reviewed by Funston et al., 2012) suggest opportunities to lower these established pre-breeding guidelines . There is great interest in hastening the onset of puberty in replacement heifers, regardless of production type. Doing so promotes herd fertility by increasing conception rates in the first breeding season (Byerley et al., 1987; Roberts et al., 2019), and could increase longevity of these females (Mulliniks et al., 2013).

Establishing Puberty and Maintaining Cyclicity

Puberty in beef cattle is defined as ovulation of an oocyte coinciding with normal luteal function and visual displays of estrus behavior (Perry, 2016), and refers to the point of sexual maturity in a heifer's life in which conception and maintenance of a pregnancy through parturition becomes possible. Despite maturational differences between breeds, the events leading up to the onset of puberty are similar across cattle. For puberty to occur, the hypothalamic-pituitary-ovarian axis must mature through the interactions of several hormones (Perry, 2016). The diminishing negative feedback of estradiol on gonadotropin-releasing hormone (GnRH) allows for a dramatic increase in the amount of luteinizing hormone (LH) and

Follicle Stimulating Hormone (FSH) which in turns stimulates follicular growth and estradiol production by follicles (Day and Anderson, 1998). The change in feedback of estradiol, from negative to positive, stimulates an initial LH surge which signals the establishment of cyclicity when followed by a luteal phase of normal length (Day and Anderson, 1998; Perry, 2016). Heifers during the peripubertal phase of development often experience short cycles immediately preceding puberty, where ovulation and estrus behavior can occur independently (Atkins et al., 2013). Oocytes ovulated during short cycles can become fertilized, but embryonic mortality rates are high due to premature corpus luteum (CL) regression, hypothesized to be the result of premature release of prostaglandins from the uterus (Perry, 2016). Ovulation during the prepubertal period can occur naturally or artificially by inducing an LH surge, however this is not a reliable indicator of puberty or fertility. Oocytes collected from prepubertal donors are subject to an increased amount of developmental incompetence, preventing or inhibiting their ability to become fertilized (Armstrong, 2001). Heifers supplied exogenous GnRH experienced elevated LH levels similar to pubertal animals; however, no luteal function followed the LH surge and these animals quickly returned to a state of anestrus (McLeod et al., 1985). Prior research has demonstrated that prepubertal heifers on restricted feed have reduced LH secretion and may fail to attain puberty (Day et al., 1986a), and severe feed restriction can cause cycling heifers to enter a state of nutritionally induced anestrus (Bossis et al., 1999, 2000). Once puberty has been attained, it is crucial that adequate nutrition be maintained, as anestrous animals require substantial amounts of time and feed before cyclicity resumes, further delaying these animals from entering the breeding herd (Bossis et al., 1999, 2000). Historically, a positive correlation has been observed between conception rates and BCS so long that BCS remains less than, or equal to 6 (Lalman et al., 1993). Decreased fertility has been observed in heifers whose BCS was

<5 prior to the breeding season (Selk et al., 1987). Recent retrospective analyses confirmed these observations and found that heifers at BCS 6 had the greatest chance to conceive to AI and had the highest pregnancy rates at the conclusion of the breeding season.

Target BW

Previous research in replacement heifers has identified an inverse relationship between post-weaning growth rate and age at puberty, indicating that increased gains during the post-weaning period often correlate to reduced age at puberty (Short and Bellows, 1971; Ferrell, 1982). Replacement heifers should be 60-65% of their mature BW, on average, to ensure the majority of a group has reached puberty prior to entering their first breeding season (Patterson et al., 1992; Whittier et al., 2005; Perry et al., 2012). This target BW varies based on breed, European derived breeds being 60%, 55% for dual-purpose breeds, and 65% for *Bos indicus* cattle (Larson, 2007). These thresholds have been utilized to establish minimum nutrient requirements for replacement heifers to achieve maximum pregnancy rates (Patterson et al., 1992; Funston et al., 2012).

There is great interest in lowering the target BW for heifers to achieve prior to breeding which would reduce development costs by decreasing feed required to establish cyclicity without sacrificing pregnancy rates in the first breeding season (Funston et al., 2012; Endecott et al., 2013). Evidence does exist that supports revisions to target BW recommendations based on changes within the complicated and interconnected relationships amongst BW, puberty, and pregnancy rate (Funston et al., 2012). It was hypothesized by Funston and others (2012) that selection emphasis on fertility to beef cattle over recent decades has reduced the pre-breeding target BW requirements without compromising reproductive efficiency after generations of continual selection. Recent studies have investigated a target of 51-57% of mature BW for

replacement heifers with varying degrees of success (Funston and Deutscher, 2004; Roberts et al., 2007, 2009; Martin et al., 2008; Larson et al., 2011). Raising replacement heifers to 51% of mature BW was shown to be more cost effective than heavier thresholds (Martin et al., 2008) and overall conception rates did not differ between groups for the first or second breeding seasons (Martin et al., 2008). However, a larger proportion of open heifers in the 51% group failed to reach puberty prior to the breeding season (78.9 vs. 45%; Martin et al., 2008), and other studies have reported a reduction in the number of females reaching puberty prior to the breeding season in heifers developed on restricted systems, approximately 10% between the two studies (Funston and Deutscher, 2004; Roberts et al., 2009). Although when comparing the total cost of development, heifers developed to a lighter BW were $22-24 cheaper to develop per pregnant heifer. Therefore, heifers may not have to reach 65% of mature BW to maximize conception and rebreeding percentages (Patterson et al., 1992). These results indicate that while plane of nutrition and BW gain during the post-weaning period play an important role in establishing cyclicity, long-term selection of breeding animals with an emphasis on fertility over the past several decades have made it possible for more cost effective heifer development techniques (Funston et al., 2012).

Hormonal Induction of Estrus

Due to the importance of establishing cyclicity before the first breeding season, hormonal manipulation has been explored to promote estrus and the onset of puberty. Administration of exogenous estrogens and progestogens were successful in hastening the onset of puberty in (Gonzalez-Padilla et al., 1974; 1975). Several studies have also indicated that older and heavier animals were more likely to respond to hormonal manipulation, reiterating the importance of age and weight in reproductive function (Wiltbank et al., 1969; Oeydipe et al., 1982; Nelsen et al.,

1985; Hall et al., 1997). Rate of gain and BW attained by a given age did not influence the ability of a progestin to induce puberty, however these did affect the number of heifers that had become cylic at the initiation of hormonal administration. Higher proportions of older animals and heifers on high rates of gain achieved puberty prior to progestin exposure and were subsequently removed from latter portions of the study (Hall et al., 1997).

Estrous synchronization protocols are commonly used to shorten calving seasons and consolidate resources, and their success involving replacement heifers depends largely on the pubertal status of animals at the initiation of these protocols. Heifers were more likely to respond to a synchronization protocol if they had already reached puberty but prepubertal heifers can be induced to ovulate (Wood-Follis et al., 2004).There is a wide variety of accepted estrous synchronization protocols that include progestins and can be used effectively to hasten the final developmental changes necessary to achieve puberty leading up to breeding. However heifers that had reached puberty prior to initiation of the protocol were more likely to conceive after fixed-time artificial insemination than prepubertal heifers (61% vs. 47%, respectively; Bridges et al., 2014). The use of hormonal induction of estrous and estrus synchronization will continue to rise in popularity as further studies refines their use.

Seasonality

Cattle are not considered seasonal breeders (Schillo et al., 1992), meaning that they do not enter a period of seasonal anestrus based on photoperiod or time of year, unlike sheep and goats, (Foster et al., 1988). However, several studies have identified variations in reproductive activity and development coinciding with seasonal differences in cattle (reviewed by Schillo et al., 1992). Mature Brahman cows had higher magnitude of LH surges and higher mid-luteal concentrations of progesterone in early spring compared to winter (Harrison et al., 1982).

Additionally, postpartum Hereford and Hereford-Holstein cows that calved in fall returned to estrus sooner than those calving in the spring (King and Macleod, 1984). Ovariectomized cows also exhibited slight seasonal fluctuations in LH secretion (Day et al., 1986b), with concentrations increasing in spring and decreasing in autumn contrasting the work of King and Macleod (1984). However, response to estradiol negative feedback did not change with season in cattle, indicating a lack of seasonal steroid-dependent control of LH secretion (Stumf et al., 1988).

Monitoring the development of replacement heifers with respect to seasonality is difficult because of the long duration necessary for these females to physically and sexually develop, which occurs over multiple seasons (Hansen, 1985). Multiple studies have shown that dairy heifers born in the spring or summer were younger at first estrus (Hawk et al., 1954; Menge et al., 1960) and were younger at puberty than heifers born during fall (Roy et al., 1980). Supplemental lighting for 16 hours daily during winter months increased growth rate and reduced age at puberty in dairy heifers (Peters et al., 1978). Another study in beef heifers found that 18 hours of daily supplemental lighting reduced age at puberty, but did not affect LH concentrations in subsequent estrous cycles (Hansen et al., 1983). Other reports in beef heifers have also demonstrated an impact of season on the onset of puberty. Heifers born in late spring or fall, months associated with longer photoperiods, were younger at the time of puberty than those born in early spring or winter (Arije and Wiltbank, 1971; Swiersta et al., 1977; Schillo et al., 1982). The environment associated with winter months has been demonstrated to delay puberty (Grass et al., 1982) in which a larger proportion of fall-born heifers did not avheiev puberty during winter and remained prepubertal until the subsequent spring. However a contrasting study (Greer, 1984) found no effect of season on age at puberty. Further contrasting

these observations, winter born heifers supplemented with melatonin to simulate a short photoperiod during summer attained puberty faster than control animals of similar age (Tortonese and Inskeep, 1990). Based on these observations, it is likely that photoperiod plays a role in influencing the timing of puberty in heifers, however this role is minimized compared to other factors such as age and nutritional status and their interactions in determining when puberty will occur. In cyclic Brahman heifers, the frequency of corpora lutea and uterine tone gradually increased in spring months, rising to a peak of reproductive activity during the summer when photoperiod is greatest, and was lowest during winter months, and age at puberty did not differ by season of birth, although weaning weight and age at puberty were correlated (Plasse et al., 1968). Months with the shortest photoperiod were also associated with the highest rates of heifers returning to a state of anestrus in previously cycling Brahman heifers, with mean luteal serum progesterone levels decreasing in winter months (Stahringer et al., 1990).

SUPPLEMENTATION

Protein

Protein composition

Given the overwhelming importance of proper nutrition for replacement heifers, it is crucial to understand the dietary components necessary for proper development. Often times, heifers graze low quality forages which do not meet nutrient requirement to achieve puberty prior to breeding (Marston et al., 1995). Increasing crude protein (CP) is a common method to ensure heifers reach pre-breeding target weights (Wiltbank et al., 1969). The benefits of supplemental CP in heifer development systems has been well documented (Granger et al., 1989; Lalman et al., 1993; Martin et al., 2007; Dickinson et al., 2019). In Brangus and Angus heifers, those receiving elevated levels of supplemental feedstuffs high in CP achieved puberty at a younger age but similar weight than diets low in CP (Granger et al., 1989). Another study by

Lalman et al., (1993) agreed with these findings and found that heifers receiving supplemental CP achieve puberty at a younger age than non-supplemented controls. A recent retrospective analysis of 6 years of heifer development records indicated that heifers receiving supplemental CP during development that achieved normal developmental parameters were more likely to become pregnant in their first breeding season than contemporaries (Dickinson et al., 2019).

There are several components that make up CP: rumen-degradable protein (RDP), rumen-undegradable protein, and non-protein nitrogen (NPN; Schwab et al., 2003). Non-protein nitrogen makes up the smallest proportion of CP and these compounds are smaller molecules including peptides, free amino acids, nucleic acids, amides and amines, nitrate, and ammonia. The two larger components are RDP and RUP. Rumen-degradable protein is broken down by the microbial organisms within the rumen and fuels the synthesis of microbial crude protein (MCP); whereas, RUP by-passes ruminal degradation and goes directly to the small intestine (NRC, 1996; Bohnert et al., 2002; Schwab et al., 2003). The main source of protein being presented to the small intestine of grazing ruminants is MCP from RDP (NRC, 1996; Bohnert et al., 2002; Schwab et al., 2003; NASEM, 2016) Microbial crude protein has the capability of providing all 10 dietary essential amino acids through microbial synthesis, allowing ruminants to utilize poor quality forages with very low protein levels (Nolan, 1993; NASEM, 2016). Metabolizable protein (MP) consists of two separate components; the overall protein needs of the animal, and the RDP needs of ruminal microorganisms in order to maximize the supply of MCP (NASEM, 2016). The proportions of the total metabolizable protein provided by these components depends largely on the feedstuffs and forages provided, but it was estimated by the NASEM (2016), that 67-75% of absorbed AA are derived from MCP, signifying the importance of maximizing microbial production.

Development and Reproduction

Protein is the first limiting nutrient in cattle production systems grazing dormant-season forage diets (Wallace, 1987), and providing increased levels can increase forage intake and enhance reproduction (Caton et al., 1988; Wheeler et al., 2002). Bahrami-yekdangi and others (2016), clarified that the objective of ruminant protein nutrition is to provide adequate amounts of RDP to optimize ruminal efficiency while minimizing the amount of dietary CP which may reduce feed costs my maximizing the amount of MCP produced, without grossly exceeding requirements. Increasing RUP to augment protein supply to the small intestine has been explored for its effect on reproductive performance. Prepubertal heifers supplemented 250 g of RUP in addition to *ad libitum* low quality hay required less total TDN to achieve similar rates of gain than other treatments, however these heifers were 7 to 8 kg heavier than control animals at the time of puberty (Lalman et al., 1993). A smaller percentage of RUP supplemented heifers were serviced in the first 21 days of the breeding season (64% vs. 76%) but overall pregnancy rates were not different between treatments (Lalman et al., 1993). Brahman heifers and cows supplemented with a 56% RUP supplement experienced higher first service conception rates than those receiving a 38% RUP supplement by 28% (Triplett et al., 1995). The study by Triplett et al., (1995), unlike Lalman and colleagues (1993), yielded differences in final pregnancy rates after a 90 day breeding season, with High and Medium RUP treated groups tending to have higher conception rates than low RUP groups (56% vs. 62% vs. 43%, respectively), indicating that protein supplementation regularly affects first-service conception, but may not always impact overall pregnancy rstes. Other investigators have found no benefit (Lalman et al., 1993) or an inhibition of performance (Elrod and Butler, 1993), while some have found positive benefits (Martin et al., 2007; Mulliniks et al., 2013). Rumen-undegradable protein also impacts

the ovary. After estrus synchronization, RUP supplemented cows had larger ovulatory follicles than controls (15.53 vs. 12.91mm; Gunn et al., 2014). Concentrations of progesterone tended to be lower in high RUP groups (Gunn et al., 2014), which may impact the ability of these females to maintain pregnancy. Progesterone has also been reported to not be impacted by RUP supplementation, however high RUP supplemented heifers had increased levels of IGFBP 2 and 4 in follicular fluid (Kane et al., 2004)., Since follicular atresia is associated with elevated levels of IGFBP proteins these data may indicate decreased competence of follicles from high RUP heifers (Kane et al., 2004). . Low and Mid RUP groups had higher levels of FSH compared with high RUP, suggesting limited benefits from supplementing feedstuffs high in RUP (Kane et al., 2004).

The use of by-product feeds has increase in recent years, and can be an economical feedstuff to satisfy nutrient requirements of beef females grazing low quality forages (Gunn et al., 2014). Distillers dried grains (DDG), for example, contains much higher levels of CP and energy than corn (Loy et al., 2003), additionally, the RUP fraction for DDG is often above 50% (Benton et al., 2006). Body weight and BCS were unaffected but DDG supplemented heifers had improved response to estrus synchronization and conception rates AI than control heifers (75% vs. 52.9%, respectively; Martin et al., 2007). Heifers developed on native range may benefit more from RUP supplementation compared to drylot developed heifers with increased conception rates, lower development costs, and increased retention rates (Mulliniks et al., 2013). Development systems where RDP requirements are not grossly exceeded may benefit from RUP supplementation in complimenting MCP for post-ruminal utilization.

Supplemental Amino Acids

In order for cattle and other animals to utilize the AA supplied through dietary and MCP (in the case of ruminants), these proteins must be digested and made available for absorption. When utilizing processed feeds, this can become problematic, as feeds can become heat-damaged during processing, inhibiting their digestibility and compromising gain in growing animals (NASEM, 2016; Nakamura et al., 1994). Free amino acids are degraded rapidly in the rumen (Veira et al., 1991), so they must be supplemented in a ruminally-protected form. Since microbial protein provides the majority of MP to beef cattle (NRC, 1985; Spicer et al., 1986; NASEM, 2016), it is logical that grazing cattle or those consuming large amounts of harvested forages with little protein would be subject to the first-limiting amino acid from MCP, which is Met in most scenarios (Storm and Ørskov, 1984) and could benefit via growth and production of AA supplementation (Veira et al., 1991; Klemesrud et al., 2000). Developing heifers were supplemented with a commercial blend of sulfur containing amino acids from either corn-gluten meal or 88% 2-hydroxy-4-methylthio butanoic acid, with high RUP values, and found that heifers receiving the blend had improved ADG over the first 30 days of the study and tended to have greater reproductive tract scores (Hersom et al., 2009). The link between growth and reproductive development has been well established; therefore, providing additional AA could elevate growth and enhance reproductive development of replacement heifers.

Energy

Carbohydrates

While protein is the first limiting nutrient in low-quality forages, energy can be limiting as well (Wallace, 1987). Forages comprise between 90 to 100% of beef cattle diets, with the exception of finishing diets in feed lot cattle (Gaylean and Goetsch, 1993). However the quality

of these forages varies greatly and is determinate of the need for supplemental protein and/or energy.Carbohydrates are the most significant source of energy to cattle, supplying digestible energy to the animal and, specifically, the rumen (NASEM, 2016). Thus carbohydrates have been investigated as effective supplements in heifer development systems. Carbohydrates can be divided into two fractions based on their function and chemical make-up. Non-fiber carbohydrates (NFC) which is typically the more digestible fraction, and neutral detergent fiber (NDF; NASEM, 2016). Neutral detergent fiber includes hemicellulose, cellulose, and lignin, and takes much longer to digest. Starch is considered a non-NDF carbohydrate, and it is the main storage carbohydrate in plants. Additionally, starch is prevalent in cereal grains, making it a useful medium to evaluate carbohydrates impact on heifer development (NASEM, 2016). Beyond the classic function of starch as an energy source, it also serves other purposes within the rumen. Starch fermentation in the rumen can help provide quality carbohydrates that aid in microbial protein synthesis once RDP requirements have been reached (Russell et al., 1992). Replacement heifers supplemented with a high starch supplement for 30 and 60 days prior to breeding were younger at puberty than controls (Ciccioli et al., 2005). Similarly, 50% of heifers fed a high starch, high concentrate diet achieved puberty before 300 days of age, compared to 0% of control heifers (Gasser et al., 2006).

Fats

The use of supplemental lipids in heifer development has been explored extensively for their effects on growth, circulating hormone concentrations, and reproduction (Lammoglia et al., 1997; Mattos et al., 2000) . Conventional diets for beef cattle provide relatively small amounts of lipids, given the low-fat content of most forages and roughages (NASEM, 2016). Recommendations for feeding supplemental fat in high forage diets is typically limited to 4-5%

of total dry matter intake (DMI; Larson, 2007; Hess et al., 2008) to avoid reductions in fiber digestibility and DMI (Coppock and Wilks, 1991). Several common feedstuffs contain higher lipid content such as; sunflower, safflower, soybeans, cottonseed, rice bran, fishmeal, tallow, corn germ, distillers grains, and calcium salts of fatty acids (NASEM, 2016) making them suitable sources of increased energy, without the risk of ruminal acidosis (Rasby and Funston, 2016) Evaluating lipid's impact on hormonal characteristics and reproductive performance beyond their use as an energy source is more difficult Dietary fat undergoes substantial compositional changes in the rumen during biohydrogenation, where fatty acids are stripped from their glycerol backbone (Funston, 2004), unsaturated fatty acids have a portion of their double bonds reduced (Mattos et al., 2000), and free-glycerol is fermented to propionic acid (Williams and Stanko, 1999). Lipid supplementation has been shown to increase the circulating concentrations of cholesterol in cattle (Staples et al., 1998), which acts as a precursor for progesterone synthesis by luteal cells in the ovary (Williams and Stanko, 1999). This is critical during early pregnancy, as adequate synthesis of progesterone is essential in establishing and maintaining pregnancy (Perry et al., 2007). Supplemental fat has been also shown to increase LH secretion in animals on a negative energy balance, however a mechanism independent of energy impacting LH production has not been established (Mattos et al., 2000). Animals on a positive plane of nutrition showed no effect of dietary fat on LH dynamics (Staples et al., 1998). These data are is not surprising given that LH secretion is partially dictated by the energy status of growing animals (Day et al., 1986a, b; Bossis et al., 1999). Reports of the effects of fat supplementation on conception rates have been mixed. Heifers supplemented high amounts of fat tended to conceive earlier in the breeding season than contemporaries (Whitney et al., 2000). Some studies report supplementing feedstuffs high in fat prior to breeding increased pregnancy

rates (Lammoglia et al., 1997; Bellows et al., 1999; Hess et al., 2008), while others reported no impact on reproductive performance (Lammoglia et al., 2000; Bellows et al., 2001; Funston et al., 2002; Shike et al., 2013). Studies reporting reproductive benefits to fat supplementation fed high-fat for at least 90 days prior to breeding (reviewed by Rasby and Funston, 2016). It has been postulated that the reproductive response is largely dependent on the type of fatty acids present in the fat source (Funston, 2004; Hess et al., 2008).However the benefits of fat supplementation may be limited in well-developed replacement heifers on a positive plane of nutrition (Funston, 2004; Rasby and Funston, 2016).

UTERINE ENVIRONMENT

In beef cattle, maternal recognition of pregnancy occurs via conceptus-derived secretions of interferon tau (IFNT) approximately 16 days after the expression of estrus (Northey and French, 1980; Betteridge et al., 1980; Bazer et al., 1997). The primary function of interferon tau is to ensure an adequate supply of progesterone is produced from the CL (Bazer, 2013). The period prior to maternal recognition is the time period in which a majority of embryonic losses occur (Dunne et al., 2000; Diskin and Morris, 2008). Embryonic losses prior to day 27 of gestation range from 20-44% in beef cattle of all embryonic loss (Humbolt, 2001; Geary, 2005). This is concerning given that fertilization rates of beef and dairy cattle are generally very high, estimated at 90% and 75-85%, respectively (Bridges et al., 2013. Dunne and colleagues (2000) concluded that embryonic loss occurs predominantly before day 14 of pregnancy, and found no differences in embryonic survival between day 14, day 30, and full term gestation.). During the pre-attachment period, the embryo resides within the uterus as a free-floating entity, thus, is entirely dependent on uterine luminal fluid, also known as histotroph, for nutrients to maintain growth and development (Bazer, 1975; Bridges et al., 2013). Uterine histotroph is composed of

enzymes, cytokines, growth factors, ions, hormones, sugars, amino acids, fatty acids, various proteins, and adhesion molecules necessary for successful pregnancy and embryonic development (Gao et al., 2009; Mullen et al., 2012; Forde et al., 2014). Uterine gland knockout models in ewes have consistently shown that restriction of glandular secretions not only hinders embryonic development but also prevents elongation of the embryo (Bartol et al., 1999; Gray et al., 2001, 2002). Thus, the uterine environment, specifically the ULF is paramount to successful establishment and maintenance of pregnancy.

Uterine pH and Urea

Given the importance of ULF in bovine embryo development, it is relevant to consider conditions that alter this environment, such as pH. The pH scale measures the dissociation of salts and acids in solution reflecting the negative logarithmic measurement of the hydrogen ions present in a solution (Pool, 2004). Changes in pH are not representative of a numeric chance in hydrogen ion concentration, rather they represent a fold-change in concentration, i.e., a decrease in pH from 7.4 to 7.2 represents a 60% reduction in hydrogen ions (Swain, 2010). The primary influencers of pH are bicarbonate and carbon dioxide, which dissolves in solution into carbonic acid (Pool, 2004; Swain, 2010), however these can be impacted by temperature, elevation and the type/concentrations of proteins in the solution (Swain, 2010; Roper, 2014). Maintaining an adequate pH suitable for embryo development is crucial in preventing reproductive inefficiency. The uterine environment pH is typically 7.0 (Elrod et al., 1993; Elrod and Butler, 1993; Kane et al., 2002; Hugentobler et al., 2007) substantially different from systemic pH of 7.4 (Elrod and Butler, 1993; Pool, 2004; Hugentobler et al., 2004, 2007; Swain, 2010; Roper, 2014). Uterine pH in cattle is typically measured by either inserting a pH probe through the cervix directly into the uterine environment (Elrod and Butler, 1993; Perry and Perry, 2008), or via a 2-way catheter and

infusing a flush media (Hersom et al., 2010; Cappellozza et al., 2015) of both dairy (Elrod and Butler, 1993) and beef cattle (Hugentobler et al., 2004; Perry and Perry, 2008). During the luteal phase of the estrous cycle, pH of ULF is relatively stable around 7.0 - 7.2 however it undergoes an acidic change to approximately 6.8 (Elrod and Butler, 1993; Perry and Perry, 2008a; b) during the follicular phase, with the lowest pH measurements recorded near the time of estrus. This decrease in pH is associated with increases in estradiol (Perry and Perry, 2008a; b). Supplementation of estradiol cypionate also decreased uterine pH and was speculated to be a potential mechanism for sperm transport (Perry and Perry, 2008a; b).

Classical studies originally reported an impact of dietary protein on uterine pH, in a study that supplied cycling dairy heifers with (Elrod and Butler, 1993). Heifers offered 150% of their CP requirements had a more acidic uterine pH (6.79) than contemporaries (7.09) on day 7 of the estrous cycle (Elrod and Butler, 1993), and observed a 21% reduction in conception rates in, potentially due to increased urea (Elrod and Butler, 1993). This phenomenon was unique to the uterus, and not mimicked in urine, saliva, or blood pH of non-pregnant, non-lactating dairy cows (Elrod et al., 1993). Lactating, dairy cows receiving urea infusions had lower uterine pH than control cows indicating that BUN exerts a rapid, direct effect on uterine pH (Rhoads et al., 2004). In contrast, increased BUN levels during the luteal phase increased uterine pH in dairy and beef-cross heifers (Grant et al., 2013) indicating either an interaction between BUN and stage of the estrous cycle on pH. Additionally, protein supplementation frequency has been shown to impact the uterine environment. Cappellozza and colleagues (2015) found that cows supplemented once weekly tended to have greater uterine flush pH values than cows supplemented thrice weekly, or daily (6.20 vs. 6.13 vs. 6.14, respectively). Though dietary protein has continually demonstrated to impact the uterine environment, attempts to alter its

effects on reproductive success have varying results (Elrod and Butler, 1993; Elrod et al., 1993; Gath et al., 2012; Crouse et al., 2019). Increasing protein in the diet of non-pregnant dairy cows from 12% to 23% altered the concentrations of Mg, K, P and Zn ULF (Jordan et al., 1983).Elevated levels of urea in blood plasma are indicative of excessive protein in the diet, since ammonia that escapes the rumen is converted to urea in the liver and is either excreted in urine or recycled into the gastrointestinal tract (GIT) to be converted back to ammonia in the rumen (NASEM, 2016). Originally hypothesized to be responsible for decreased fertility associated with high protein intake, (Elrod and Butler, 1993; Elrod et al., 1993) However, beef heifers and cows with higher concentrations of BUN (>16mg/dL) tended to have higher conception rates than those with lower levels (Gunn et al., 2016). These results suggest that fertility can be negatively impacted by high amounts of dietary protein (Elrod and Butler, 1993; Elrod et al., 1993) it is not always (Gunn et al., 2016).The reproductive inefficiency associated with protein may only occur in conjunction with maintenance of an acidic change in the uterine environment following ovulation. Confirmed by recent research where high quality embryos were transferred into beef heifers receiving 250 g of urea per day, and had no effect on conception rates on day 35 of gestation compared to controls (Gath et al., 2012).

Ocon and Hansen (2003) observed deleterious developmental effects of acidic culture media (pH 7.0 or below) on in vitro cultured embryos, implying that an acidic pH in-utero, often associated with excess protein intake, would illicit similar developmental retardation. Urea only inhibited development prior to fertilization, suggesting that BUN may not be the mechanism associated with reduced fertility in acidic uterine pH 7 days after breeding, although increased pH due to high BUN may alter nutrient uptake by the embryo (Ocon and Hansen, 2003). Developing embryos are largely dependent on their environment, however they are capable of

adjusting to physiological fluctuations in pH. Lane and Bavister (1999) determined the presence of an alkaline (bicarbonate/chlorine exchanger) and two acidic (sodium dependent, bicarbonate/chlorine exchanger; sodium/hydrogen antiporter) pH correction mechanisms in 8-16 cell bovine embryos. The presence of these mechanisms indicates that embryos can accommodate both an alkaline or acidic environment. These data indicated that embryos are capable of combatting acidosis more efficiently, while alkaline environments were harsher on embryos and hindered development more severely (Lane and Bavister, 1999). Additionally, certain amino acids have been shown to buffer embryonic intracellular pH in-vitro (Edwards et al., 1998).

Amino Acids' in ULF

As previously discussed, the ULF must create an environment for proper embryonic development and establishment of pregnancy until maternal circulation can take over (Bazer, 1975; Gray et al., 2001, 2002; Hugentobler et al., 2007). Uterine luminal fluid contains a multitude of constituents necessary to support the nutritional and physiological needs of the embryo during this free-floating period (Hugentobler et al., 2007), and a classical study by Stanke et al. (1974) identified all 20 proteogenic amino acids in oviduct fluid Holstein heifers throughout the estrous cycle. Amino acids in ULF were historically characterized by their role in protein synthesis (Morris et al., 2002; Sturmey et al., 2008; Leese, 2012). However, there is an increasing amount of literature indicating multiple functions of amino acids during early embryonic development (Rieger and Guay, 1988; Leese, 1991; Bavister and McKiernan, 1993; Edwards et al., 1998; Steeves and Gardner, 1999; Van Winkle, 2001). Amino acids have classically been divided into two categories; non-essential, which can be synthesized by the organism, and essential, which much be provided through the diet because they cannot be

synthesized (Sturmey et al., 2008). In vitro studies have been performed on embryos of several species to evaluate the function of specific amino acids in regards to embryonic development.

Estrous-Temporality/Pre-attachment

Recent studies have focused on characterizing the physiological concentrations of amino acids over the course of the estrous cycle in cattle (Hugentobler et al., 2007; Forde et al., 2014). These experiments have worked towards defining the changes in AA's over the estrous cycle, which can be extrapolated to the pre-attachement period of early pregnancy, prior to when maternal recognition and embryonic attachment occurs (Hugentobler et al., 2007). These observations are useful in determining the availability of AA for use by the developing embryo, previously only estimated in culture media in-vitro (Hugentobler et al., 2007). This is difficult since amino acid utilization and requirements are not static and change over the course development in bovine embryos (Partridge and Leese, 1996; Morris et al., 2002). Concentrations of amino acids varied in ULF throughout the estrous cycle, with a majority of amino acids increasing as ovulation grew closer and 10 AA's (7 non-essential, 3 essential) exhibited higher concentrations in ULF compared with blood plasma (Hugentobler et al., 2007). This demonstrates a difference between systemic and uterine amino acid profiles. In agreeance with previous studies (Hugentobler et al., 2007; 2010; Forde et al., 2014) a temporal increase in concentrations of ULF amino acids from day 7 to 13 of the estrous cycle, with the exception of aspartate and asparagine (Mullen et al., 2014).This temporal increase in amino acid concentrations is not surprising, and complements the findings of Sturmey and colleagues (2010), in which it was determined that embryos further along the course of development (i.e., expanding blastocyst) depleted more amino acids in culture medium than less developed

counterparts (i.e., compacted blastocyst), regardless of how they were derived (*in-vivo* vs. *in-vitro*).

Adequate levels of progesterone are critical for the establishment and maintenance of pregnancy. Exogenous progesterone increased pregnancy rates in cows with low levels of progesterone during the subsequent luteal phase following breeding (Starbuck et al., 2001). Elucidating the impact of progesterone on ULF may yield potential opportunities for intervention in creating an optimally receptive uterine environment. However, only Valine was modulated by progesterone on day 6 of the estrous cycle in ULF (Hugentobler et al., 2010). This trend was not mimicked in oviduct AA in the same study and concentrations of 9 oviductal AA concentrations were increased after receiving supplementary progesterone at day 3 of the estrous cycle, with the largest increase seen in glycine, which nearly doubled. Worth noting is that on day 3, the embryo still resides within the oviduct and is dependent on its secretions for maintained development. Consistent with these observations, dairy heifers with low levels of progesterone during the luteal phase had reduced concentrations of Asn , His and Thr in ULF on d 13 of the estrous cycle (Mullen et al., 2014). Progesterone supplemented for 7 days post-estrus increased the expression of the endometrial transporters SLC1A1, SLC1A4, SLC7A1, SLC7A7, SLC38A7 and SLC6A14, which are responsible for movement of amino acids into ULF indicating that proper embryonic development may be inhibited if circulating progesterone levels are low (Forde et al., 2014). Additionally, the concentrations of Asp, Arg, Gln, His, Lys, Ile, Leu, Phe, and Tyr increased temporally with gestation in pregnant heifers and were higher at day 19 of pregnancy than day 16 of the estrous cycle in non-pregnant heifers (Forde et al., 2014). These findings are in agreeance with other studies in cattle and livestock species (Gao et al., 2009; Crouse et al., 2019). In sheep, a significant increase in the total amino acid content has been recorded in ULF

of ewes between days 10 and 16 of pregnancy (Gao et al., 2009). A recent study reported a pregnancy-dependent increase of essential amino acids at day 15 and 18 post-insemination in ULF of beef heifers (Groebner et al., 2011). This accumulation of luminal amino acids in the uterus is occuring around the time of maternal recognition of pregnancy (Groebner et al., 2011; Forde at al., 2014).

Post-Attachment

While characterizations of amino acids in ULF have been performed during the estrous cycle (Hugentobler et al., 2007) and during the pre-attachment period of gestation (Forde et al., 2014), few studies have investigated further into early gestation or maternal plane of nutrition's on histotrophic components. Important to note, during the preimplantation period, the embryo is free floating within the uterus and entirely dependent on the histotroph for nutrients (Bazer 1975). Through 50 days of gestation, 17 amino acids were influenced by day of gestation in at least one of the utero-placental fluids, histotrophic, allantoic, and amniotic, of beef heifers fed for no gain or to gain 0.45 kg per day (Crouse et al., 2019). Most amino acids tended to increase as pregnancy progresses to support the increased requirements of the developing conceptus (Crouse et al., 2019). Additionally, the AA Ala, Gln, His, Leu, Lys, Met, Orn, Pro, and Val in histotroph were higher post-attachment at day 34 or 50 than day 16. Plane of nutrition also impacted amino acid requirements, and heifers fed a restricted diet, balanced for maintenance requirements but no gain, reported higher mean concentrations of Asn in histotroph and lower concentrations of Gly, Ser, and Thr in blood serum (Crouse et al., 2019). Geppert and others (2017a) demonstrated that different byproduct feeds supplemented to mature beef cows grazing corn stocks altered the circulating concentrations of the amino acids Phe and Leu without altering total plasma AA content. In this study non-pregnant beef cows were provided isonitrogenous diets at 150% of

maintenance requirements with either corn-gluten meal (moderate RUP), or soybean meal (low RUP) and measured blood plasma for amino acid concentrations. Total concentrations of amino acids did not differ but several were found to be differentially expressed between the two diets. Cows receiving corn-gluten meal also had larger ovulatory follicles and tended to have lower progesterone levels 7 days after estrus, suggesting protein degradability impacts systemic amino acid concentrations in addition to other reproductive parameters (Geppert et al., 2017a). The second study utilized only the corn-gluten meal supplement, supplied at either 125% or 150% of MP requirements for maintenance. Follicular characteristics were enhanced in the 150% group, which boasted larger ovulatory follicles, greater CL volume at day 7, and a higher antral follicle count than the lower protein group. Additionally, the proportion of branched-chain amino acids in plasma at ovulation was higher in cows receiving 150% of their MP requirements, which the authors proposed may be responsible for the increase in ovarian function, since estradiol levels were not different between treatments (Geppert et al., 2017b). Since concentrations of amino acids are higher in ULF than those found in plasma (Hugentobler et al., 2007), it is possible that these differences may have been exacerbated had ULF samples been taken.

In-vitro

Inclusion of supplemental amino acids in IVF culture media has shown benefits to embryo production in a number of species including cattle. Inclusion Gly and Ala in culture media improved the number of bovine embryos progressing to the blastocyst stage mimicking the secretion of these amino acids by the oviduct (Moore and Bondioli, 1993). Supplemental amino acids, specifically non-essential and Gln, also increase the cell number, blastocysts formation, and hatching rate *in-vitro* for sheep (Gardner et al., 1994). Similarly, other studies found benefits to including AA, and determined the highest percentage of blastocyst formation in

cattle embryos were cultured with a multitude of supplemental amino acids (Pinyopummintr and Bavister, 1996) and taurine (Liu and Foote, 1997).

Rieger and Guay (1988) evaluated bovine embryos at day 7 and determined that Gln is metabolized and serves as an energy substrate during early development, while another group reiterated glutamine's role as an energy source, and determined that it also acts as an osmolyte to protect embryos from osmotic stress during the first 3 days of development and stimulated the uptake of dietary non-essential amino acids, and its removal reduced blastocyst development at day 7 (Steeves and Gardner, 1999). Glycine and taurine were proposed as buffers transported in and out of the 8-cell embryo to maintain pH in hamsters (Bavister and McKiernan, 1993) in agreeance with reduced acidification of mouse embryos observed when cultured with Gln and a combination of dietary non-essential amino acids up to the 4-cell stage (Edwards et al., 1998). The essential amino acids, Leu and Arg, have been identified as prolific signaling molecules which increase the nutrients supplied to the embryo after the 8-cell stage, and increasing the rate of protein synthesis and the number of cells in the inner cell mass (ICM) (Van Winkle, 2001). The non-essential amino acids, Ala, Asn, Asp, Glu, Gly, Pro, Ser and the essential amino acid Gln, function primarily in cleavage of the zygote and early development (Land and Gardner, 1997).Whereas, essential amino acids are utilized more in the development of the inner cell mass between the 8-cell stage and implantation in mice, or attachment in bovine and other ruminant species (Lane and Gardner, 1997). These studies largely suggest that as long as AA concentrations in culture medium are relatively consistent with those reported for physiological levels in the uterus and oviduct, inhibitory effects are rarely seen. The benefits of individual addition or subtraction of amino acids varied between medium types, and further research is needed to elucidate these interactions.

SUMMARY

Proper nutritional management of replacement heifers remains a top priority for producers from the time they are weaned, through the completion of their first breeding season. Growth and maturation during this key period have long-term implications regarding longevity and ultimately whether heifers remain in the cow herd or are culled. Successful heifer development begins with the timely establishment of puberty. The impacts of nutritional status on puberty in beef cattle has been well documented, which has led producers to target pre-breeding BW's based on estimations of mature size (Patterson et al., 1992; Funston et al., 2012). Supplementing feedstuffs high in protein and energy to heifers consuming low-quality forages can increase growth and help ensure proper development prior to the breeding season. Promoting cyclicity prior to breeding allows for increased conception early in the breeding season which is paramount for longevity of heifers (Roberts et al., 2019). The levels and types of dietary nutrients have consistently been shown to influence ULF and impact reproductive performance (Elrod and Butler, 1993; Bridges et al., 2013, Crouse et al., 2019). Additionally, characterizations of the uterine environment across the estrus cycle and early pregnancy have been performed for pH and various amino acids. Maternal plane of nutrition has also been identified to play a significant role in determining the composition of histotroph (Crouse et al., 2019), however more research is needed to determine how individual feedstuffs, with varying nutrient composition and characteristics influence uterine amino acids. While characterizations of amino acids in ULF of cycling animals have been performed (Hugentober et al., 2007), further studies evaluating dietary influence of ULF before puberty are needed to evaluate changes in the uterine environment near the establishment of cyclicity. Elucidating the mechanisms through which diet impacts the uterine environment will increase the understanding of how these manipulations can impact reproductive efficiency during heifer development and early gestation.

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CHAPTER THREE

AMINO ACID CONCENTRATIONS IN UTERINE LUMINAL FLUID OF DEVELOPING BEEF HEIFERS SUPPLEMENTED WITH VARYING LEVELS OF CRUDE PROTEIN

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ABSTRACT

Replacement heifer development is one of the most critical components of productive beef operations. By identifying the ideal uterine environment in pre-breeding heifers, fertility and reproductive efficiency can be maximized. Our hypothesis was that different levels of protein supplementation would affect the rate of development and uterine environment of beef heifers. To test the effects of dietary supplementation on these outcomes, a randomized complete block design with repeated measures was implemented. Commercial Angus heifers (n=60) were blocked by BW into 4 weight classes. Within each weight class, three pens of 5 heifers each were randomly assigned to one of three treatment groups of supplemental protein (10% [**CON**], 20% [**P20**], and 40% [**P40**]). All heifers were allowed access to ad libitum grass hay. Bodyweight and BCS were collected every 2 weeks to monitor heifer development. Uterine fluid samples were collected monthly by flushing with sterile saline to evaluate components of the uterine environment. Relative uterine pH was calculated by subtracting saline pH from the uterine flush pH. Concentrations of non-normal AA's were log transformed. Separate mixed model ANOVAs and mean separation via PROC GLIMMIX (SAS 9.4, Cary, NC) were used to determine if protein supplementation treatments, time and their interactions influenced BW, BCS, or uterine environment. Bodyweight of the control group was significantly lower ($P<0.05$) than P20 or P40 groups from December to March. There was an interaction between treatment and time for relative uterine pH ($P=0.002$). In November, the P40 group exhibited less change in pH compared to the control group (0.33±0.1 vs. 0.74±0.1; $P<0.05$). Whereas in December the P40 (0.23±0.1) group showed more basic uterine environment compared to the P20 (-0.22±0.1) or control (-0.13±0.1) groups ($P<0.05$). Concentrations of AA's varied across development with most, highest at sample 7 ($P<0.0001$). Protein supplementation impacted concentrations of Trp

and Orn. In conclusion, higher protein supplementation does improve heifer growth and development, as well as alters the pH of the ULF from weaning to first breeding.

INTRODUCTION

Replacement heifer development is one of the most critical components of beef production with nearly 6 million entering the United States beef herd annually (National Agricultural Statistics Service; USDA NASS, 2019). The overarching goal of development strategies is to maximize the percentage of heifers that conceive early in the first breeding season and maintain a pregnancy. Replacement heifers should weigh 50 to 65% of their mature bodyweight (BW; Patterson et al., 1992; Funston et al., 2012) to maximize the number of cyclic females entering the breeding season, since puberty is primarily dictated by age and weight (Wiltbank et al., 1969; Nelsen et al., 1982; Oeydipe et al., 1982). Heifers conceiving earlier in their first breeding season have longer recovery time before the next breeding season resulting in improved overall longevity in the herd (Day and Nagueira, 2013). Replacement females must incorporate both genetic progress and phenotypic traits to maximize production. During development, a positive correlation between body condition score and reproductive success has been observed, provided that heifers do not become over conditioned (Utter et al., 1993). Heifers supplemented with additional protein experience elevated growth and fertility rates compared to counterparts developed without protein supplementation (Short and Bellows, 1971). Conversely, overfeeding protein can delay the onset of puberty, reduce pregnancy rates at first service, and cause an abnormally low uterine pH which may negatively impact fertility (Ferrell, 1982; Elrod and Butler, 1993; Lalman et al., 1993; Kane et al., 2004).

In addition to influencing growth and development, supplementation may also influence the uterine environment of replacement heifers (Elrod and Butler, 1993; Crouse et al., 2019). By identifying the ideal uterine environment in pre-breeding heifers, maximal productivity via increased fertility and reproductive efficiency can be obtained. Developing management practices that lead to an ideal uterine environment will allow producers to identify and more

efficiently raise successful replacement females. Establishment and maintenance of pregnancy is dependent on proper interaction of the uterine environment and embryo to support development until hemotropic exchange can be established (Forde et al., 2014). Deficiencies of energy substrates or key constituents within the uterine environment are a major contributor to embryonic mortality (Bridges et al., 2013). The largest percentage of embryonic mortality occurs prior to day 14 post insemination (Dunne et al., 2000). The uterine environment is comprised of secretions and transported molecules that can be quantified and described as the maternal contribution to uterine luminal fluid (ULF; Forde et al., 2014). Prior to maternal recognition, which occurs around day 16 of gestation in beef cattle, the embryo is free floating within the uterus and entirely dependent on ULF for nutrients (Bazer 1975).

Several recent studies have analyzed AA concentrations within ULF throughout the estrous cycle (Hugentobler et al., 2007), during pre-attachment (Groebner et al., 2011; Forde et al., 2014), and at the time of maternal recognition of pregnancy and early gestation (Crouse et al., 2019). Amino acids have been demonstrated to increase prior to embryonic attachment to provide adequate supply to the growing embryo in a temporal manner (Groebner et al., 2011; Forde et al., 2014), and were reduced in ULF of sub-fertile cattle at day 17 of pregnancy (Meier et al., 2014), indicating a relationship between AA concentrations and successful establishment of pregnancy. Despite the potential relationship between maternal nutrition and reproductive efficiency, few studies have analyzed maternal nutrition's impact on AA concentrations in ULF. Crouse and colleagues (2019) reported that animals receiving a restricted diet had altered utero-placental AA concentrations from day 16 to 50 of pregnancy, a time point of development in which the embryo is still largely dependent on ULF. Therefore, our hypothesis was that different levels of supplemental CP would impact the AA concentrations within the ULF of developing

heifers without altering reproductive development; specifically, pubertal onset and overall conception rates. The objectives of the current study were to quantify the AA concentrations and pH in ULF while meeting developmental goals and utilizing thresholds consistent with industry standards. All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee.

MATERIALS AND METHODS

All animal procedures were approved by the University of Tennessee Institutional Animal Care and Use Committee for this experiment.

Animals

Commercial Angus heifers (n=60) one month after weaning (266.2 ± 12.2kg) were utilized for completion of objectives and sample collection. All heifers had *ad-libitum* access to mixed grass hay baled on site (95.62% dry matter, 7.60% crude protein, and 1.97% fat), trace mineral supplement, and water. Heifers received a single experimental supplement for the entirety of the study, provided 4 times per week (Marston et al., 1995) to maintain adequate protein supply. Due to the interaction between energy and protein, an average daily gain of 0.68 kg was targeted to minimize the potential impacts of different intake levels. Heifers were weighed on two consecutive days, blocked by BW into 4 weight classes, and randomly assigned to one of three supplemental treatments: 1) control (CON) consisting of 100% cracked corn resulting in a 10% CP supplement (87.5 % dry matter, 8.2% CP, 3.77% fat, 60.8% RUP) 2) 20% CP supplement (P20) consisting of 25% corn and 75% dried distillers grains (DDGs; 91.6% dry matter, 23.6% CP, 7.4% fat, 75.1% RUP), and 3) 40% CP supplement (P40) consisting of 25% DDGs and 75% soybean meal (90.2% dry matter, 47.1% CP, 2.8% fat, 45.9% RUP). This resulted in balanced diets calculated to contain CP of approximately 11, 15, and 19% for CON, P20 and P40 heifers, respectively. Heifers were housed in three-acre pens with 5 heifers each

resulting in 4 pens per treatment. Body weight, BCS (1 = emaciated; 9 = obese; Wagner et al., 1988), and a jugular blood sample were collected every two weeks (**Figure 1**; adapted from Seay et al., 2020) to monitor growth and maintain adequate development of all heifers. Before final sample collection, all heifers underwent a 7-Day CO-Synch + CIDR estrus synchronization protocol (Figure 1; Geary et al., 1998). Heifers received an initial 100 µg intramuscular (i.m.) injection of GnRH (Cystorelin®, Boehrenger Ingleheim, Duluth, GA) and a controlled internal drug release (CIDR) device (1.38 g progesterone; Zoetis Animal Health, Florham Park, NJ) was inserted into the vagina for 7 days. Upon CIDR removal, heifers received an i.m. injection of 500 µg $PGF_{2\alpha}$ (Cloprostenol sodium; Synchsure™, Boehrenger Ingleheim, Duluth, GA). Fixed time artificial insemination was performed by a single technician beginning approximately 54 h after CIDR removal, with an injection of GnRH given simultaneously.

Uterine Fluid Collection

Uterine fluid was collected from each animal at monthly intervals for a total of 7 samples/animal (Figure 1) via a 14 Fr Foley catheter, with the first sample being collected at the initiation of supplementation. Samples were obtained by passing the catheter through the cervix and injecting 20 mL of sterile saline (Baxter Healthcare Coorporation, Deerfield, Il) in the uterine lumen. Saline was allowed to equilibrate throughout the uterus before being withdrawn into a syringe using massage/palpation of the uterus. Saline pH for each bag used was recorded prior to use for comparison to retrieved Saline + ULF, which was measured for pH immediately upon removal. Following pH measurements, uterine fluid samples were vortexed for 10 seconds and separated into two mL aliquots and stored at -80°C until analysis.

Progesterone and Puberty

Serum samples were collected via jugular venipuncture into 10-mL Serum-separating Vacutainer tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and allowed to clot at room temperature before being centrifuged at 5000 ×g for 20 minutes at 4^{o}C. Serum was separated from blood constituents and stored in 2 mL aliquots at -80℃. Blood samples were then analyzed to quantify progesterone concentration via radioimmunoassay (RIA) using the Double Antibody RIA Kit (MP Biomedicals, Irvine, CA) as described previously (Pohler et al., 2015) estimate the onset of puberty. Onset of puberty was determined when progesterone exceeded 1.0 ng/mL in two consecutive samples that coincided with a normal estrous cycle. Birthdates for each animal were used to in conjunction with progesterone data to determine age at puberty. Intrassay coefficient of variation was 5.22%. Seven heifers were removed from puberty analyses due to discrepancies involving their progesterone levels and an inability to determine the onset of puberty.

Amino Acid Analysis

Twenty five amino acids and intermediary metabolites; Alanine (**Ala**), Arginine (**Arg**), Aspartic Acid (**Asp**), Citrulline (**Cit**), Cystine (**CC**), Glutamine (**Gln**), Glycine (**Gly**), Histidine (**His**), Isoleucine (**Ile**), Leucine (**Leu**), Lysine (**Lys**), Methionine (**Met**), Phenylalanine (**Phe**), Proline (**Pro**), Serine (**Ser**), Threonine (**Thr**), Tryptophan (**Trp**), Tyrosine (**Tyr**), Valine (**Val**) and intermediates 1-Methyl-Histidine (**1MH**) , alpha-Aminoadipic acid (**AAAA**), alpha-Aminobutyric acid (**AABA**), delta-Hydroxylysine (**DHL**), Ornithine (**Orn**), and Sarcosine (**Sar**) were quantified within ULF via liquid chromatography-mass spectrometry (LC/MS). Prior to LC/MS, solid phase extraction was performed by the EZfaast Amino Acid Analysis Kit (Phenomenex, Torrance, CA). Following solid phase extraction, amino acids were derivatized in

solution for further separation from components that may interfere with quantification. After derivatization, samples were re-dissolved in aqueous mobile phase before analysis and quantification on a LC/MS system. Concentrations were determined by comparing the area under the curve for each peak to certified standards, included with the extraction kit (EZ:faast; sensitivity 1 nmol/mL).

Statistical Analyses

Initial pH of saline used for uterine flushes was subtracted from final pH after flushing to normalize values and is reported as pH change. This calculation reflects the overall change in saline pH after exposure to the uterine environment and mixing with ULF. To test the effects of dietary supplementation on heifer BW, BCS, incidence of puberty, ULF pH, and ULF AA profile, a randomized complete block design with repeated measures was utilized. Because BW, BCS and uterine fluid was collected from each animal, an individual heifer represents the sampling unit. However, the experimental unit was pen (n = 12; 4 per treatment). Separate mixed model ANOVAs and mean separation (LSD) via PROC GLIMMIX (SAS 9.4, Cary, NC) was used to determine if protein supplementation, time, or their interactions influenced BW, BCS, pH, incidence puberty, or concentrations of amino acids. Bodyweight, BCS, pH, and progesterone levels were included in previous models as covariates and removed if not significant ($P > 0.05$). Log transformation was performed when AA concentrations did not yield normally distributed residuals. Shapiro-Wilk's statistic and visual assessment of residual plots were assessed to determine normality ($W > 0.8$). Reported concentrations and standard error of the mean were back calculated to raw data and reported in µM. Amino Acid concentrations were converted to binary format with a value of one representing the AA was present, and 0 for not present. The frequency procedure was used to determine combinations of AA's and progesterone

occurring together in ULF during each sample. Progesterone values >1.0 ng/mL indicating a functional CL were converted to a value of 1 for frequency analyses.

RESULTS

There was a significant main effect of sample day ($P < .0001$) as a result of continued BW accumulation, in addition to a treatment by sample day interaction ($P < .0001$). Results were sliced in SAS by sample day to analyze treatment effects over time. The BW of CON heifers (291.1 ± 12.72kg) was lower ($P = 0.02$) than P20 or P40 heifers (306.7 ± 12.72kg vs. 314.6 ± 12.72kg, respectively) starting at Day 42, and continued through the final BW measurement at Day 163 for CON, P20 and P40 heifers (338.4 ± 12.72kg, 376.5 ± 12.72kg, 377.9 ± 12.72kg, respectively; **Figure 2**). Heifer development with regards to body energy reserves, BCS, was impacted by treatment at several sample days ($P < .0001$; **Figure 3**), and there was a significant sample day by treatment interaction ($P < .0001$). Similar to BW, results were sliced in SAS by sample day to analyze treatment effects at individual sample days. Average BCS for CON, P20, and P40 heifers was 5.4 ± 0.09, 5.5 ± 0.09, and 5.6 ± 0.09 respectively. However, BCS was more variable over time, and there was a significant main effect of sample day ($P < .0001$). Initial BCS for all groups was between 5.1-5.2 at study initiation. Heifers on P20 and P40 had higher BCS (5.3 ± 0.09 and 5.4 ± 0.09, respectively) than CON heifers (5.0 ± 0.09) at Day 28 ($P < 0.001$) and there were no differences at Day 42 ($P = 0.63$). During both the 4^{th} and 5^{th} measurements, from Day 56-70, P20 supplemented heifers were intermediary between CON and P40 groups (CON = 5.3± 0.09; P20 = 5.5± 0.09; P40 = 5.5± 0.09 for measurement 4 ($P = 0.05$); CON = 5.4± 0.09; P20 = 5.5± 0.09; P40 = 5.6± 0.09 for measurement 5; $P = 0.09$). Heifers provided P20 and P40 had higher BCS (5.8± 0.09) than CON heifers (5.5± 0.09) at Day 84 and maintained that difference through Day 98 ($P = .002$; $P= .0002$). Supplementation with P20

resulted in higher BCS (5.7± 0.09) than CON or P40 groups (5.3± 0.09; 5.5± 0.09, respectively) at the 8th observation at Day 112 (P=.0006). Heifers given P20 and P40 supplement had higher BCS (5.7± 0.09, 5.9± 0.09) than CON heifers (5.4± 0.09) at Day 126 (P<.0001). Heifers supplemented with P40 supplement had higher BCS (5.9± 0.09) than CON or P20 groups (5.5± 0.09; 5.6± 0.09, respectively) at the Day 140 BCS measurement (P<.0001). At the 11th and final sample collection on Day 163, the P40 and P20 groups were significantly higher (5.8± 0.09) than CON heifers (5.5± 0.09; P=.0001. All groups maintained a positive plane of nutrition and concluded the study in excellent condition. These differences in BCS, while significant, are largely negligible and all treatments concluded the study in adequate condition to enter a breeding season.

Age and Serum progesterone at puberty

Identification of potential cyclicity via determination of the initial luteal phase for each animal was signified by the presence of at least 1 ng/mL serum progesterone on 2 consecutive sample days, coinciding with normal luteal function. Although heifers receiving CON supplement weighed less than contemporaries through the majority of the study, treatment did not influence age at puberty (P > 0.98). Onset of puberty occurred at 340.9 ± 16.3, 344.3 ± 15.0, and 344.0 ± 14.4 days of age for CON, P20, and P40 supplemented groups, respectively, after approximately 100 days of supplementation on average. Serum progesterone levels at the time of detected puberty (**Figure 4**) was greater (P = 0.02) in P20 heifers (4.87 ± 0.59) compared with P40 and CON heifers (2.72±0.59; 3.12±0.59, respectively). The goal of this study was to maintain similar development across treatments which was achieved, indicated by similar age at puberty.

pH

There was an interaction between treatment and time for relative change in pH (P = 0.002). By calculating a relative change in pH, we were able to observe pH changes of the uterine environment without the confounding effects of different saline pH. Initial pH of flush saline ranged widely from 6.04-7.72, although most values were near the overall average, which was 6.56. Results for relative change in pH are displayed in **Figure 5**. At Day 28, the P40 group exhibited less relative change in pH compared to the CON group (0.33 ± 0.1 vs. 0.74 ± 0.1; P =0.01). Whereas at Day 56, the P40 heifers (0.23 ± 0.1) had a more basic uterine environment compared to the P20 (-0.22 ± 0.1) or control (-0.13 ± 0.1) groups (P = 0.005). Over the final four sampling periods (Day 84-163), all heifers exhibited a basic relative pH change but no differences were observed amongst treatments (P > 0.05). At the onset of puberty, CON heifers had a greater (P = 0.054; **Figure 6**) raw uterine pH (7.15 ± 0.1) when compared to P40 heifers (6.96 ± 0.09) and P20 heifers were not different from either group (7.02 ± 0.09). Relative uterine pH at the time of detectable puberty was not different among treatments (P = 0.18). Raw pH of the uterine environment at the time of detectable puberty differed between weight classes regardless of treatment. Heifers blocked by BW in weight class 1 (heaviest at the beginning of the study) had a decreased (P < 0.01) pH (6.89 ± 0.06) compared with heifers in weight class 3 (7.18 ± 0.06) or 4 (7.11 ± 0.06) and heifers in weight class 2 (6.96 ± 0.06) had an intermediate pH.

Amino Acids

Concentrations during Development

Of the 25 examined AA and intermediates, none had an interaction (P > 0.26) between sample month and protein supplement. Therefore, only main effects will be reported and

discussed. The CON and P20 (0.12± 0.03 µM and 0.07± 0.03 µM, respectively) heifers had greater (P = 0.03) Orn than P40 heifers (0.03± 0.03 µM). However, P40 heifers had greater (P = 0.03) Trp in ULF (0.18± 0.03 µM) than CON or P20 heifers (0.07± 0.03 and 0.07± 0.03 µM, respectively; **Figure 7**). Arginine concentrations increased (P < 0.001) during development, but concentrations were very low until Day 56. Concentrations of Arg were affected by BCS, Progesterone and pH and were greatest at Day 163 (17.96 ± 1.5 µM; **Figure 8A**). Serine was relatively nonexistent until Day 163 (27.83 ± 7.74 µM) and lowest at Day 112 (0.02 ± 7.74 µM); **Figure 8B**). Glycine, like Arg, appears to increase during development (P < 0.001) and was highest at Day 163 (47.08 ± 8.61 µM; **Figure 8C**). Aspartic Acid fluctuated throughout the study (P < 0.001) but peaked at Day 84 (108.75 ± 19.33 µM; **Figure 8D**). Glutamine was affected by progesterone and pH, decreased (P < 0.001) from Day 28-112 and was highest at Day 163 (9.73 ± 1.62 µM; **Figure 9A**). Only pH affected Ala, was present throughout but highest (P < 0.001) at Day 163 (5.84 ± 0.59 µM; **Figure 9B**). Body condition score and pH affected Val concentrations, which were highest (P < 0.001) at Day 163 (4.38 ± 0.62 µM; **Figure 9C**) but relatively stable (0.97-1.54 µM). Leucine was solely affected by pH and was highest at Day 163 (3.13 ± 0.38 µM; **Figure 9D**) but otherwise never exceeded 0.83 µM (P < 0.001). Proline ranged from 0.16-0.69 µM (P < 0.001) until Day 163 (2.76 ± 0.31 µM; **Figure 10A**). Lysine was affected by progesterone and pH, and was present in low levels until Day 163 (2.01 ± 0.28 µM; **Figure 10B**). Lysine concentrations fluctuated for Day 0-140, never exceeding 0.85 µM (P < 0.001). Isoleucine was also low and only affected by pH (**Figure 10C**). Methionine concentrations were influenced by pH and extremely low throughout but appeared to increase (P < 0.001) over the course of development peaking at Day 163 (0.48 ± 0.08 µM; **Figure 10D**). Phenylalanine concentrations were present in small amounts and fluctuated throughout (P <

0.001). Concentrations of Phe were affected by pH, and highest at Day 163 (0.99 ± 0.12 µM; **Figure 11A**). Tryptophan also was present in low amounts and affected by pH (**Figure 11B**). Threonine concentrations fluctuated throughout development ($P < 0.001$), peaking at Day 163 (1.82 ± 0.44 µM; **Figure 11C**). Tyrosine was affected by pH and only exceeded 1.0 µM at Day 163 (1.03 ± 0.12 µM; **Figure 11D**). Body weight affected CC which was only present in low levels until Day 56, peaked at Day 140 (26.5 ± 3.2 µM; **Figure 12A**), and ranged from 13.21-26.5 µM ($P < 0.001$). Citrulline was affected by BCS, progesterone and pH, present in low levels throughout and was highest at Day 163 (0.44 ± 0.08 µM; **Figure 12B**), and increased throughout development ($P < 0.001$). Histidine was affected by only pH and peaked ($P < 0.001$) at Day 28 (1.09 ± 0.28 µM; **Figure 12C**) but appeared in ranges from .77-1.09 µM. 1-Methyl-Histidine was not present for most of development (Day 0, 112; 0 µM; data not shown). Alpha-Aminoadipic acid interestingly, was highest at Day 112 (4.29 ± 0.31 µM) and lowest the following month at Day 140 (0.36 ± 0.31 µM; $P < 0.001$; data not shown). Alpha-Aminobutyric acid was affected by pH and concentrations were very low fluctuating throughout ($P < 0.001$), never exceeding 1 µM, highest at Day 84 (0.89 ± 0.11 µM; data not shown) and lowest at Day 112 (0.11 ± 0.11 µM). Alpha-Aminobutyric acid concentrations ranged from 0.73-0.89 µM. Delta-Hydroxylysine was very low and also affected by pH, highest at Day 163 (0.19 ± 0.03 µM; data not shown) and lowest at Day 112 (0.03 ± 0.03 µM) and only exceeded 0.1 µM at Day 28, 84, and 163 ($P < 0.001$). Ornithine was affected by progesterone and pH but concentrations were extremely low ($P < 0.001$) only exceeding 0.1 µM again at Day 28 and 56 (data not shown). Sarcosine was affected solely by pH, and levels were very low (data not shown).

Amino Acid Frequency Analyses

These results indicate the frequencies at which the 14 most prevalent AA appear with each other (**Table 1**) within each sampling period during supplementation. Due to the interconvertibility and multi-functionality of many AAs, frequency analyses was performed to determine the commonality of AAs being found together.

Day 0

Alanine was present in 59 of 60 heifers and always occurred with Gln, Ile, Leu, and Val, with frequencies of 100%. Arginine was present in 58 of 60 heifers and always occurred with Gln, Ile, Leu, and Val, with frequencies of 100%. Aspartic Acid was not as prevalent, present in only 12 heifers but was present 100 % of the time with Ala, Arg, Lys, Met, Orn, Val, CC, Ile, Leu, Phe, Gln, and Trp. Cystine was only present in 12 heifers and occurred most often with Asp for a frequency of 100%. Glutamine was common and present in 59 heifers and occurred most often with Ala, with a frequency of 100%. Glycine was present in 52 heifers and occurred most often with Gln, Ile, Leu, and Val, with frequencies of 100%. Isoleucine was present in all heifers and occurred most frequently with Gln, Leu, and Val, with frequencies of 100%. Leucine was present in all heifers and occurred most frequently with Gln, Ile, and Val, with frequencies of 100%. Lysine was present in 34 heifers and occurred with a multitude of AA's; most frequently with Ala, Arg, Gln, Leu, Ile, and Val, with frequencies of 100%. Methionine was present in in 28 heifers and occurred most frequently with Ala, Arg, Gln, Leu, Ile, Phe, and Val, with frequencies of 100%. Ornithine was detected in a subset of heifers, present in 21 heifers but occurred most frequently with several AA's; Ala, Arg, Gln, Ile, Leu, Lys, Met, Phe, Trp, and Val, with frequencies of 100%. Phenylalanine was present in 42 heifers and occurred most frequently with

Ala, Arg, Gln, Ile, Leu, and Val, with frequencies of 100%. Tryptophan was present in 26 heifers and occurred most frequently with Ala, Arg, Gln, Ile, Leu, Phe, and Val, with frequencies of 100%. Valine was present in all heifers and occurred most frequently with Gln, Ile, and Leu, with frequencies of 100%. Progesterone exceeded 1.0 ng/mL in 10 heifers, occurring most frequently with Arg, Gln, Gly, Ile, Leu, and Val with frequencies of 100%.

Day 28

Alanine, Arg, Gln, Ile, Leu, Val were present in all 60 heifers and occurred with each other with a frequency of 100%. Aspartic Acid was present in 12 samples and occurred most frequently with Ala, Arg, Gln, Gly, Ile, Leu, and Val, with a frequency of 100%. Cystine was rarely detected, present in 16 heifers and occurred most frequently with Ala, Arg, Gln, Ile, Leu, and Val, with frequencies of 100%. Glycine was present in 54 heifers and occurred most frequently with other highly occurring AA's; Ala, Arg, Gln, Ile, Leu, and Val, with frequencies of 100%. Lysine was present in 48 heifers and occurred most frequently with Ala, Arg, Gln, Ile, Leu, and Val, with frequencies of 100%. Methionine was detected with a multitude of AA's, present in 43 heifers and coincided with the presence of Ala, Arg, Gln, Ile, Leu, Lys, Orn, Trp and Val (100%). Ornithine was present in 47 heifers and coincided with the presence of other prevalent AA"s; Ala, Arg, Gln, Ile, Leu, Phe, and Val (100%). Phenylalanine was present in 49 heifers and coincided with the presence of Ala, Arg, Gln, Ile, Leu, and Val (100%). Tryptophan was present in 45 heifers and coincided with the presence of Ala, Arg, Gln, Ile, Leu, Phe, and Val (100%). Progesterone exceeded 1.0 ng/mL in only a few animals, 10 heifers, occurring most frequently with Ala, Arg, Gln, Ile, Leu, and Val (100%).

Day 56

Alanine, Arg, CC, Gln, Gly, Ile, Leu, Val were present in all 60 heifers (100%). Phenylalanine was present in 56 heifers, and coincided with the presence of Ala, Arg, CC, Gln, Gly, Ile, Leu, and Val (100%). Lysine and Methionine were present in 52 heifers and both coincided with the presence of Ala, Arg, CC, Gln, Gly, Ile, Leu, and Val (100%). Ornithine was present in 45 heifers and coincided with the presence of Ala, Arg, CC, Gln, Gly, Ile, Leu, and Val (100%). Tryptophan was present in 43 heifers and coincided with the presence of Ala, Arg, CC, Gln, Gly, Ile, Leu, and Val (100%). Aspartic Acid was rarely detected, only present in 12 heifers and coincided with the presence of Ala, Arg, CC, Gln, Gly, Ile, Leu, and Val (100%). Progesterone exceeded 1.0 ng/mL again in a small number of females, only10 heifers, occurring most frequently with Ala, Arg, CC, Gln, Gly, Ile, Leu, and Val (100%).

Day 84

Alanine, Leu, and Val were present in all 60 heifers (100%). Glycine and Gln were present in 59 heifers and coincided with the presence of Ala, Leu, and Val (100%). Isoleucine was present in 58 heifers and coincided with the presence of Ala, Gln, Leu, and Val (100%). Arginine was present in 54 heifers and coincided with the presence of Ala, Gln, Leu, and Val (100%). Phenylalanine was present in 51 heifers and coincided with the presence of Ala, Gln, Leu, and Val (100%). Lysine was present in 50 heifers and coincided with the presence of Ala, Gln, Leu, and Val (100%). Methionine was present in 49 heifers and coincided with the presence of Ala, Gln, Leu, and Val (100%). Tryptophan and Orn were present in just over half of all animals, 38 heifers and coincided with the presence of several AA's; Ala, Arg, Gln, Leu, Met, and Val (100%). Aspartic acid and CC were present in over half of all animals, 34 heifers and coincided with the presence of sveral AA's; Ala, Arg, Gln, Leu, and Val (100%). Progesterone

exceeded 1.0 ng/mL in under 25% of females, only 14 heifers, occurring most frequently with Ala, Gln, Ile, Leu, and Val (100%).

Day 112

Valine was the most prevalently detected AA, present in 58 heifers, and coincided with the presence of Ala with a frequency of 98.25% (56/57). Leucine and Ile were present in 55 heifers and coincided with the presence of Val (100%) and with each other (98.18%; 54/55). Glycine was present in 52 heifers and coincided with the presence of Val with a frequency of 98.08% (51/52). Glutamine was present in 50 heifers and coincided with the presence of rarely detected AA's; Orn (15/15), Met (30/30), and Lys (26/26) with frequencies of 100%. Cystine was present in 49 heiferss and coincided with the presence of Ile and Leu with frequencies of 91.84% (45/49). Phenylalanine was present in a majority of heifers, 47 samples and coincided with the presence of Gln with a frequency of 91.49% (43/47). Arginine was present in only 32 heifers and coincided with the presence of Ala, Gln, Leu, and Valine with frequencies of 100%. Tryptophan was present in only 14 heifers and coincided with the presence of Ala, Arg, Ile, Leu, Met, Phe, and Val in frequencies of 100%. Aspartic Acid was not present in any heifers at sample 5. Progesterone exceeded 1.0 ng/mL in a quarter of animals, 15 heifers, occurring most frequently with the sole AA Val (100%).

Day 140

Glutamine, Ile, and Val were present in all 60 heifers (100%). Glycine was only absent in one sample, present in 59 heifers and coincided with the presence of Gln, Ile and Val (100%). Alanine was present in 57 heifers and coincided with the presence of Gln, Ile and Val (100%). Methionine was highly prevalent, present in 55 heifers and coincided with the presence of Gln,

Ile and Val (100%). Arginine and Leu were both highly detected, present in 48 heifers and coincided with the presence of Ala, Gln, Ile, Val and each other (100%). Phenylalanine was present in 42 heifers and coincided with the presence of Ala, Gly, Gln, Ile, Leu, Lys (39/39) and Val (100%). Cystine was present in 35 heifers and coincided with the presence of Ala, Gln, Ile, Leu and Val (100%). Ornithine was only present in 33 heifers and coincided with the presence of Ala, Gln, Ile, and Val (100%). Tryptophan was present in just 26 heifers and coincided with the presence of Ala, Gln, Ile, and Val (100%). Aspartic Acid was present in 23 heifers and coincided with the presence of Ala, Gln, Gly, Met, Ile, Leu, and Val (100%). Progesterone exceeded 1.0 ng/mL in 33.33% of animals, 20 heifers, occurring most frequently with Gln, Ile, and Val (100%).

Day 163

Alanine, Gln, and Gly were present in all 59 heifers with a recorded sample (100%). Arginine was highly prevalent present in 58 heifers and coincided with the presence of Ala, Gln and Gly (100%). Phenylalanine was present in 57 heifers and coincided with the presence of Ala, Gln and Gly (100%). Methionine was present in 54 heifers and coincided with the presence of Ala, Arg, Gln and Gly and Phe (100%). Valine and Leu were present in 53 heifers and coincided with the presence of Ala, Arg, Gln, Gly and Phe (100%), but not each other. Isoleucine was present in 51 heifers and was also found with Ala, Gln, Gly and Phe (100%). Lysine was found in 50 heifers and coincided with the presence of several AA's; Ala, Arg, Gln, Gly, Leu, Met and Phe (100%). Tryptophan was prevalent, found in 44 heifers and coincided with the presence of several AA's; Ala, Arg, Gln, Gly, Leu, Met and Phe (100%). Ornithine was present in 2/3 of animals, 40 heifers and coincided with the presence of Ala, Arg, Gln, Gly, Leu, Lys, Met and Val (100%). Cystine was present in less than half of all females, only 25 samples and coincided

with the presence of Ala, Arg, Gln, and Gly (100%). Aspartic Acid was rarely detected present in 8 heifers and coincided with the presence of all other AA's for which frequency analyses were performed. Progesterone exceeded 1.0 ng/mL in a majority of animals, 53 heifers; AA's coinciding with progesterone were Ala, Gln, Gly, and Ile**.**

DISCUSSION

In the current experiment, heifers receiving CON supplement achieved puberty at approximately the same age as contemporaries, despite lower BW. This is in agreement with a previous study where heifers receiving a high starch intake experienced lower BW at puberty (Ciccioli et al., 2005). Age at which puberty occurs in developing replacement heifers in relation to the beginning of the breeding season has a large impact on the lifetime productivity of these animals. Day and Nogueira (2013) determined that heifers which conceived early in their first breeding season were more productive and economically efficient than females conceiving later in their first breeding season. This concept reinforced the importance of developing females that are pubertal and cycling before the breeding season (Perry, 2012). Previous research has demonstrated that heifers bred after at least one previous ovulation experience elevated conception rates compared with heifers bred at first estrus (Byerley et al., 1987). Traditionally producers have developed heifers targeting a BW threshold of 60-65% of mature BW (Engelken, 2008) in hopes of maximizing the number of cycling females at the beginning of the breeding season. Protein was demonstrated as a limiting factor for growth during development in CON supplemented groups, despite adequate energy for a maintained positive plane of nutrition. This is likely responsible for the decreased BW attained by CON supplemented groups during the latter portion of the trial, despite intake adjustment attempts to maintain similar rates of gain between treatments. When developing replacement heifers, BCS at the beginning of the breeding

season is critical, as reductions in fertility have been noted for females with a BCS < 5 (Selk et al., 1987) or > 6 (Utter et al., 1993). This BCS goal was attained by all heifers in the current experiment. This likely explains why our model did not influence development in terms of cylicity, as BCS was highly variable between treatments, but a positive plane of nutrition was maintained for all groups, despite P20 and P40 groups having higher BCS at the final sampling. Heifers receiving P20 supplement, containing 75% DDGS, had more progesterone than CON or P40 heifers at detection of puberty, similar to results published by Gunn et al., (2014), in which feeding DDGS increased circulating progesterone levels in addition to altering follicular growth and estradiol production. Increased RUP values in addition to elevated fat content in DDGS have both been hypothesized as the mechanism for these changes, however further research is necessary to improve supplementation strategies (Martin et al., 2007; Gunn et al., 2014).

In order for proper embryo development to take place, pH within the uterine environment must be closely regulated to maintain developmental competence (Ocon and Hansen, 2003). A majority of cellular processes are regulated by pH, and dysregulation can inhibit proper development if pH exceeds (Ocon and Hansen, 2003), or falls below (Edwards et al., 1998; Lane and Bavister, 1999), physiological intracellular pH of 7.4. High protein intake has been demonstrated to reduce fertility in gilts (Dyck, 1991), ewes (Wallace et al., 1994), as well as cattle (Jordan and Swanson, 1983; Elrod and Butler, 1993). Specifically in cattle, high levels of intake protein resulted in lower uterine pH and subsequent conception rates than controls (Elrod and Butler, 1993). This change in pH is unique to the uterus during the luteal phase, and does not manifest in any other bodily fluid measured in previous studies (Elrod and Butler, 1993; Hugentobler et al., 2007). Uterine pH returns to approximately 6.8 at estrus, to provide an isotonic environment to spermatozoa (Elrod and Butler, 1993). Heifers receiving the highest

amounts of supplemental protein (P40) in the current experiment did have a more acidic uterine environment than other treatments at the second sample. This was the only instance where higher levels of protein supplementation coincided with reduced uterine pH and may indicate and adaptive mechanism to long term supplementation. Results of the current study are similar to those observed by Grant et al. (2013) where high levels of N supplementation failed to illicit a decrease in uterine pH during the luteal phase, and actually caused a subtle increase in uterine pH. This reinforces the concept that heifers receiving higher levels of supplemental protein had adequate amounts for maintained growth and development, without the negative impacts on fertility historically associated with higher amounts of protein supplementation.

Overall, AA concentrations in the present study were much lower than those reported in previous studies using similar flush methods to analyze ULF (Hugentobler et al., 2007, 2010; Groebner et al., Forde et al., 2014; Crouse et al., 2019). Unlike previous studies, the kit utilized for AA extraction measured only free AAs and intermediary metabolites, while allowing larger proteins and other interfering compounds to pass through in a wah solution. These results suggest prior reports may have included a portion of degraded proteins broken down to their AA base. In previous studies, heifers were all cyclic prior to AA analysis. In the current study, nearly 40% of heifers did not achieve cyclicity until the final sample, 14 days post-AI, following estrus synchronization. These results indicate that AA's may increase in prevalence after puberty and their transporters may not become active until cyclicity has been established. Of the 25 examined AA's and intermediates, 18 were highest at sample 7. Previous studies in cycling heifers have shown Gly to be the most prevalent AA in ULF (Hugentobler et al., 2007; Forde et al., 2014; Crouse et al., 2019). In the current study Gly was found in the highest concentration only at sample 7, when most heifers had achieved cyclicity. The AA's Ala, Arg, CC,Gln, Gly, Ser, Val,

and the intermediary metabolite alpha-aminoadipic acid were found in the highest concentrations in the current study, in agreement with previous studies (Hugentobler et al., 2007; Groebner et al., 2011; Crouse et al., 2019), suggesting these AA's may be utilized most readily by the embryo upon arrival in the uterine environment. Amino acids were detected consistently across samples 6 of the 7 samples. Sample 5 was lowest concentration for 23 of the 25 examined AA's, with the exception of alpha-aminoadipic acid and CC, although further investigation is necessary to elucidate the cause of these low concentrations. Protein supplementation affected the concentrations of 2 AA's, 1 of which had the highest values for P40 supplemented heifers (Trp). These observations are in agreeance with a study (Geppert et al., 2017), that found heifers supplemented different protein sources had different levels of Phe and Leu in serum. Given that AA's in ULF are higher in ULF than blood (Hugentobler et al., 2007), it is logical that AA differences were detected in the current study. The combination of different feedstuffs and increasing protein levels in the supplements may be the cause of the differences in our dataset.

In summary, protein supplementation impacted weight at puberty, but did not affect age at puberty. Variation in final weights was not intended however these results are useful in signifying the importance of overall nutritional plane on successful heifer development, although it is likely increased supplementation to CON heifers to compensate decreased growth elevated overall feed costs. Further research is necessary to determine if lower weights at puberty, achieved by a slower ADG, can be achieved using supplements similar to P20 or P40 without compromising reproductive success. Supplement type did affect the amount of progesterone at the attainment of puberty and altered the concentrations of Trp and Orn in ULF of developing heifers, supporting the hypothesis that supplements with different characteristics, in this case CP, are transported post-digestion in varying amounts into the uterine environment. Further studies

are needed to determine if Trp and Orn are consistently affected by different feed types and how this transport may affect fertility.

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APPENDIX

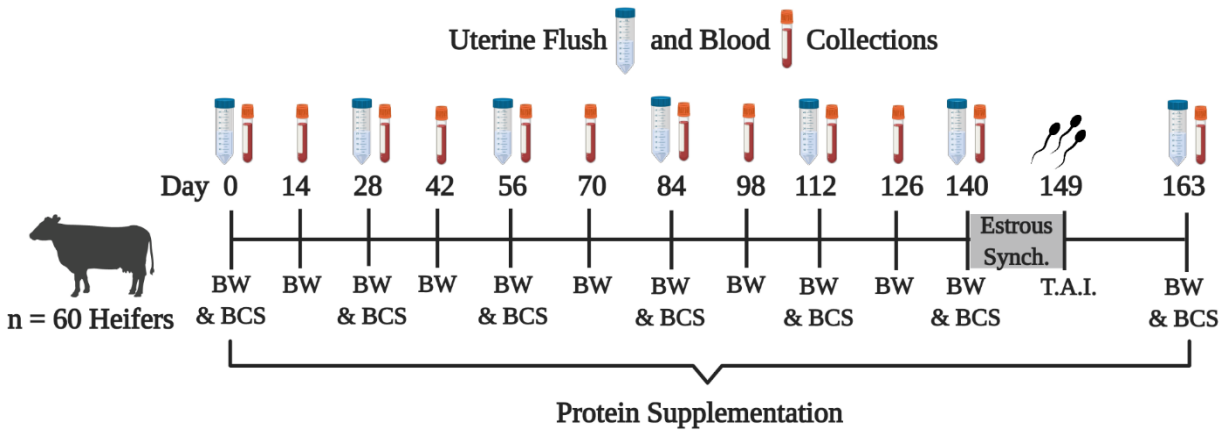


Figure 1. Project timeline with sample collection overview and supplementation timeline (adapted from Seay et al., 2020).

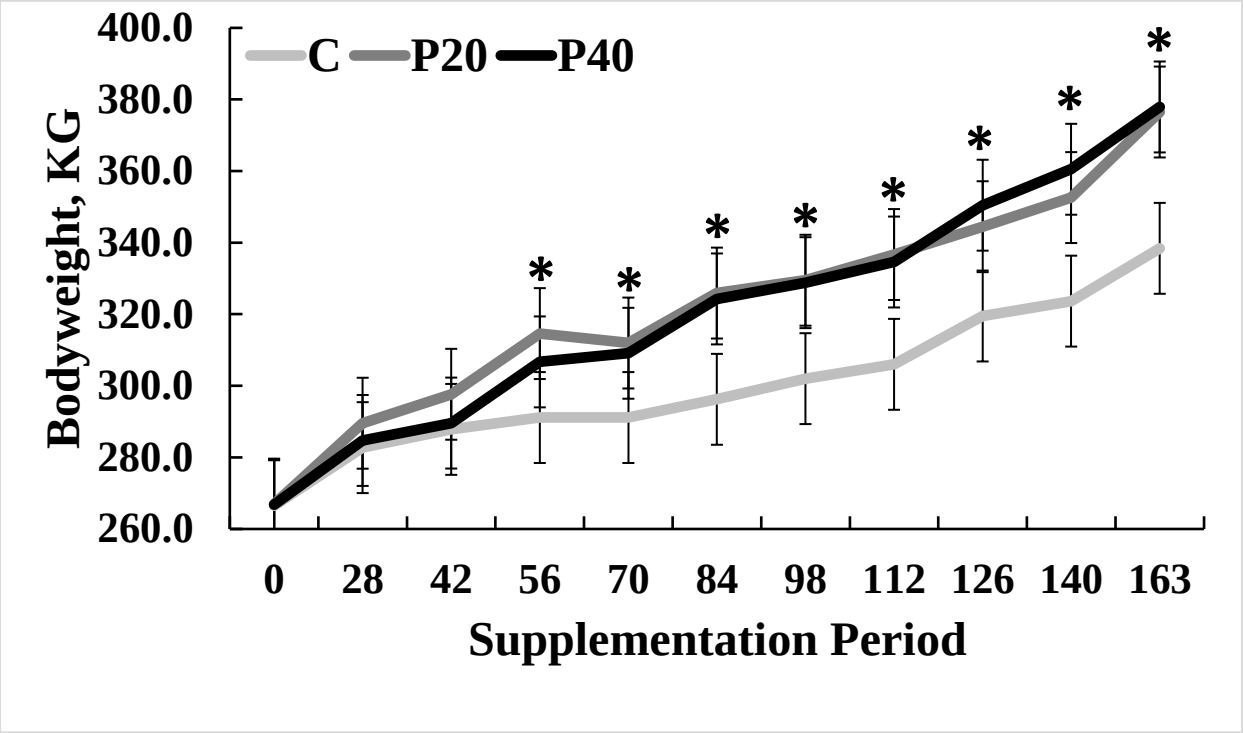


Figure 2. Average bodyweight of each treatment (C, P20, P40), kg, at each sample collection day (0-163) over the duration of supplementation. Samples with * symbol indicates significant differences between treatments (P20, P40 > CON; *P*<0.05).

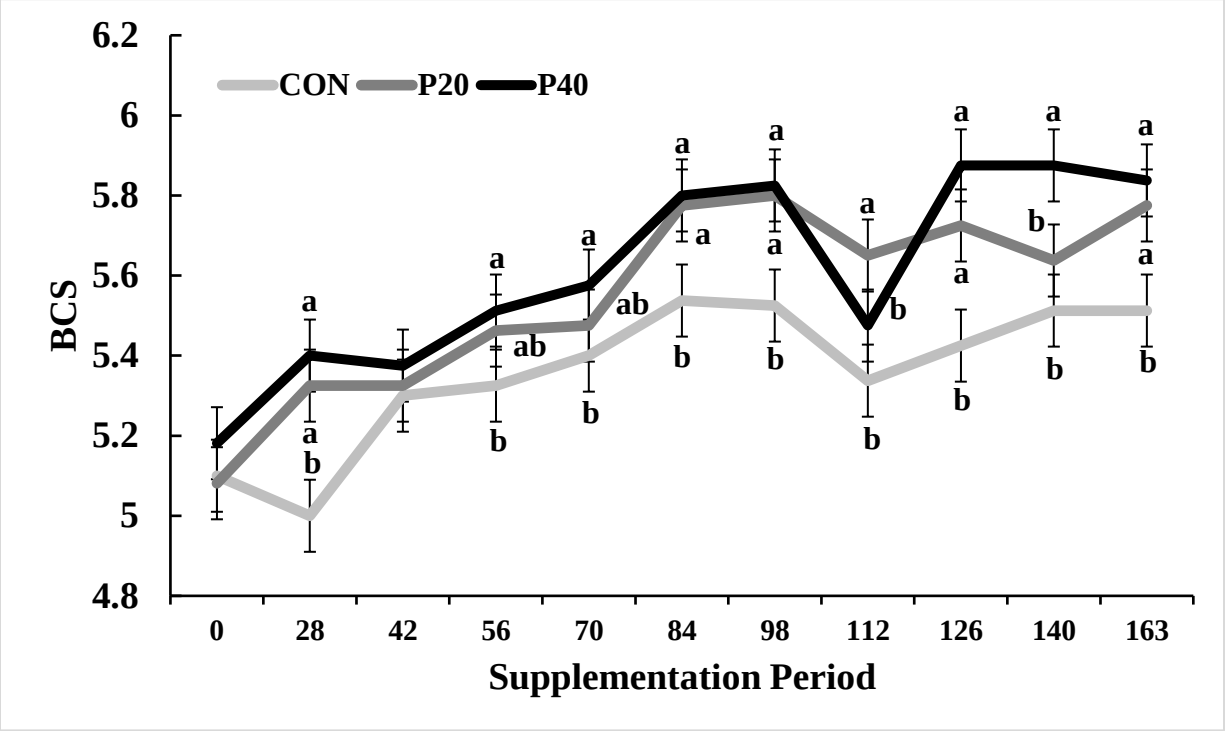


Figure 3. Changes in BCS (0-9) by treatment (Con, P20, P40) at each observation over the duration of supplementation. Means with different letter groupings indicate significant differences between treatments (*P*<0.05). There were no differences in BCS at study initiation, and although differences fluctuate throughout, all treatments maintained a positive plane of nutrition, accumulating BCS as supplementation progressed.

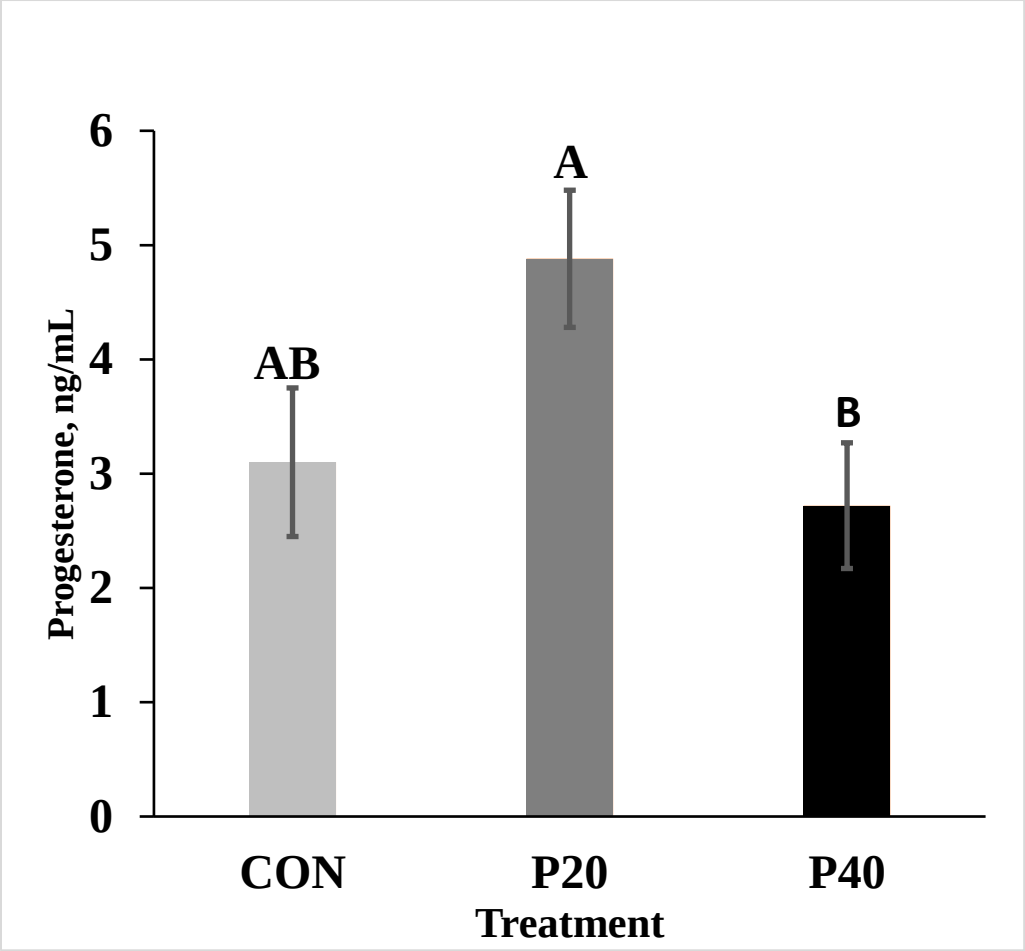


Figure 4. Average progesterone content in blood serum, ng/ml, by treatment (Control, P20, P40), at the detection of pubertal onset. Consecutive measurements of 1.0 ng/mL were used to determine the onset of cyclicity for each heifer. Means with different letter groupings indicate significant differences between treatments (*P*=0.02)

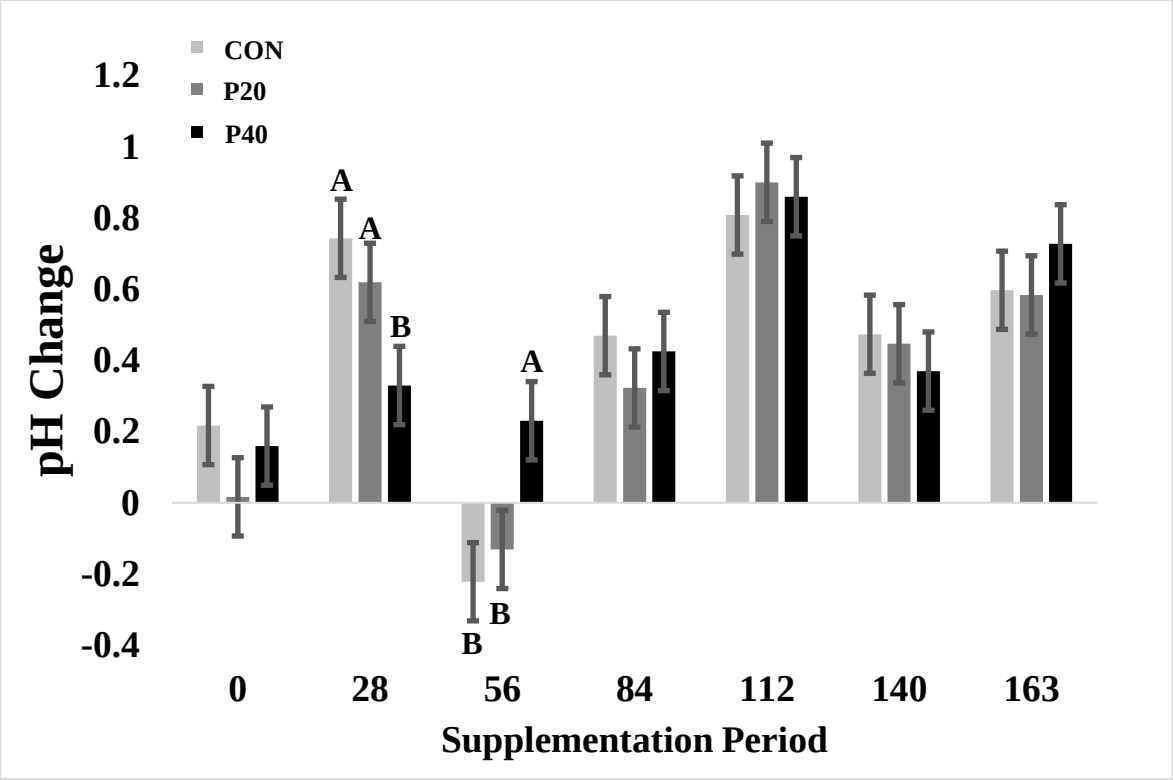


Figure 5. Average change in flush pH between treatments at each sample collection over the supplementation period. Calculated by subtracting initial pH of flush saline from final uterine flush pH to quantify the uterine environments impact on pH, as well to correct for variability in saline pH. Means with different letter groupings indicate significant differences between treatments (*P*<0.05).

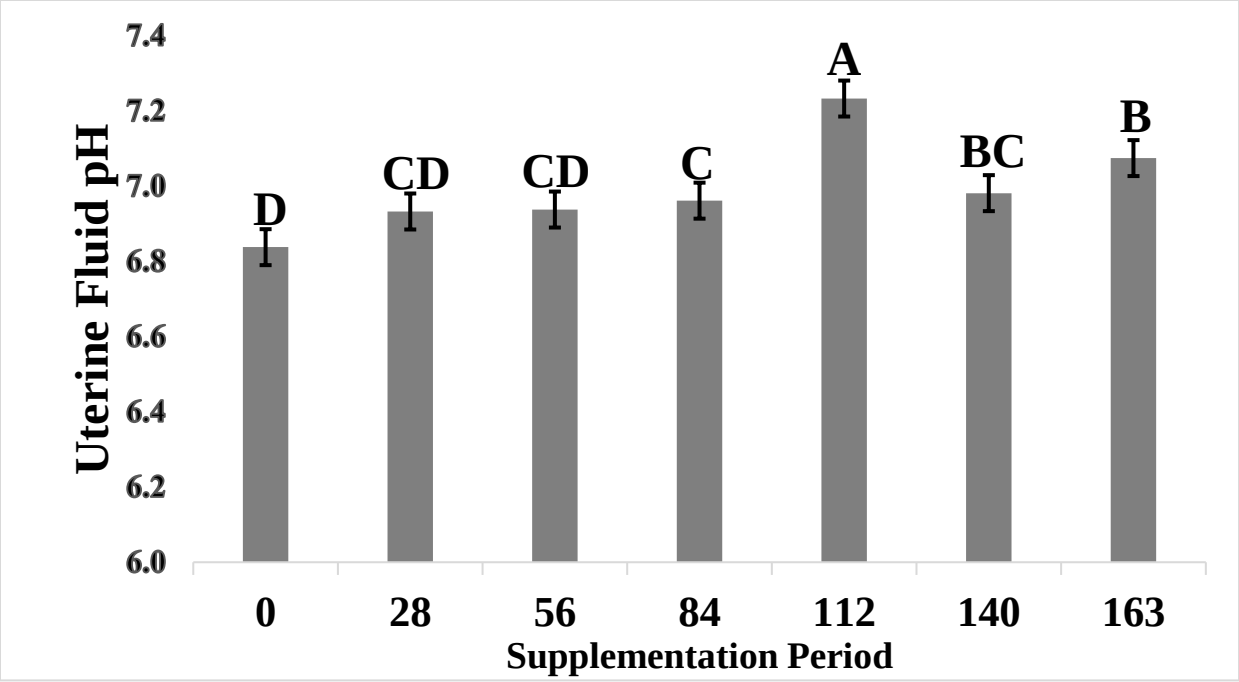
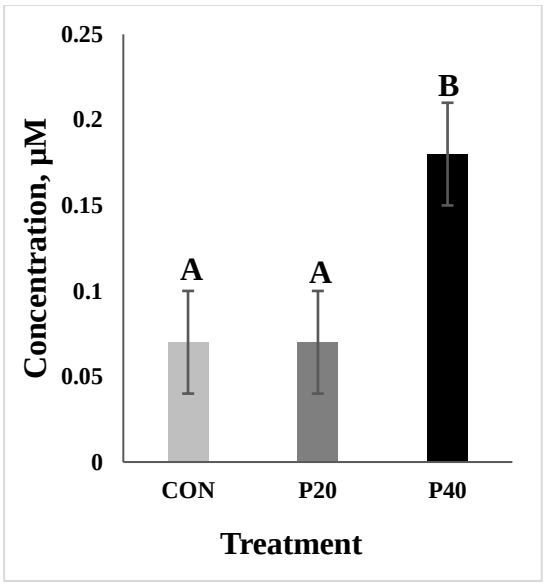


Figure 6. Average uncorrected (raw) uterine pH of all heifers for each sample collection over the course of the supplementation period. No differences were detected between treatments (*P* = 0.94) although uterine pH differed between sample days during the supplementation period (*P* < .0001). Means with different letter groupings indicate significant differences in pH between samplings (*P*<0.05).

A.



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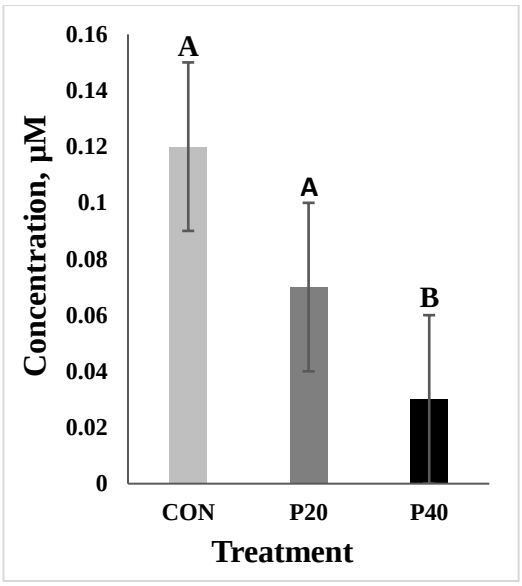

Figure 7. Impacts of protein supplement on amino acid concentrations in uterine luminal fluid of developing heifers. A) Concentrations of tryptophan (Trp) and B) concentrations of ornithine (Orn). Means with different letter groupings indicate significant differences between treatments (*P*<0.05).

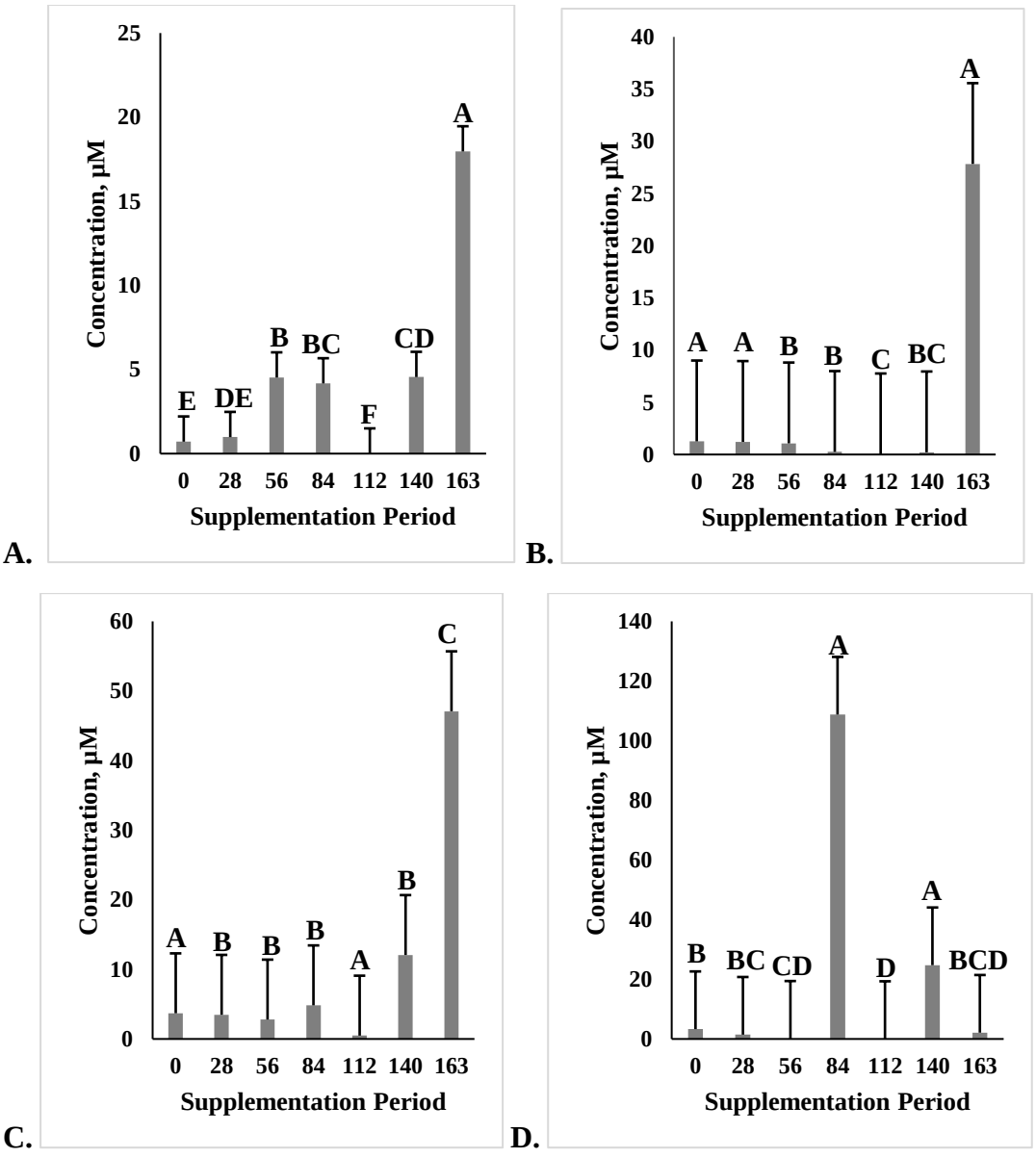

Figure 8. Impacts of sample date on amino acid concentrations in uterine luminal fluid of developing heifers over the supplementation period. A) Concentrations of Arginine (Arg) and B) concentrations of Serine (Ser). C) Concentrations of Glycine (Gly). D) Concentrations of Aspartic Acid (Asp). Means with different letter groupings indicate significant differences between samples (P<0.05).

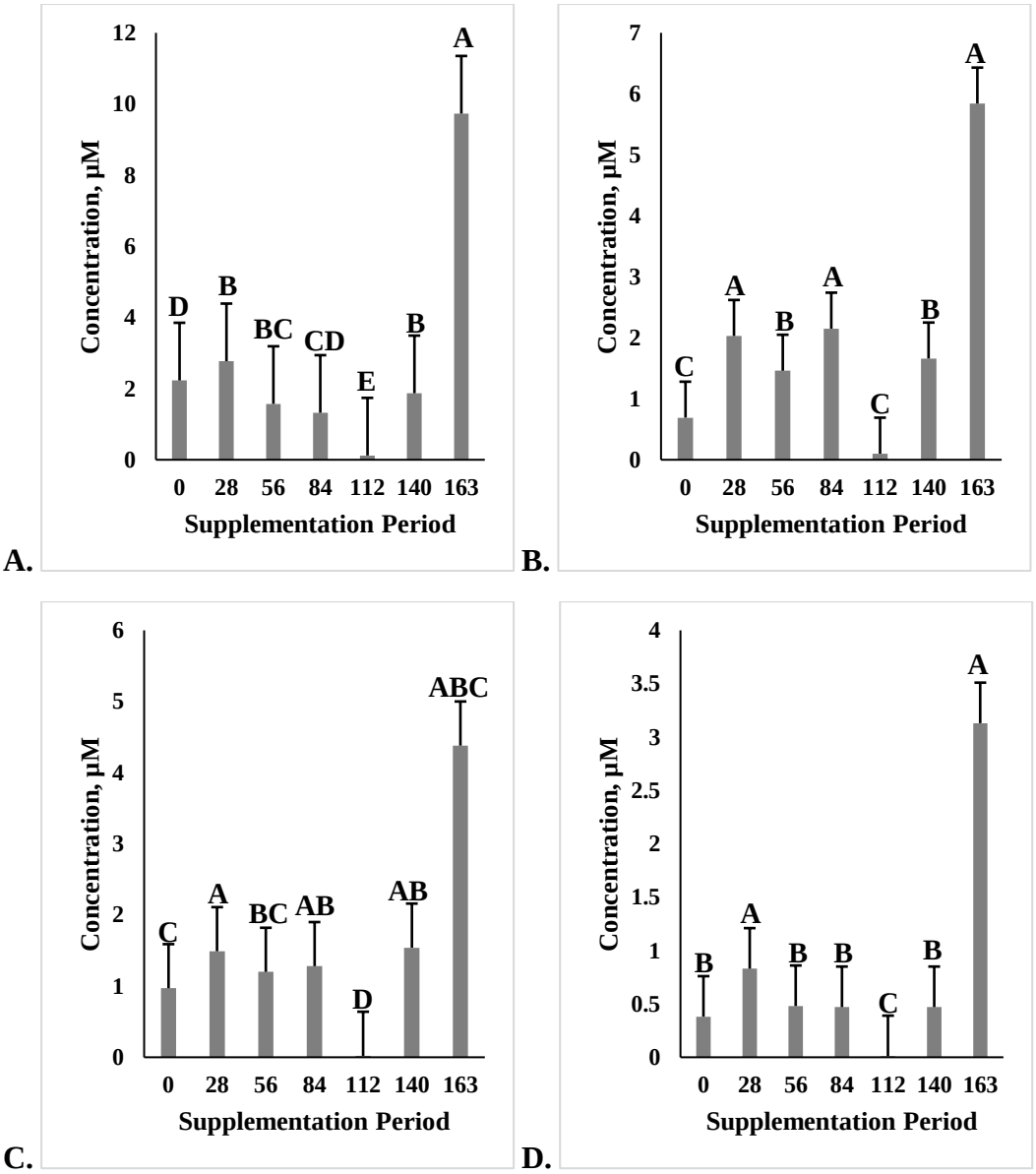

Figure 9. Impacts of sample date on amino acid concentrations in uterine luminal fluid of developing heifers over the supplementation period. A) Concentrations of Glutamine (Gln) and B) concentrations of Alanine (Ala). C) Concentrations of Valine (Val). D) Concentrations of Leucine (Leu). Means with different letter groupings indicate significant differences between samples (P<0.05).

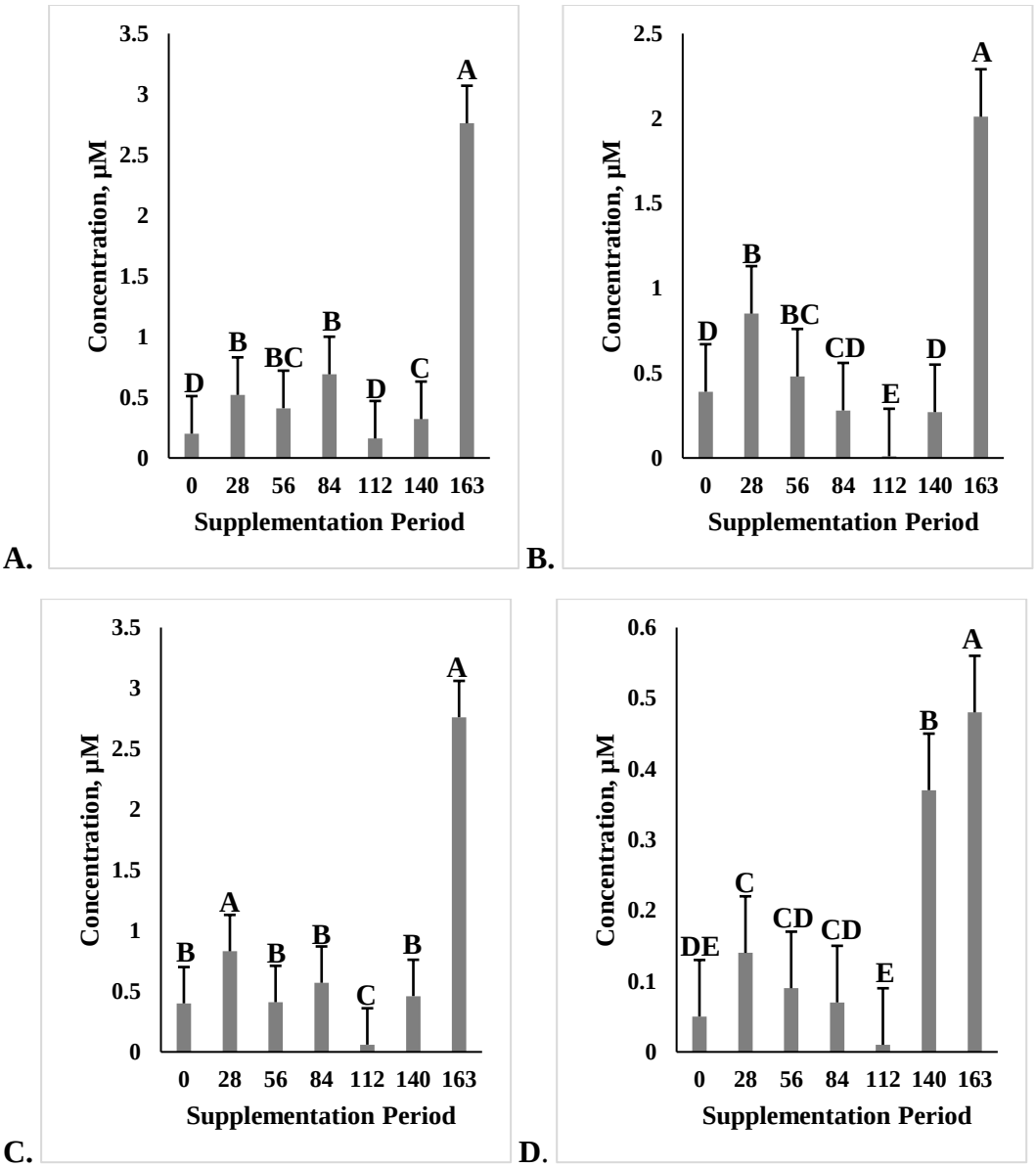

Figure 10. Impacts of sample date on amino acid concentrations in uterine luminal fluid of developing heifers over the supplementation period. A) Concentrations of Proline (Pro) and B) concentrations of Lysine (Lys). C) Concentrations of Isoleucine (Ile). D) Concentrations of Methionine (Met). Means with different letter groupings indicate significant differences between samples (P<0.05).

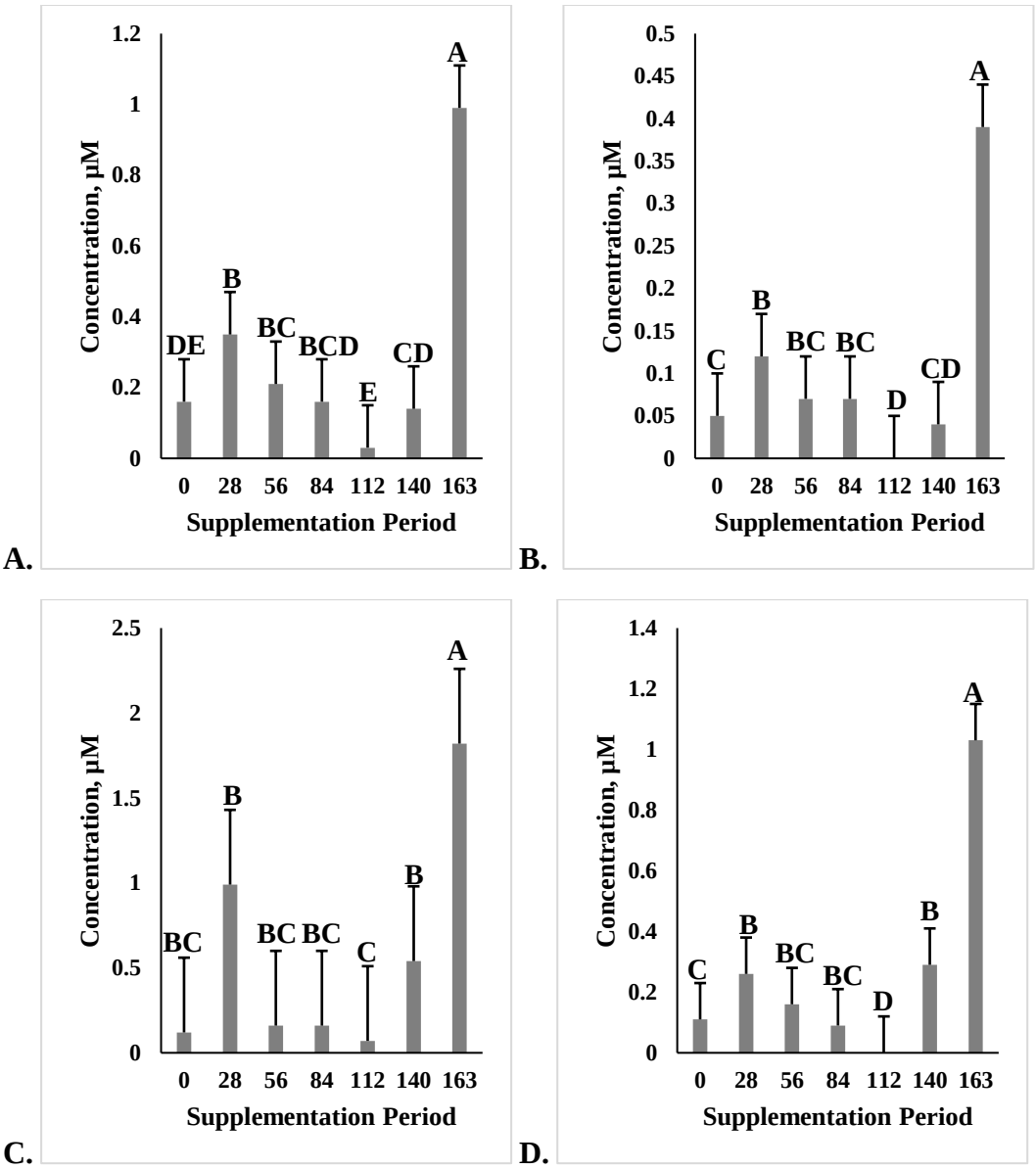

Figure 11. Impacts of sample date on amino acid concentrations in uterine luminal fluid of developing heifers over the supplementation period. A) Concentrations of Phenylalanine (Phe). B) Concentrations of Tryptophan (Trp. C) Concentrations of Threonine (Thr). D) Concentrations of Tyrosine (Tyr). Means with different letter groupings indicate significant differences between samples (P<0.05).

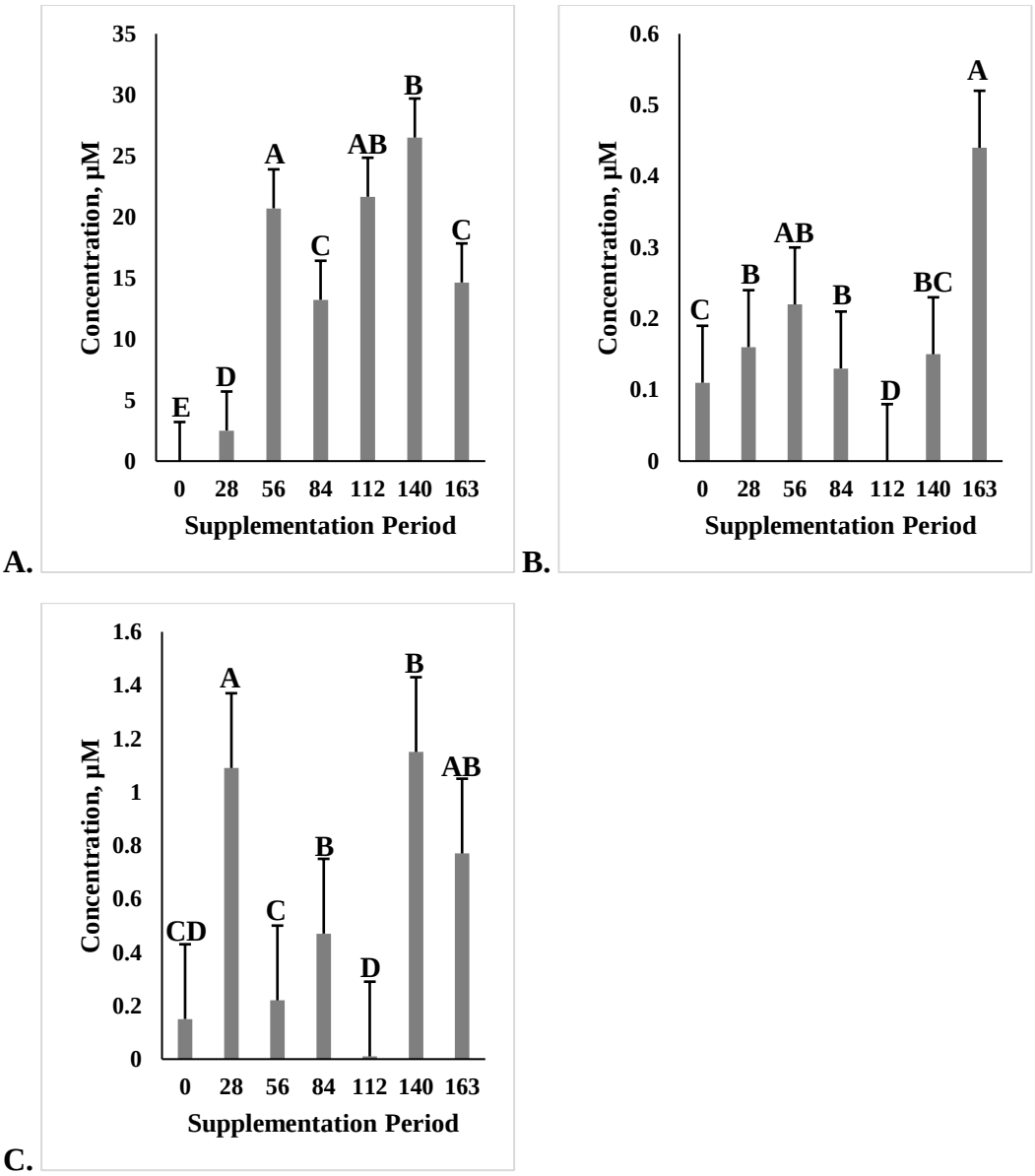

Figure 12. Impacts of sample date on amino acid concentrations in uterine luminal fluid of developing heifers over the supplementation period. A) Concentrations of Cystine (CC) and B) concentrations of Citrulline (Cit) and C) concentrations of Histidine (His). Means with different letter groupings indicate significant differences between samples (P<0.05).

TABLE 1. FREQUENCIES OF AA'S OCCURRING IN THE PRESENCE OF 12 OTHER PREVALENT AA'S AND PROGESTERONE

Present	Present	Day 0 [60][5]		Day 28 [60]		Day 56 [60]		Day 84 [60]		Day 112 [60]		Day 140 [60]		Day 163 [59]	
		Obs[1]	Freq[2] (%)	Obs	freq (%)	Obs	freq (%)	Obs	freq (%)	Obs	freq (%)	Obs	freq (%)	Obs	freq (%)
Arg	Ala	57[3](58)[4]	98.28	60(60)	100	60(60)	100	54(54)	100	32(32)	100	48(48)	100	58(58)	100
	Asp	12(58)	20.69	12(60)	20	12(60)	20	34(54)	62.96	0(32)	0	23(48)	47.92	8(58)	13.79
	Gly	51(58)	87.93	54(60)	90	60(60)	100	53(54)	98.15	27(32)	84.38	47(48)	97.92	58(58)	100
	Lys	34(58)	58.62	48(60)	80	52(60)	86.67	48(54)	88.89	26(32)	81.25	39(48)	81.25	50(58)	86.21
	Met	28(58)	48.28	43(60)	71.67	52(60)	86.67	47(54)	87.04	27(32)	84.38	43(48)	89.58	54(58)	93.1
	Val	58(58)	100	60(60)	100	60(60)	100	54(54)	100	32(32)	100	48(48)	100	52(58)	89.66
	CC	0(58)	0	16(60)	26.67	60(60)	100	28(54)	51.85	23(32)	71.88	35(48)	72.92	25(58)	43.1
	Ile	58(58)	100	60(60)	100	60(60)	100	54(54)	100	31(32)	96.88	48(48)	100	50(58)	86.21
	Leu	58(58)	100	60(60)	100	60(60)	100	54(54)	100	32(32)	100	48(48)	100	53(58)	91.38
	Phe	42(58)	72.41	49(60)	81.67	56(60)	93.33	50(54)	92.59	29(32)	90.63	42(48)	87.5	56(58)	96.55
	Gln	58(58)	100	60(60)	100	60(60)	100	54(54)	100	32(32)	100	48(48)	100	58(58)	100
	Trp	26(58)	44.83	45(60)	75	43(60)	71.67	38(54)	70.37	14(32)	43.75	19(48)	39.58	44(58)	75.86
	P4	10(58)	17.24	19(60)	31.67	18(60)	30	13(54)	24.07	12(32)	37.5	13(48)	27.08	52(58)	89.66
Ala	Asp	12(59)	20.34	12(60)	20	12(60)	20	34(60)	56.67	0(57)	0	23(57)	40.35	8(59)	13.56
	Gly	51(59)	86.44	54(60)	90	60(60)	100	59(60)	98.33	49(57)	85.96	56(57)	98.25	59(59)	100
	Lys	34(59)	57.63	48(60)	80	52(60)	86.67	50(60)	83.33	26(57)	45.61	39(57)	68.42	50(59)	84.75
	Met	28(59)	47.46	43(60)	71.67	52(60)	86.67	49(60)	81.67	30(57)	52.63	52(57)	91.23	54(59)	91.53
	Val	59(59)	100	60(60)	100	60(60)	100	60(60)	100	56(57)	98.25	57(57)	100	53(59)	89.83
	CC	0(59)	0	16(60)	26.67	60(60)	100	34(60)	56.67	46(57)	80.7	35(57)	61.4	25(59)	42.37
	Ile	59(59)	100	60(60)	100	60(60)	100	58(60)	96.67	53(57)	92.98	57(57)	100	51(59)	86.44
	Leu	59(59)	100	60(60)	100	60(60)	100	60(60)	100	53(57)	92.98	48(57)	84.21	53(59)	89.83
	Phe	42(59)	71.19	49(60)	81.67	56(60)	93.33	51(60)	85	46(57)	80.7	42(57)	73.68	57(59)	96.61

Table 1 Continued.															
	Gln	59(59)	100	60(60)	100	60(60)	100	59(60)	98.33	50(57)	87.72	57(57)	100	59(59)	100
	Trp	26(59)	44.07	45(60)	75	43(60)	71.67	38(60)	63.33	14(57)	24.56	26(57)	45.61	44(59)	74.58
	P4	9(59)	15.25	19(60)	31.67	18(60)	30	14(60)	23.33	19(57)	33.33	14(57)	24.56	53(59)	89.83
Asp	Gly	8(12)	66.67	11(12)	91.67	12(12)	100	33(34)	97.06	0(0)	0	23(23)	100	8(8)	100
	Lys	12(12)	100	9(12)	75	11(12)	91.67	31(34)	91.18	0(0)	0	21(23)	91.3	8(8)	100
	Met	12(12)	100	5(12)	41.67	11(12)	91.67	29(34)	85.29	0(0)	0	23(23)	91.3	8(8)	100
	Val	12(12)	100	12(12)	100	12(12)	100	34(34)	100	0(0)	0	23(23)	100	8(8)	100
	CC	12(12)	100	7(12)	58.33	12(12)	100	10(34)	29.41	0(0)	0	10(23)	43.48	8(8)	100
	Ile	12(12)	100	12(12)	100	12(12)	100	34(34)	100	0(0)	0	23(23)	100	8(8)	100
	Leu	12(12)	100	12(12)	100	12(12)	100	34(34)	100	0(0)	0	23(23)	100	8(8)	100
	Phe	12(12)	100	8(12)	66.67	11(12)	91.67	33(34)	97.06	0(0)	0	22(23)	95.65	8(8)	100
	Gln	12(12)	100	12(12)	100	12(12)	100	34(34)	100	0(0)	0	23(23)	100	8(8)	100
	Trp	12(12)	100	7(12)	58.33	5(12)	41.67	24(34)	70.59	0(0)	0	8(23)	34.78	36(51)	70.59
	P4	1(12)	8.33	6(12)	50	1(12)	8.33	11(34)	32.35	0(0)	0	7(23)	30.43	7(8)	87.5
Gly	Lys	29(52)	55.77	43(54)	79.63	52(60)	86.67	49(59)	83.05	22(52)	42.31	39(59)	66.1	50(59)	84.75
	Met	23(52)	44.23	38(54)	70.37	52(60)	86.67	48(59)	81.36	26(52)	50	55(59)	93.22	54(59)	91.53
	Val	52(52)	100	54(54)	100	60(60)	100	59(59)	100	51(52)	98.08	59(59)	100	53(59)	89.83
	CC	0(52)	0	15(54)	27.78	60(60)	100	34(59)	57.63	46(52)	88.46	34(59)	57.63	25(59)	42.37
	Ile	52(52)	100	54(54)	100	60(60)	100	57(59)	96.61	51(52)	98.08	59(59)	100	51(59)	86.44
	Leu	52(52)	100	54(54)	100	60(60)	100	59(59)	100	50(52)	96.15	47(59)	79.66	53(59)	89.83
	Phe	35(52)	67.31	44(54)	81.48	56(60)	93.33	50(59)	84.75	43(52)	82.69	42(59)	71.19	57(59)	96.61
	Gln	52(52)	100	54(54)	100	60(60)	100	58(59)	98.31	43(52)	82.69	59(59)	100	59(59)	100
	Trp	21(52)	40.38	40(54)	74.07	43(60)	71.67	37(59)	62.71	13(52)	25	26(59)	44.07	44(59)	74.58
	P4	10(52)	19.23	17(54)	31.48	18(60)	30	13(59)	22.03	17(52)	32.69	14(59)	23.73	53(59)	89.83
Lys	Met	26(34)	76.47	43(48)	89.58	51(52)	98.08	48(50)	96	24(26)	92.31	39(39)	100	50(50)	100
	Val	34(34)	100	48(48)	100	52(52)	100	50(50)	100	26(26)	100	39(39)	100	49(50)	98

Table 1 Continued.															
	CC	0(34)	0	14(48)	29.17	52(52)	100	25(50)	50	19(26)	73.08	27(39)	69.23	20(50)	40
	Ile	34(34)	100	48(48)	100	52(52)	100	50(50)	100	26(26)	100	39(39)	100	48(50)	96
	Leu	34(34)	100	48(48)	100	52(52)	100	50(50)	100	26(26)	100	39(39)	100	50(50)	100
	Phe	33(34)	97.06	47(48)	97.92	51(52)	98.08	49(50)	98	26(26)	100	39(39)	100	50(50)	100
	Gln	34(34)	100	48(48)	100	52(52)	100	50(50)	100	26(26)	100	39(39)	100	50(50)	100
	Trp	25(34)	73.53	45(48)	93.75	42(52)	80.77	38(50)	76	13(26)	50	19(39)	48.72	44(50)	88
	P4	5(34)	14.71	16(48)	33.33	15(52)	28.85	12(50)	24	9(26)	34.62	11(39)	28.21	45(50)	90
Met	Val	28(28)	100	43(43)	100	52(52)	100	49(49)	100	30(30)	100	55(55)	100	52(54)	96.3
	CC	0(28)	0	12(43)	27.91	52(52)	100	24(49)	48.98	23(30)	76.67	31(55)	56.36	21(54)	38.89
	Ile	28(28)	100	43(43)	100	52(52)	100	49(49)	100	30(30)	100	55(55)	100	50(54)	92.59
	Leu	28(28)	100	43(43)	100	52(52)	100	49(49)	100	30(30)	100	43(55)	78.18	53(54)	98.15
	Phe	28(28)	100	43(43)	100	52(52)	100	47(49)	95.92	30(30)	100	41(55)	74.55	54(54)	100
	Gln	28(28)	100	43(43)	100	52(52)	100	49(49)	100	30(30)	100	55(55)	100	54(54)	100
	Trp	25(28)	89.29	43(43)	100	41(52)	78.85	38(49)	77.55	14(30)	46.67	26(55)	47.27	44(54)	81.48
	P4	4(28)	14.29	14(43)	32.56	15(52)	28.85	11(49)	22.45	12(30)	40	13(55)	23.64	48(54)	88.89
Val	CC	0(60)	0	16(60)	26.67	60(60)	100	34(60)	56.67	47(58)	81.03	35(60)	58.33	19(53)	35.85
	Ile	60(60)	100	60(60)	100	60(60)	100	58(60)	96.67	55(58)	94.83	60(60)	100	51(53)	96.23
	Leu	60(60)	100	60(60)	100	60(60)	100	60(60)	100	55(58)	94.83	48(60)	80	52(53)	98.11
	Phe	42(60)	70	49(60)	81.67	56(60)	93.33	51(60)	85	46(58)	79.31	42(60)	70	53(53)	100
	Gln	60(60)	100	60(60)	100	60(60)	100	59(60)	98.33	50(58)	86.21	60(60)	100	53(53)	100
	Trp	26(60)	43.33	45(60)	75	43(60)	71.67	38(60)	63.33	14(58)	24.14	26(60)	43.33	44(53)	83.02
	P4	10(60)	16.67	19(60)	31.67	18(60)	30	14(60)	23.33	20(58)	34.48	15(60)	25	48(53)	90.57
CC	Ile	0(0)	0	16(16)	100	60(60)	100	32(34)	94.12	45(49)	91.84	35(35)	100	18(25)	72
	Leu	0(0)	0	16(16)	100	60(60)	100	34(34)	100	45(49)	91.84	35(35)	100	20(25)	80
	Phe	0(0)	0	14(16)	87.5	56(60)	93.33	25(34)	73.53	39(49)	79.59	30(35)	85.71	23(25)	92
	Gln	0(0)	0	16(16)	100	60(60)	100	33(34)	97.06	40(49)	81.63	35(35)	100	25(25)	100

Table 1 Continued.															
	Trp	0(0)	0	14(16)	87.5	43(60)	71.67	15(34)	44.12	11(49)	22.45	15(35)	42.86	18(25)	72
	P4	0(0)	0	9(16)	56.25	18(60)	30	7(34)	20.59	18(49)	36.73	6(35)	17.14	23(25)	92
Ile	Leu	60(60)	100	60(60)	100	60(60)	100	58(58)	100	54(55)	98.18	48(60)	80	50(51)	98.04
	Phe	42(60)	70	49(60)	81.67	56(60)	93.33	51(58)	87.93	46(55)	83.64	42(60)	70	51(51)	100
	Gln	60(60)	100	60(60)	100	60(60)	100	58(58)	100	47(55)	85.45	60(60)	100	51(51)	100
	Trp	26(60)	43.33	45(60)	75	43(60)	71.67	38(58)	65.52	14(55)	25.45	26(60)	43.33	44(51)	86.27
	P4	10(60)	16.67	19(60)	31.67	18(60)	30	14(58)	24.14	19(55)	34.55	15(60)	25	46(51)	90.2
Leu	Phe	42(60)	70	49(60)	81.67	56(60)	93.33	51(60)	85	46(55)	83.64	42(48)	87.5	53(53)	100
	Gln	60(60)	100	60(60)	100	60(60)	100	59(60)	98.33	47(55)	85.45	48(48)	100	53(53)	100
	Trp	26(60)	43.33	45(60)	75	43(60)	71.67	38(60)	63.33	14(55)	25.45	19(48)	39.58	44(53)	83.02
	P4	10(60)	16.67	19(60)	31.67	18(60)	30	14(60)	23.33	20(55)	36.36	13(48)	27.08	48(53)	90.57
Phe	Gln	42(42)	100	49(49)	100	56(56)	100	51(51)	100	43(47)	91.49	42(42)	100	57(57)	100
	Trp	26(42)	61.9	45(49)	91.84	42(56)	75	38(51)	74.51	14(47)	29.79	19(42)	45.24	44(57)	77.19
	P4	6(42)	14.29	16(49)	32.65	17(56)	30.36	13(51)	25.49	17(47)	36.17	11(42)	26.19	51(57)	89.47
Gln	Trp	26(60)	43.33	45(60)	75	43(60)	71.67	38(59)	64.41	14(50)	28	26(60)	43.33	44(59)	74.58
	P4	10(60)	16.67	19(60)	31.67	18(60)	30	14(59)	23.73	18(50)	36	15(60)	25	53(59)	89.83
Trp	P4	4(26)	15.38	15(45)	33.33	13(43)	30.23	10(38)	26.32	6(14)	42.86	6(26)	23.08	39(44)	88.64

[1]Obs= observations for a given sample where;

[2]Freq = frequency at which AA's occurred together for a given sample

[3]free number is the number of actual observations

[4]() parentheses hold total number for row on a given sample

[5][] brackets hold total observations for a given sample

CHAPTER FOUR

CONCLUSIONS

Proper nutritional management of replacement heifers remains a top priority for producers from the time they are weaned, through the completion of their first breeding season. Growth and maturation during this key period have long-term implications regarding longevity and ultimately whether heifers remain in the cow herd or are culled. Successful heifer development begins with the timely establishment of puberty. Heifers in the current study all attained puberty at a similar age and had similar final pregnancy rates. The impacts of nutritional status on puberty in beef cattle has been well documented, which has led producers to target pre-breeding BW's based on estimations of mature size (Patterson et al., 1992; Funston et al., 2012). All heifers regardless of treatment achieved acceptable pre-breeding BW, which largely explains the lack of drastic differences in terms of reproductive success. Supplementing feedstuffs high in protein and energy to heifers consuming low-quality forages can increase growth and help ensure proper development prior to the breeding season, exacerbated in the current study by the increased weight in P20 and P40 heifers at puberty. Promoting cyclicity prior to breeding allows for increased conception early in the breeding season which is paramount for longevity of heifers (Roberts et al., 2019). The levels and types of dietary nutrients have consistently been shown to influence ULF and impact reproductive performance (Elrod and Butler, 1993; Bridges et al., 2013, Crouse et al., 2019). Concentrations of two AA's were impacted in the current study, reinforcing dietary

impact on ULF. Additionally, characterizations of the uterine environment across the estrus cycle have been performed for pH and various amino acids, however the current study provides novel insight identifying AA concentrations prior to the detection of cyclicity. Maternal plane of nutrition has also been identified to play a significant role in determining the composition of histotroph (Crouse et al., 2019), however more research is needed to determine how individual feedstuffs, with varying nutrient composition and characteristics influence uterine amino acids, specifically Trp and Orn, which were altered by supplement type in the present study. Elucidating the mechanisms through which diet impacts the uterine environment will increase the understanding of how these manipulations can impact reproductive efficiency during heifer development and early gestation.

Vita

Kiernan James Brandt was born in Powell, Wyoming on May 29th, 1996 to Kenny and Rebecca Brandt. Kiernan grew up in Cheyenne, Wyoming and became interested in animal agriculture, specifically beef production, in high school. These early interests led him to pursue an Associate of Science degree in Animal Science from Laramie County Community College after studying for a year at Eastern Wyoming College. Upon graduating, Kiernan completed his Bachelor of Science degree at The University of Wyoming with a focus in Production Agriculture. While there, he realized his passion for reproductive physiology and integrating these sciences into commercial beef production, and chose to pursue a Master of Science degree in Reproductive Physiology and Heifer Development from The University of Tennessee.