SUPPLEMENTAL LEUCINE IN MILK REPLACER FED TO NEONATAL CALVES AND LAMBS: EFFECTS ON GROWTH, DEVELOPMENT, AND METABOLISM

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Animal Sciences

April 2021

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Maximizing growth during the neonatal period in preruminants can enhance long-term growth and productivity, increasing producer profits and input efficiency. Meeting the requirements for growth can be achieved through feeding singular amino acids, specifically leucine, to enhance protein synthesis in muscle tissues. In order to evaluate the impact of supplemental leucine in milk replacer fed to neonatal preruminants, three experiments were designed. Experiment 1 examined short-term effects of supplemental leucine administration on calf and organ growth, nutrient digestibility and nitrogen retention, serum amino acid and metabolite profiles, and regulators of protein synthesis. Experiment 2 determined long-term effects of supplemental leucine administration during the pre-weaning phase on pre- and post-weaning lamb growth, pre-weaning serum amino acid and metabolite profiles, feedlot performance, and carcass characteristics. Experiment 3 evaluated effects of supplemental leucine on the pancreatic enzymes α-amylase and trypsin, and the small intestinal carbohydrases maltase, isomaltase, glucoamylase, and lactase using tissue samples from experiments 1 and 2. Overall, supplemental leucine was able to affect animal productivity in the short- and long-term in a species-specific manner. In experiment 1, calf growth was unaffected by treatment; however, select tissue masses were affected by supplementation. Digestibility of milk replacer and nitrogen retention were also unaffected. Supplemental leucine was able to alter serum amino acid and metabolite profiles, but did not alter the activity of proteins involved in protein synthesis. In experiment 2, supplemental leucine increased pre-weaning lamb growth and average daily gain, but did not alter animal performance in the feedlot. Organ masses were unaffected, and lambs receiving supplemental leucine carried more fat in the carcass. In experiment 3, supplemental leucine had no effect on pancreatic enzyme activity, but had both a short- and long-term effect

on small intestinal carbohydrase activities in both species, diminishing the activity of maltase and isomaltase in the jejunum. In calves, leucine also decreased lactase activity in the jejunum. These results suggest that leucine supplementation to milk replacer may affect short-term growth and development of neonatal preruminants, and does have a short- and long-term effects on intestinal carbohydrase activities, and future research should evaluate its effects on carbohydrate digestibility.

ACKNOWLEDGEMENTS

I would like to deeply thank all the people who helped make the results of this dissertation a reality. I am extremely grateful to my advisor, Dr. Kendall Swanson, for allowing me the opportunity to work in his lab with these fun and challenging projects, as well as for his insight, encouragement, and guidance as I worked to complete my dissertation. Thank you for giving me the opportunities to showcase my passion for this work both domestically and internationally. I would also like to thank Drs. Kasey Maddock-Carlin, Christopher Schauer, Michael Steele, and Katie Reindl, my committee members, for their advice and wisdom during this process.

Thank you to the other faculty members I have had the ability to work with (Drs. Kim Vonnahme, Marc Bauer, Travis Hoffman, and Carl Dahlen. I enjoyed learning from each of you and absorbing the knowledge and passion you brought to the department. I am especially grateful for the chance to participate in cannulation surgeries and presenting information and data to sheep producers in North Dakota and my home state of Iowa, opportunities I never would have had without an invitation.

Thank you to Marsha Kapphahn, Laurie Geyer, Jena Bjertness, Jim Kirsch, Wanda Keller, Skip Anderson, and the staff at the Animal Nutrition and Physiology Center for their assistance in sample processing, laboratory analyses, animal care, and for their willingness to teach me the technical skills I needed during my time at NDSU.

A deep appreciation also goes to my lab mates, Ananda, Leo, Ron, Kafi, Macie, and Tommy. I could not have asked for a better group of people to help with bleeding and weighing calves, getting to the sheep unit before sunup for blood draws, all the tissue collections, and the valued input into my writing. I would not have been able to complete my work at NDSU without

you, and am forever grateful that I had the opportunity to work with you and share in our labs' growth.

I would also like to thank Felipe, Alex, Khaled, Karissa, Megan, and Ashley for their help with my projects, as well as all the grad students from office 128 over the past four years (Laura, Michaella, James, Elfren, Jerica, Aaron, Rike, and Jacob) for the time spent chatting, attempting stats homework together, and the card games to help ease the stress of school and projects. Thank you for always being there.

I would like to thank my parents Brad and Tracy and my siblings Alex and Faith for always being a phone call away. I am always appreciative of your willingness to listen and provide support in any way you can, even from a distance. I am so thankful to have your support during these past years, and without you this journey would not have been possible.

Finally, I would like to thank Claudia and Dan Putnam. I had no idea what I was getting myself into when I stepped onto your farm 14 years ago, and I am immensely thankful that you took in this city kid with no experiences in agriculture. It was with your family that I discovered my passion for sheep, and I can only hope that one day I am able to allow the same opportunities for others.

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LIST OF ABBREVIATIONS

4E-BP1.....Elongation initiation factor 4E binding protein 1

AA.....Amino acid

ADF.....Acid detergent fiber

ADG.....Average daily gain

Ala.....Alanine

Asn.....Asparagine

Asp.....Aspartate

Arg.....Arginine

BCAA.....Branched chain amino acid

BCTRC.....Boneless closely trimmed retail cuts

BF.....Backfat

BW.....Body weight

BWT.....Body wall thickness

ºC.....Degree Celsius

CP.....Crude protein

Cys.....Cysteine

d.....Day

DM.....Dry matter

DMB.....Dry matter basis

DMI.....Dry matter intake

EAA.....Essential amino acid

EE.....Ether extract

eIF4E.....Elongation initiation factor 4E

Gln.....Glutamine

Glu.....Glutamate

Gly.....Glycine

h.....Hour

HCW .....Hot carcass weight

His .....Histidine

HPLC .....High performance liquid chromatography

Ile .....Isoleucine

Leu .....Leucine

LMA.....Longissimus muscle area

Lys.....Lysine

ME.....Metabolizable energy

Met .....Methionine

mM.....Millimolar

MP.....Metabolizable protein

MR .....Milk replacer

mTOR .....Mammalian target of rapamycin

N.....Nitrogen

NDF.....Neutral detergent fiber

NEAA .....Nonessential amino acid

OM .....Organic matter

p4E-BP1.....phosphorylated elongation initiation factor 4E binding protein 1

Phe.....Phenylalanine

Pro .....Proline

Ser .....Serine

Thr.....Threonine

Trp.....Tryptophan

Tyr.....Tyrosine

U.....Unit

UPLC .....Ultra performance liquid chromatography

Val.....Valine

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1. LITERATURE REVIEW

1.1. Neonatal Ruminant Digestive Physiology

At birth, neonatal calves and lambs are classified as preruminants as their digestive system functions similarly to that of nonruminants, and are developmentally in the liquid-feeding phase. The reticulum, rumen, and omasum of neonatal calves and lambs is small and nonfunctional; this is due to the neonates consuming liquid feed in the form of milk or milk replacer, which, when ingested, forces the closure of the esophageal groove and the ingested milk is shunted directly to the abomasum, the initial site of milk coagulation as well as protein and fat digestion (Lyford Jr., and Huber, 1988). The abomasum is the most developed part of the stomach during the neonatal period, and appears to be as functional and developed as the abomasum observed in functional ruminants.

In many production systems, newborn ruminants are allowed access to pasture or solid feed formulated for their growing needs. By the age of 3 weeks, if access to dry feed is allowed, neonatal preruminants enter a transitional phase of stomach development – the intake of solid feed is displacing milk intake, and nutritional needs are being met by both decreasing amounts of milk and increasing amounts of solid feed, as well as environmental exposure to microbial populations that will aid in ruminal development (Lyford Jr., 1988). As the animal continues to ingest increasing amounts of solid feeds and lesser amounts of milk, the abomasum will shift to the right side of the body to allow more space for the rapidly growing and developing rumen. When the neonatal ruminant is between 6-9 weeks old, the rumen will occupy three quarters of the available space in the abdominal cavity (Lyford Jr., 1988).

The final stage of stomach development is the ruminant phase, when intake of milk has ceased and all nutrient requirements are met through intake of solid feed that reaches the

reticulorumen. The reticulum and rumen are often described as one unit, the reticulorumen. Ingested feed is fermented in the rumen by the microbial population to produce volatile fatty acids (i.e., acetic, propionic, and butyric acids) for energy. Lipids in the rumen undergo lipolysis and biohydrogenation; ruminal fates of dietary protein are discussed in-depth below. Larger particles will remain in the rumen until they have been decreased to a sufficiently small particle size. The reticulum will collect small feed particles from the rumen and pass them to the omasum. This part of the stomach has numerous folds to aid in the absorption of water exiting with digesta from the reticulum. Feed then moves into the abomasum for final digestion and preparation for absorption in the small intestine.

1.2. Enzymes of the Pancreas and Small Intestine

1.2.1. α-Amylase

In cattle, approximately 18 to 42% of starch escapes ruminal degradation (Owens et al., 1986) and passes to the small intestine. This escaped starch is then broken down by pancreatic and small intestinal enzymes. Intestinal digestion of starch is 42% more efficient than that of ruminal digestion (Huntington et al., 2006), but the small intestine is limited in its ability to digest starch (Harmon, 2009), likely due to insufficient enzyme secretion (Cao et al., 2018a). Pancreatic α-amylase initiates starch digestion in the small intestine by cleaving amylose and amylopectin chains into maltotriose and maltose (Coombe and Siddons, 1973; Van Soest, 1994) for further breakdown by other enzymes. In preruminant calves, secretion of pancreatic α-amylase is low and activity in calves under 1 month of age is insignificant as starch content of milk or colostrum is low (Radostits and Bell, 1968; Guilloteau et al., 2009). Enzyme activities do increase within the first month of life and after weaning, by as much as 2400% for α-amylase,

and this increase can be stimulated by diet offered to calves (Guilloteau et al., 2009), whether milk-based or a starch-based starter feed.

Pancreatic secretions of α-amylase can also be increased in mature animals. Brannon reported that α-amylase content and synthesis increased in nonruminants when dietary carbohydrate intake increased (Brannon, 1990); however, this was not observed to occur in ruminants (Harmon and Swanson, 2020). It was suggested that regulatory signals for the synthesis of pancreatic α-amylase are not conserved between ruminants and nonruminants (Harmon, 1993). In a report by Kreikemeier et al (1990), α-amylase activity was greater in calves consuming a greater amount of feed and those on a forage-based diet. Further, growing steers receiving an intestinal infusion of starch and differing levels of casein, pancreatic α-amylase linearly increased with increasing amounts of infused casein (Richards et al., 2002). It was also reported that infusion of casein and essential amino acids (EAA) increased α-amylase secretion, while infusion of nonessential amino acids (NEAA) and Glu increased starch digestibility (Brake et al., 2014). Infusions of Leu into the duodenum also was reported to increase α-amylase activity, although this increase was only seen within the first 20% of the small intestine (Yu et al., 2014).

1.2.2. Trypsin

Trypsin is an enzyme secreted by the pancreas that aids in the degradation of protein, breaking protein down into peptides and individual amino acids (AA). Protein exiting the abomasum in ruminants is either protein that escapes ruminal degradation or is microbial protein. Trypsin activity is not present at birth in lambs, but sharply increases up to 14 days post-birth, and is constant up to 42 days post birth (Brown and Perry, 1981).

Pancreatic secretions can be altered by numerous factors, including animal age, insulin concentrations, cholecystokinin, and dietary energy and protein levels. Therefore, the amount of trypsin secreted into the small intestine can be increased by increasing pancreatic secretions, and the method garnering the greatest research interest is dietary protein and AA content (Cao et al., 2018a). Yu et al. (2014) reported that duodenal infusions of Leu and Phe in yearling goats increased trypsin activity in the small intestine. Specifically, a greater amount of infused Leu increased trypsin activity in the first 40% of the small intestine, though there was no apparent increase further along in the small intestine (Yu et al., 2014). A separate study examining duodenal infusions of Phe or two levels of Leu in yearling goats reported that Phe infusions decreased trypsin activities while the greater Leu infusion increased trypsin activity, and both Leu infusions resulted in greater trypsin activity than the Phe treatment (Cao et al., 2018b). Another study examining the supplementation of Phe, Leu, or both in milk to calves reported no changes in trypsin activity (Cao et al., 2018a), though the supplementation amount was less than previous studies. Together, these studies indicate that alterations to trypsin activity may be dependent on quantity of the select AA, with the greatest effect due to Leu.

1.2.3. Sucrase-Isomaltase

The sucrase-isomaltase enzyme complex comprises approximately 10% of the small intestinal microvillous membrane proteins, but constitutes up to 80% of the maltase activity in non-ruminants (Galand, 1989; Van Beers et al., 1995). This enzyme cleaves maltose and isomaltose into two glucose units, allowing for the intestinal absorption of the end product. Following weaning and the introduction of greater quantities of dietary starch, the production of isomaltase in the small intestine increases (Guilloteau et al., 2009). Additionally, activity of isomaltase is highest in the jejunum, slightly lower in the ileum, and lowest in the duodenum

(Coombe and Siddons, 1973). The sucrase subunit, although present, is not active in ruminants (Galand, 1989).

Sucrase-isomaltase is sensitive to substrate regulation as a decrease in dietary intake of starch will lead to a decrease in the enzymatic activity of sucrase-isomaltase. However, an animal in a state of starvation will exhibit an increase in sucrase-isomaltase activity (Van Beers et al., 1995). Harmon and Swanson (2020) reported that small intestinal carbohydrase activities, including sucrase-isomaltase, are not affected by changes in diet. Most data on this enzyme complex are on functional ruminants and adult animals, with minimal data from neonatal animals. Galand (1989) reported that isomaltase activity in suckling rats is low. Data on sucrase-isomaltase activities in neonatal ruminants are limited, as are studies on dietary influences on activities of the individual subunits.

1.2.4. Maltase-Glucoamylase

The maltase-glucoamylase enzyme complex comprises approximately 2% of the small intestinal microvillous membrane proteins, but accounts for the remaining 20% of maltase activity (Galand, 1989). In addition to cleaving maltose, the glucoamylase subunit is the only subunit responsible for removing individual glucose units from amylose and/or amylopectin (Van Beers et al., 1995). Maltase activities in neonatal calves is insignificant (Radostits and Bell, 1968), but has a reported activity of 1-3 U/g protein in calves that were 28 days old (Guilloteau et al., 2009). Additionally, maltase activity is fully developed at birth and its activity remains low throughout the life of sheep and cattle (Siddons, 1968; Coombe and Siddons, 1973; Galand, 1989). However, there are some developmental differences between these species: maltase activities are uniformly distributed throughout the small intestine in neonatal calves, but lambs have decreased maltase activity from the duodenum to ileum on day 1 of life, but by day 7

activity is similar to that observed in calves (Guilloteau et al., 2009). Similar to isomaltase, maltase production increases after weaning (Guilloteau et al., 2009).

With the small intestinal carbohydrases being limiting for small-intestinal starch digestion (Van Beers et al., 1995), increasing their activity would lead to increased digestibility of starch flowing to the small intestine. However, as reported above, carbohydrase activities in the small intestine are not typically influenced by dietary changes (Harmon and Swanson, 2020).

1.2.5. Lactase

Lactase is the enzyme responsible for breaking lactose into glucose and galactose, and is the most important enzyme in neonatal animals as lactose is the major carbohydrate source in milk (Van Beers et al., 1995). The activity of lactase is at its highest after birth and within the first 7 days of life in calves (Guilloteau et al., 2009), and decreases at 43 days of age in calves, remains constant up to 114 days of age, then decreases again in adults (Siddons, 1968). Reasons for the decrease are unclear as lactase activity was similar between 4-month-old calves that were maintained on a milk diet and 4-month-old calves that were weaned at 6 weeks of age (Siddons, 1968). Further, activity of lactase is greater in the duodenum than jejunum and ileum (Hembry et al., 1967).

While lactase activity is high when lactose is consumed, excess intake of lactose does not increase lactase activity, but high concentrations of glucose can slightly increase its activity in rats. It was also determined that the gene expression of lactase increases in the fetus prior to parturition and high levels of lactase activity was observed shortly after birth, suggesting that lactase activity is predetermined in the neonate and cannot be influenced by diet or starvation post-birth (Van Beers et al., 1995). Additionally, alterations in diet type (forage or grain) and

level of feed intake does not affect lactase activities in functional ruminants (Kreikemeier et al., 1990).

1.3. Proteins and Amino Acids

1.3.1. Protein and Amino Acid Supply and Requirements

In ruminant diets, protein is often measured as crude protein (CP: N × 6.25). The CP system can also be allocated into rumen degradable protein (RDP) and rumen undegradable protein (RUP). Protein that is degradable in the rumen is protein that is available to ruminal microbes for their maintenance and growth. As these microbes die or pass into the small intestine, they are digested into absorbable AA and often referred to as microbial crude protein (MCP). Both RUP and MCP contribute AA to the animal, and the AA from these protein fractions are available for the maintenance, growth, and production of the ruminant.

A more accurate measure of dietary protein available to ruminants is metabolizable protein (MP), which is composed of MCP and AA derived from the RUP fraction that is absorbed in the small intestine. While increasing MP supply to the small intestine increases AA available for absorption, the MP system does not account for specific AA needs. For instance, sheep require greater amounts of AA containing sulfur to accommodate wool growth (NRC, 2007). Additionally, AA needs for muscle growth, fetal development, and lactation are similar between sheep, beef cattle, and dairy cattle, and require Arg, His, Ile, Leu, Lys, Met, Phe, Thr, and Val (NRC, 2000; NRC, 2007).

Twenty amino acids used for protein synthesis can be classified as either nonessential AA (NEAA) or essential AA (EAA; Wu et al., 2014). Amino acids that can be synthesized in the body in sufficient amounts to meet the animals' physiologic needs are classified as NEAA, whereas EAA are not synthesized in the body in sufficient amounts to meet maintenance and

growth requirements, and need to be supplemented in the diet to meet those needs (Wu et al., 2013; Wu et al., 2014). Due to their limited synthesis in the body, some EAA may be limiting for growth and production. These limiting AA need to be supplemented at greater levels in the diet to compensate for limitations in the diet. In ruminants consuming corn-based diets, these limiting AA are typically either Lys or Met (NRC, 2001); limiting AA in preruminants are not well understood.

1.3.2. Fates of Proteins and Amino Acids in the Rumen and Small Intestine

The RDP fraction of protein is degraded by ruminal microbes into AA or small peptides which are deaminated into ammonia (NH_3; Selinger et al., 1996). This end product is then utilized by microbes as a N source to generate MCP, which passes into the abomasum and small intestine. Within the abomasum, MCP is broken down and reaches the small intestine as AA and small peptides. The RUP fraction of protein remains unchanged by ruminal microbes and reaches the abomasum as intact proteins, and at that point will be degraded along with MCP and pass into the small intestine.

In the rumen, the majority of free AA are deaminated into NH_3 for use by ruminal microbes, and will not reach the small intestine as intact AA (Chalupa, 1976; Wu and Papas, 1997). Therefore, supplementation of specific AA to the diet of functional ruminants will not result in an increase in supply of the limiting AA. However, preruminants without a functional rumen can utilize AA supplemented to the diet as the AA bypass the rumen and enter the small intestine intact.

Once protein reaches the abomasum, the digestion and absorption processes are similar to that of nonruminants. Trypsin, chymotrypsin, and carboxypeptidase break down proteins in the duodenum into di- and tripeptides and AA for absorption in the jejunum and ileum as either

intact AA or are broken down to urea and carbon dioxide (Merchen, 1988; Owens and Zinn, 1988). Peptides reaching the small intestine are transported into intestinal epithelial cells via the intestinal peptide transporter 1 (PepT1) where they are subsequently cleaved for transport into the circulatory system as free AA (Merchen, 1988; Wang et al., 2017).

1.3.3. Peptide Transporters in the Small Intestine

Intestinal peptide transporter 1 (PepT1) is the major transporter of di- and tripeptides in the small intestine, accepting over 400 dipeptides and 8,000 tripeptides (Boudry et al., 2010). This transporter is localized to the brush border membrane within the intestine and has the greatest expression in the duodenum, which decreases towards the colon (Boudry et al., 2010). The expression of PepT1 is regulated by dipeptides, with no effect from free AA, but can also increase its ability to absorb peptides when a protein-free diet is provided to the animal (Boudry et al., 2010). This difference in substrate regulation was reported by Zhang et al. (2013), where piglets fed a low protein diet had a greater expression level of PepT1 compared to piglets receiving a normal level of protein in the diet and piglets receiving a low protein diet supplemented with BCAA. PepT1 can also be used to aid in meeting AA needs through uptake of peptides as they are cleaved into AA (Merchen, 1988; Bröer and Fairweather, 2019). This is supported by the observation that the uptake of peptides by PepT1 is quicker than the uptake of AA by their transporters (Merchen, 1988; Daniel, 2004).

1.3.4. Amino Acid Transporters in the Small Intestine

Five types of AA transporters exist in the small intestine, and are specific to the classification of AA (e.g. neutral, cationic, anionic, imino, and β-AA; Bröer, 2008). However, only the four proteogenic AA transporters will be discussed.

1.3.4.1. Neutral Amino Acid Transporters

The main AA transporters of neutral AA include sodium-coupled neutral AA transporter 2 (SNAT2), system L AA transporter 2 (LAT2), and B^0 AA transporter (B^0AT1). SNAT2 senses low cellular AA concentrations and will uptake Gln from the intestinal lumen (Bröer, 2008). In addition, SNAT2 is also present in skeletal muscle as a regulator of Gln concentration. (Dodd and Tee, 2012). LAT2 maintains a presence in the jejunum and ileum where it exchanges smaller AA for branched-chain AA (BCAA) such as Leu, Ile, and Val, or aromatic AA; however, it has no affinity for Pro (Bröer and Fairweather, 2019). The transporter B^0AT1 is the major transporter for all neutral AA, but has the greatest affinity for Met and the branched-chain AA (BCAA) Leu, Ile, and Val, and has the lowest affinity for Lys (Bröer, 2008; Bröer and Fairweather, 2019).

1.3.4.2. Cationic Amino Acid Transporters

The main cationic AA transporters in the small intestine are rBAT/$b^{0,+}$AT, y^+LAT1, y^+LAT2, and CAT-1 (Bröer, 2008; Bröer and Fairweather, 2019). These AA transporters exchange neutral AA for cationic AA. The major AAs involved in this exchange are Leu and Lys, where greater concentrations of the neutral AA Leu will drive the uptake of Lys in cells (Bröer and Fairweather, 2019).

1.3.4.3. Anionic Amino Acid Transporters

There are two recognized transporters for the anionic AA, EAAT3 and AGT1. Both of these transporters exchange Na^+ for Glu; however, only AGT1 is involved in both Glu and Asp uptake, as observed by an uptake rate that was five times greater for Glu than Asp when extracellular concentrations were equal (Bröer, 2008; Bröer and Fairweather, 2019). It was also determined that neutral AA, as well as Phe, can inhibit the transport capability of the anionic AA transporters (Bröer and Fairweather, 2019). Bröer (2008) has also reported that the majority of

Glu absorbed in the small intestine is metabolized into α-ketoglutarate for further metabolic use by the body, and very little Glu or Asp reach circulation.

1.3.4.4. Imino Amino Acid Transporters

Several imino AA transporters have been observed in the small intestine: the proton AA transporter (PAT1), the system IMINO transporter (SIT1), and a neutral AA transporter, B^0 (Bröer and Fairweather, 2019). The PAT1 transporter is one of the dominant imino transporters in the small intestine, and cotransports a hydrogen atom with each AA, including Pro and Gly, but also transports Ala, β-Ala, as well as non-proteinogenic AA, with uptake of Pro being greater than that of Gly (Bröer, 2008; Bröer and Fairweather, 2019). SIT1 is another transporter of Pro, but can also transport neutral AA such as Phe with no ability to transport Gly (Bröer, 2008). The B^0 transporter has limited ability to transport Gly, as it is easily inhibited by Met, the AA it has the greatest affinity for (Bröer and Fairweather, 2019).

1.4. Leucine

Leucine is one of the three (BCAA) and is an EAA as it is not synthesized by the body in sufficient quantities to meet needs of the animal. However, this AA is not typically considered as a limiting AA for production as it is found in greater quantities in feed. It is considered a functional AA, in that it has the ability to regulate metabolic pathways involved in maintenance, growth, and reproduction (Wu et al., 2009). Leucine is a potent regulator of protein synthesis and also has significant roles in tissue regeneration, metabolism, lipid decomposition, and insulin secretion, as well as ATP generation (Pedroso et al., 2015; Mao et al., 2019).

Leucine stimulates protein synthesis through affecting the initiation of mRNA translation and through the suppression of proteolysis in cells (Dodd and Tee, 2012; Pedroso et al., 2015).

This alteration of protein synthesis and proteolysis is carried out through the mammalian target of rapamycin (mTOR) pathway.

1.5. mTOR Pathway

1.5.1. mTORC1 and mTOR

The mammalian target of rapamycin (mTOR) is the protein involved in the mTOR pathway, which controls protein synthesis in tissues, either through an increase in cell number or in cell volume (Roux and Topisirovic, 2018). This protein senses cellular concentrations of growth factors, amino acids, and energy in the form of ATP to either upregulate protein synthesis when these levels are adequate or downregulate protein synthesis when these levels are not sufficient (Mahoney et al., 2009). This degree of oversight by mTOR is necessary as protein synthesis has a high energy cost to maintain (Dodd and Tee, 2012). mTOR is found in two separate complexes, mTOR complex (mTORC) 1 and mTORC2. mTORC1 is comprised of mTOR, raptor, GTPase β-subunit like protein, and deptor (Roux and Topisirovic, 2018). mTORC1 is sensitive to the AA Leu, Arg, and Gln, all of which can stimulate mTORC1 activity, and therefore increase protein synthesis. The AA Leu has the strongest effect of these three AA in liver (Dennis et al., 2011). This complex is able to achieve this through the regulation of the eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BP), and the ribosomal S6 kinases (S6K).

1.5.2. Proteins Upregulating the mTOR Pathway

Eukaryotic translation initiation factor 4F (eIF4F) is a complex comprised of eIF4E, eIF4G, and eIF4A. Together, these proteins aid in protein translation, and the bound combination of eIF4E and eIF4G is needed to maintain protein synthesis in cells. However, in periods of protein downregulation, the 4E-BPs will split the eIF4E-eIF4G complex and bind to the eIF4E

complex, inhibiting protein translation. When protein translation continues again, the 4E-BPs will become phosphorylated (p4EBP) and disassociate from eIF4E, allowing it to reassociate with eIF4G (Roux and Topisirovic, 2018).

The S6K protein is also involved in the mTOR pathway as an upregulator of protein synthesis. This protein, when activated by mTORC1, will increase cell growth by targeting ribosomal protein S6 (rpS6) and eukaryotic elongation factor 2 (eEF2) kinase (eEF2K; Mahoney et al., 2009; Roux and Topisirovic, 2018). eEF2K inhibits the protein synthetic protein eEF2, and the inhibition of eEF2K allows eEF2 to aid in protein synthesis along with eIF4B and the eIF4E-eIF4G protein complexes.

1.6. Neonates, Leucine, mTOR, and More

1.6.1. Neonatal Amino Acid Needs

While dietary protein requirements for ruminants and preruminants is well described, little information is available on specific AA needs of preruminants. Because protein is an expensive nutrient in feed, including specific AA instead of increasing dietary protein content could better meet the AA needs of animals without increasing the amount of wasteful protein or nonlimiting AA, leading to increased N in waste. This is further complicated by the fact that skeletal muscle tissue is rapidly growing during the neonatal period (Davis and Fiorotto, 2009), and AA requirements will change to meet the body's needs for proper growth and development. Rogers and Egan (1975) noted that lambs are sensitive to dietary AA imbalances, and growth can be diminished if the appropriate AA are not present. Williams and Hewitt (1979) described methods to determine Lys requirements in milk-fed calves.

However, it is known that Leu is an AA involved in the mTOR pathway, which can increase protein synthesis in tissues. It is not known if the Leu in milk or milk replacers fed to

calves and lambs is at the maximal concentrations to enhance protein synthesis in neonatal muscle tissues. Escobar et al. (2005) reported that plasma Leu levels would have to double before protein synthesis would be at its maximum in piglets. Most research evaluating the effects of Leu on muscle protein growth and the activation status of the mTOR pathway has been completed in piglets.

1.6.2. Leucine Studies in Pigs

Boutry et al. (2016) examined the effects of continuous feeding of milk replacer via a gastrostomy tube with pulses of either Leu or Ala on body growth, protein synthesis rate, the eIF4E-eIF4G complex, and the phosphorylation of the mTORC1 complex. Ala was selected for this experiment to control for N intake as Ala does not contribute to protein synthesis. Within the 21-d experimental period, piglets receiving pulses of Leu experienced greater body weight gain compared to piglets receiving pulses of Ala (Boutry et al., 2016). This gain was also broken down to determine whether gain was primarily lean muscle tissue gain or fat gain. Piglets receiving Leu showed more lean tissue gain and less fat gain than Ala supplemented piglets (Boutry et al., 2016). This was further supported by increased mass in the longissimus dorsi (LD), gastrocnemius, and soleus muscles in Leu supplemented piglets compared to piglets receiving Ala (Boutry et al., 2016). It was also reported that these tissues, along with the heart, showed greater protein synthesis rates. The eIF4E-eIF4G complex abundance, an indicator of increased protein synthesis, was also greater in the LD, gastrocnemius, and soleus muscles of piglets receiving Leu compared to those supplemented with Ala (Boutry et al., 2016). The phosphorylation of mTORC1, S6K1, and 4E-BP1 was also increased in Leu supplemented piglets, further supporting that Leu pulses given to piglets are able to upregulate the mTOR

pathway and generate an increase in protein synthesis in muscle tissues, allowing for greater weight gain due to increased muscle growth.

Boutry et al. (2013) also evaluated different feeding methods of piglets with or without Leu. Piglets fitted with orogastric tubes were assigned to either a continuous feeding, continuous feeding with Leu pulses, or bolus feeding of milk replacer. This study also evaluated protein synthesis rates, the abundance of the eIF4E-eIF4G complex, and proteins involved in the mTOR pathway. Similar to the previous study, protein synthesis rates in the LD, gastrocnemius, and soleus muscles were greater in Leu piglets compared to the continuously fed piglets; however, piglets receiving meals in bolus doses had even greater protein synthesis than Leu piglets in the LD and soleus, and were greater than the other treatments in the small intestine (Boutry et al., 2013). Additionally, the abundance of the eIF4E-eIF4G complex was greater in Leu piglets than continuously fed piglets, as was the phosphorylation of S6K1 and 4E-BP1; however, piglets receiving a bolus meal showed greater phosphorylation of S6K1 than Leu piglets, while the phosphorylation of 4E-BP1 and abundance of the eIF4E-eIF4G complex was similar (Boutry et al., 2013). This suggests that Leu can enhance growth in piglets when a diet is continuously fed, but the delivery of a meal in larger, less frequent doses can exceed the effects of Leu.

Columbus et al. (2015) evaluated the effects of milk replacer diets fed to piglets that were high protein, low protein, or low protein with supplemental Leu on growth, protein synthesis, and effects on the mTOR pathway. Piglets on the Leu-supplemented diet showed a greater body weight than low protein fed piglets at the end of the experiment with the piglets receiving the high protein diet having the greatest body weight (Columbus et al., 2015). Piglet weight gain was also broken into lean and fat growth, where the low protein piglets had a lower lean gain than high protein piglets with the Leu-supplemented piglets intermediate to both. There were no

differences in fat gain among treatments. This trend continued with protein synthesis in the LD, heart, liver and kidney where protein synthesis was greatest in the high protein piglets, lowest in the low protein piglets, and intermediate in the Leu-supplemented piglets. However, protein synthesis in the soleus muscle was greatest in the high protein piglets, while low protein and Leu-supplemented piglets were not different from each other (Columbus et al., 2015). Finally, the abundance of the eIF4E-eIF4G complex and phosphorylation of 4E-BP1 was greatest in the LD of Leu-supplemented piglets, lowest in the low protein piglets, and intermediate in the high protein piglets, whereas phosphorylation of S6K1 was similar among treatments (Columbus et al., 2015). These results show that Leu can be used as a supplement to overcome dietary protein deprivation and increase the activity of the mTOR pathway. Because the Leu-supplemented diet was formulated to have similar Leu levels as the high protein diet, this shows that while Leu was not the AA limiting growth, there may have been a deficiency in other AA, which led to a suppressed growth of piglets.

Most neonatal research examining the effects of Leu on growth, protein metabolism, and the regulation of the mTOR pathway has been reported in pigs. The purpose of utilizing pigs has been to model effects on human neonates in attempts to determine methods to aid in survivability and protein synthesis in low-birth weight children. However, this information can also add to knowledge for livestock producers as increasing muscle protein synthesis or body growth during the neonatal period may decrease the number of days for livestock to reach market weight, decreasing the amount of feed they consume, thereby increasing producer productivity. It is unlikely that this research could be used in functioning ruminants as the rumen deaminates AAs and any Leu supplemented to the diet would likely not reach the small intestine intact. Therefore,

supplementation to neonatal preruminants would be of greater benefit and would have the greatest chance of seeing effects of Leu supplementation.

1.6.3. Leucine Studies in Preruminants

Guo et al. (2018) evaluated the effects of increasing amounts of Leu on cultured pancreatic acinar cells of dairy calves on mTOR pathway proteins. The moderate level of Leu provided to cells (0.45 mM) showed the greatest level of phosphorylation of mTOR and S6K1 compared to the control, 0.23, and 0.90 mM treatments, whereas the phosphorylation of 4E-BP1 was unaffected by treatment (Guo et al., 2018). Guo et al. (2019) performed a similar experiment in Holstein pancreatic acinar cells with either 0 or 0.45 mM Leu added to the culture. This study reported an increase in both total and phosphorylated forms of mTOR, S6K1, and 4E-BP1 (Guo et al., 2019). These results show that in an in vitro system, pancreatic cells of dairy calves can be sensitive to Leu supplementation; however, the amount of Leu that elicited the greatest response was 0.45 mM Leu, or double the normal plasma Leu concentration found in dairy cow plasma. This agrees with the findings of Escobar et al. (2005), where Leu levels in the plasma of piglets needed to be double the normal concentration to observe maximal protein synthesis.

Zheng et al. (2018) determined the effects of Leu supplementation fed to Holstein calves via milk replacer on the length, area, and protein concentration in the LD and the activation of the mTOR pathway. Calves were fed milk replacer with or without Leu for 7 weeks, and were also provided starter feed for the final 4 weeks of the experiment. Calves receiving milk replacer with supplemental Leu had a longer LD and the LD had a greater concentration of protein (Zheng et al., 2018). Additionally, the phosphorylation of mTOR, S6K1, and 4E-BP1 was greater in calves receiving supplemental Leu compared to control calves. The results from Zheng et al. (2018) suggest that Leu supplemented to neonatal calves is able to elicit an increase in

protein synthesis in muscle, which was likely facilitated by the inhibition of 4E-BP1 and activation of mTOR and S6K1 to enhance protein synthesis.

Cao et al. (2019) determined the effects of Leu supplemented to raw milk-fed Holstein calves for a period of 7 weeks and its effects on growth and intestinal enzyme activity. Initial and final BW, as well as average daily gain (ADG) during the experimental period did not differ between control and Leu-supplemented calves. Lactase and α-amylase activity in the intestines were also not different between control and Leu-supplemented calves. There was an increase in intestinal trypsin activity where Leu-supplemented calves had greater activity of trypsin at 20% of the intestinal length past the pyloric sphincter (Cao et al., 2019). These results suggest that Leu concentration in raw milk was likely at a concentration that would not affect calf BW or enzymatic activity, and Leu was likely not an AA limiting in the body for protein synthesis.

Mao et al. (2019) examined effects of supplemental Leu in milk replacer fed to lambs from d 11 to 30 of life on pre- and post-weaning growth, feed intake, and carcass characteristics. Leucine supplementation occurred in the first of three milk feedings during the day; lambs in this study were also allowed access to starter pellets and hay during the milk-feeding period. Lamb BW at d 10, 20, and 30 of life, ADG, and intake of starter and hay were not different between treatments (Mao et al., 2019). Final finishing weight, ADG, and intake of feed by lambs during the finishing period were not different between treatments. Carcass weight and the depth of muscle and fat of the carcass at the 12^{th} rib was also similar between treatments; however, lambs receiving supplemental Leu pre-weaning did show an increase in dressing percent compared to control lambs, and drip loss, a measure of water retention by the carcass, was less in Leu-supplemented lambs compared to control lambs (Mao et al., 2019). This could suggest an increase in protein synthesis, even though the muscle and fat depth at the 12^{th} rib was similar. It

is possible that carcass conformation may have been different, such as in the leg, which could contribute to a greater dressing percent. Further, an increase in protein synthesis in muscle tissues could lead to a decrease in drip loss due to the proteins and structures involved in water retention of the carcass (Huff-Lonergan and Lonergan, 2005).

1.7. Summary and Research Objectives

The objectives of these studies were to 1) determine the short-term effects of leucine supplementation to milk-fed calves on whole body and organ growth, nutrient digestibility and nitrogen balance, serum AA and metabolite profiles, and the quantification of proteins involved in the mTOR pathway (Chapter 2), 2) evaluate the long-term effects of leucine supplementation of milk-fed lambs on pre- and post-weaning growth, serum metabolite and AA profiles, finishing performance, organ growth, and carcass characteristics (Chapter 3), and 3) examine the short- and long-term effects of leucine supplementation on the digestive enzyme activities of the pancreas and small intestine (Chapter 4).

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2. EFFECTS OF SUPPLEMENTAL LEUCINE IN MILK REPLACER ON GROWTH, SERUM AMINO ACIDS, AND MUSCLE AND VISCERAL TISSUE MASS IN HOLSTEIN BULL CALVES

2.1. Abstract

To determine the effects of leucine supplementation in milk replacer to pre-weaned calves, 23 Holstein bull calves (43.3 ± 1.16 kg; 11.3 ± 0.57 days of age) were used in a randomized complete block design. Leucine was supplemented at 0, 0.4, 0.6, or 0.8 g Leu/kg body weight (BW) per day for 28 d. Calf BW, tissue mass, nutrient digestibility, serum AA and metabolites, and protein abundance of p4E-BP1 and eIF4E were determined. Data were analyzed using the MIXED procedure of SAS. Leucine supplementation did not affect calf BW ($P = 0.73$), and digestibility of N ($P = 0.21$), organic matter ($P = 0.28$), and dry matter ($P = 0.28$). Masses relative to BW of the pancreas ($P = 0.04$), omasum ($P < 0.01$), and spleen ($P = 0.01$) were quadratically affected by treatment where tissue mass decreased at 0.4 g Leu/kg BW and increased at 0.6 and 0.8 g Leu/kg BW. Semitendinosus mass relative to BW tended ($P = 0.07$) to be quadratically affected, as tissue mass increased at 0.4 g Leu/kg BW, and decreased at 0.6 and 0.8 g Leu/kg BW. Serum Leu concentration increased linearly ($P = 0.002$; day × time × treatment) across days and after feedings with greater levels of supplemental Leu. Serum glucose concentration tended ($P = 0.10$) to be quadratically affected by treatment where glucose decreased in the 0.4 and 0.6 treatments. Increasing amounts of supplemental Leu linearly decreased serum Ala ($P < 0.01$), Arg ($P = 0.04$), Ile ($P = 0.02$), Met ($P < 0.01$), and Pro ($P = 0.05$) concentrations, and quadratically affected serum Glu ($P = 0.04$) and Lys ($P = 0.03$) concentrations where serum Glu and Lys concentrations were decreased at 0.4 g Leu/kg BW and increased at 0.6 and 0.8 g Leu/kg BW. Quadratic tendencies were observed for treatment for

serum Asp (P = 0.08), Phe (P = 0.06), Thr (P = 0.07), and Val (P = 0.09) concentrations where serum Asp and Thr concentrations decreased at 0.4 g Leu/kg BW then increased at 0.6 and 0.8 g Leu/kg BW, and serum Phe and Val concentrations increased at 0.4 g Leu/kg BW and decreased at 0.6 and 0.8 g Leu/kg BW. There was no effect of treatment on protein abundance in p4E-BP1 or eIF4E proteins. The changes in serum AA profile and tissue masses relative to BW suggest that supplementation of Leu at lower levels may result in increased AA proportional use for muscle protein synthesis.

2.2. Introduction

During early life, milk or milk replacer is the primary component of the diet for calves, as the rumen of the calf is not yet developed, which limits pre-gastric microbial fermentation of ingested feed. This results in milk-based feeds bypassing the rumen, and being digested in the abomasum and intestines (Abe et al., 1979). Soberon et al. (2012) and Bach (2012) reported that increased pre-weaning ADG and plane of nutrition, respectively, in Holstein heifers during the pre-weaning period was associated with an increase in future milk production. These results suggest a developmental programming effect on long-term productivity. Skeletal muscle protein synthesis and growth is rapid in the pre-weaning period (Wray-Cahen et al., 1998; Zheng et al., 2019) and requires adequate AA supply. Also, pre-weaned lambs are sensitive to dietary imbalances of AA (Rogers and Egan, 1975) and oversupply of non-limiting AA results in increased catabolism, leading to a delay in muscle growth.

Leucine is an essential AA as sufficient quantities are not able to be synthesized within the body to meet metabolic needs. However, Leu is not typically considered limiting in diets, but it is a functional AA because Leu has numerous effects on metabolism (Wu, 2009; Dodd and Tee, 2012; Millward, 2012). Leucine is believed to be the primary AA that signals for increasing

skeletal muscle protein synthesis (Pedroso et al., 2015). Leucine also influences plasma AA profiles in weaned piglets (Wiltafsky et al., 2010) and increases AA transport into cells (Bröer, 2008) allowing for greater protein synthesis. In pre-weaned pigs, Leu has been shown to increase protein synthesis in skeletal muscle (Escobar et al., 2010) and BW and mass of the longissimus dorsi muscle (Columbus et al., 2015). Current research on the effects of Leu in pre-weaned ruminants is limited.

We hypothesized that increasing amounts of supplemental Leu in milk replacer fed to Holstein bull calves would increase N retention, tissue mass, serum Leu concentrations, and influence serum AA concentrations. Therefore, the objectives of this study were to determine the effects of increasing amounts of supplemental Leu in milk replacer on pre-weaned calf growth and tissue masses, nutrient digestibility, and serum AA and metabolites.

2.3. Materials and Methods

2.3.1. Animals, Facilities, and Experimental Design

All procedures involving use of animals were approved by the North Dakota State University Institutional Animal Care and Use Committee. Twenty-three pre-weaned Holstein bull calves (43.3 ± 1.16 kg; 11.3 ± 0.57 days of age) were used. Calves were sourced from one commercial dairy. Calves were individually housed (0.91 × 1.2 m pens) in a temperature-controlled room (14ºC) on Tenderfoot flooring with rubber stall mats and no bedding, and fed twice daily (0730 and 1630 h) for 28 d. Calves received milk replacer (Nurture, Cargill Inc., Minneapolis, MN, USA) at 2.5% (DM basis) of their initial BW split evenly between daily feedings. Milk replacer was reconstituted with 1.5 L warm tap water at each feeding. The nutrient and AA composition of the milk replacer is reported in **Table 2-1**. Water was offered for ad libitum intake and starter was not provided to calves.

Table 2-1. Nutrient profile of calf milk replacer

	Treatment, g Leu/kg BW			
Item, %	0	0.4	0.6	0.8
DM, % of as fed	23.8	23.8	23.4	23.4
OM, % of DM	93.4	93.3	93.3	93.3
CP, % of DM	21.2	22.6	22.9	23.7
Crude fat, % of DM	20.0	18.6	18.3	17.5
AA[1], % of DM				
Glu	3.08	3.02	3.00	2.97
Lys	2.68	2.63	2.61	2.58
Leu	1.89	3.69	6.19	9.83
Asp	1.79	1.76	1.74	1.73
Pro	1.25	1.23	1.22	1.21
Thr	1.13	1.11	1.10	1.09
Val	1.11	1.09	1.08	1.07
Ile	1.10	1.08	1.07	1.06
Ser	0.87	0.85	0.85	0.84
Ala	0.86	0.84	0.84	0.83
Phe	0.73	0.72	0.71	0.70
Arg	0.59	0.58	0.57	0.57
Tyr	0.57	0.56	0.55	0.55
Met	0.52	0.51	0.51	0.50
Gly	0.47	0.46	0.46	0.45
His	0.42	0.41	0.41	0.40

[1]From analysis of milk replacer powder. Amino acid concentrations were calculated for treatments with added Leu from AA concentration in milk replacer and the amount of added Leu for each treatment.

Calves were blocked by arrival date (n = 4 per block except for 1 block with n = 3) into one of four treatment groups: milk replacer with no supplemental Leu or with 0.4, 0.6, or 0.8 g/kg initial BW added Leu (99.7% purity; Ajinomoto, Raleigh, NC, USA). Daily Leu allotments were split evenly between feedings and mixed with milk replacer prior to feeding. Amounts of supplemental Leu were chosen to include values above and below (0.63 g/kg BW) the amounts provided parenterally by Boutry et al. (2013) to piglets that resulted in increased skeletal muscle protein synthesis.

2.3.2. Sample Collection

Calves were weighed for 2 d at the beginning of the experiment (d 0 and d 1), and on d 18, 22, 28, and immediately prior to tissue collection on d 29. Blood samples were collected via jugular venipuncture prior to and 2 h post-feeding on d 1, 18, 22, and 28 for analyses of serum metabolites and AA. Blood samples were allowed to sit at room temperature to clot for 30 min prior to being placed on ice and transferred to the laboratory. Serum was harvested by centrifugation (3,000 × g at 4ºC) for 20 min, transferred to microcentrifuge tubes, and stored at -20ºC until analyses.

To determine effects of increasing amounts of supplemental Leu on nutrient digestibility and N retention, calves were adapted to metabolism crates from d 19 to 21; one calf from the 0.6 g Leu/kg BW treatment was removed as it did not adapt to the crate. Milk replacer (100 mL) was sampled from d 21 to 28 and total collection of feces and urine occurred from d 21 to 28, as described previously (Swanson et al., 2000, 2004). Daily fecal output was recorded composited across days within animal, and stored at -20ºC until laboratory analyses. Urine was collected into 10-L jugs containing 100 mL of 6 *N* HCl to prevent N volatilization. A total of 10% of daily urine output was retained, composited across days within animal, and stored at -20ºC until laboratory analyses. Nitrogen retention was calculated as N intake minus N output in urine and feces.

On d 29, calves were removed from metabolism crates, weighed, and stunned via captive bolt before exsanguination. Contents of the digestive tract were removed and masses of reticulorumen, omasum, abomasum, small intestine, colon, cecum, liver, pancreas, spleen, visceral fat, kidneys, heart, lungs, semimembranosus, semitendinosus, and longissimus dorsi were recorded. Tissue masses are reported on an absolute (g) and proportional (g/kg BW) basis.

2.3.3. Sample Analyses

Feces and milk replacer were thawed at room temperature (18ºC) and subsequently dried (60ºC) in a forced-air oven for 48 h, ground to pass a 1-mm screen, and analyzed (AOAC Int., 2006) for DM (method 934.01), OM (method 942.05), and N (method 2001.11). Urine was analyzed for N (method 2001.11, AOAC Int., 2006). Crude protein concentration was calculated as 6.25 × N. Amino acid concentration of milk replacer was analyzed by HPLC (method 982.30, AOAC Int., 2006).

Serum glucose concentration was measured using the hexokinase/glucose-6-phosphate dehydrogenase method (Farrance, 1987) using the Infinity Glucose hexokinase kit (Thermo Trace, Louisville, KY, USA). Urea N concentration in serum and urine was measured (Jung et al., 1975) using the QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, USA) according to manufacturer's instructions.

Serum free AA concentrations were analyzed by reversed phase UPLC after pre-column derivatization of AA with aminoquinolyl-n-hydroxysuccinimidyl carbamate (Salazar et al., 2012). Chromatography was achieved on a BEH C_{18} column (2.1 × 150 mm; 1.7 µm; Waters Corp., Milford, MA, USA). The combined flow rate of the mobile phase was constant at 0.2 mL/min. The initial mobile phase was composed of ultrapure water containing 30 mmol/L ammonium acetate and acetonitrile (containing 0.15% (v/v) formic acid) at a flow rate of 0.7 mL/min. The column was maintained at 43ºC and injection volumes were 1 µL. Concentrations of AA were quantified in reference to norvaline and measured at 260 nm (Acquity UPLC; Waters Corp., Milford, MA, USA).

Serum insulin concentrations were analyzed using a bovine insulin ELISA kit (RK00485; ABclonal, Woburn, MA, USA) on a spectrophotometer (Synergy H1, BioTek, Winsooki, VT, USA) according to manufacturer instructions.

For western blot analysis, approximately 500 mg of frozen semitendinosus (ST) and longissimus dorsi (LD) tissue was homogenized in 4 mL RIPA buffer (R0278; Sigma-Aldrich Corp., St. Louis, MO) which included a protease inhibitor cocktail (1%; #5871; Cell Signaling Tech., Danvers, MA) and a phosphatase inhibitor solution (1%; #5870; Cell Signaling Tech.) using a polytron (Brinkmann Instruments Inc., Westbury, NY, USA) and immediately put on ice afterwards. The samples were then centrifuged (13,000 × g, 4 °C, 15 min) and the supernatant was stored at -20 °C. Concentration of protein was evaluated using the bicinchoninic acid procedure with BSA as the standard (Keomanivong et al., 2017).

2.3.3.1. Analysis of p4E-BP1 and eIF4E in Semitendinosus and eIF4E in Longissimus Dorsi

From each homogenate, 112.5 µg protein for later detection of p4E-BP1 and eIF4E was separated on SDS-PAGE using a 5% stacking and 15% separating SDS-gel, and electrotransferred to a PVDF membrane (0.2 µm; #1704156; Bio-Rad Life Science, Hercules, CA) using a Trans Blot Turbo Transfer System (#1704150; Bio-Rad Life Science). In each SDS-PAGE, a 10 to 250 kDa molecular weight marker (#1610375; Bio-Rad Life Science) was separated in order to evaluate the size of detected proteins. The amount of protein selected for loading was within the linear range of band intensity. Blocking was done in 5% goat serum (#100371-988; VWR, Radnor, PA) in PBST for 1 h at room temperature for p4E-BP1 detection in the ST or, for eIF4E detection in the ST, in 5% non-fat dry milk (NFDM) powder in PBST for 1 h at room temperature. Blocking was done in 5% goat serum (#100371-988; VWR) in PBST for 1 h at room temperature for eIF4E detection in the LD. Afterwards, membranes were washed

3 × 5 min in 1x PBST. The membranes were incubated with primary antibodies against p4E-BP1 (#9459; Cell Signaling Tech.) or eIF4E (#9742; Cell Signaling Tech.) at a dilution of 1:5000 in 5% heat-inactivated goat serum in 1x PBST for p4E-BP1in ST, in 1x PBST for eIF4E in ST, or in 5% heat-inactivated goat serum in 1x PBST for eIF4E in LD at 4 °C overnight. The membranes were washed 3 × 10 min in 1x PBST, and then incubated at room temperature with a horseradish peroxidase (HRP)-linked goat anti-rabbit IgG secondary antibody (#7074; Cell Signaling Tech.) at a dilution of 1:10,000 and a StrepTactin-HRP conjugate (#1610380; Bio-Rad Life Science) at a dilution of 1:100,000 for 1 h in 5% heat-inactivated goat serum in 1x PBST for p4E-BP1 in ST, in 1x PBST for eIF4E in ST, or 5% heat-inactivated goat serum in 1x PBST for eIF4E in LD. Membranes were washed 3 × 10 min in 1x PBST. Afterwards, blots were developed by Amersham ECL Prime Western Blotting Detection Reagent (RPN2232; Cytiva Life Sciences, Marlborough, MA) for 5 min at room temperature, and the intensities of the specific bands were detected with a FluorChem FC2 system (Protein Simple, San Jose, CA, USA) after an exposure time of 1.5× the recommended exposure time, and quantified by AlphaEaseFC (Protein Simple) software.

2.3.4. Statistical Analysis

Data were analyzed as a randomized complete block design (block = arrival date group; n = 6 blocks). Nutrient intake, excretion, disappearance, and digestibility, and N retention, tissue masses, and protein abundance were analyzed using the MIXED procedure in SAS (SAS 9.4, SAS Institute Inc., Cary, NC, USA). The experimental unit was calf, and the model included effects of block and treatment. Linear and quadratic effects of treatment were determined using orthogonal contrast statements. Because of unequal spacing of supplemental Leu treatments, the IML procedure of SAS was used to develop contrast coefficients. Serum AA and metabolites

were analyzed using the MIXED procedure in SAS with repeated measures. The experimental unit was calf, the repeated effect was day, arrival date served as block, and the model included effects of day, treatment, time, and all interactions. Linear and quadratic effects of treatment were determined by orthogonal contrasts. Appropriate (minimize information criterion) covariance structures were used (Wang and Goonewardene, 2004). Significance was declared at $P \leq 0.05$ and a tendency at $0.05 < P < 0.10$.

2.4. Results

Initial and final BW, and ADG were not influenced by supplemental Leu treatment (**Table 2-2**). Dry matter and OM intake, excretion, disappearance, and digestibility were not influenced by supplemental Leu treatment (**Table 2-3**). Intake, excretion, and digestibility of N were not influenced by supplemental Leu treatment, but there was a linear increase ($P = 0.04$) in N disappearance. Nitrogen retention also did not differ among treatments.

Table 2-2. Body weight of calves fed milk replacer with increasing amounts of supplemental leucine

	Treatment, g Leu/kg BW					Contrasts	
Item	0	0.4	0.6	0.8	SEM[1]	Linear	Quadratic
Initial BW, kg	43.8	43.0	44.5	43.2	2.28	0.96	0.97
Final BW, kg	58.7	57.4	60.5	60.0	3.51	0.73	0.78
ADG, kg/d	0.533	0.516	0.574	0.601	0.059	0.38	0.56

[1]Standard error of the mean (n = 6 for 0, 0.6, and 0.8 g Leu/kg BW treatments; n = 5 for 0.4 g Leu/kg BW treatments).

Table 2-3. Nutrient intake, excretion, disappearance, digestibility, and N retention of calves fed milk replacer with increasing amounts of supplemental leucine

	Treatment, g Leu/kg BW					Contrasts	
Item	0	0.4	0.6	0.8	SEM[1]	Linear	Quadratic
Intake, g/d							
DM	1052	1063	1063	1100	55.4	0.58	0.78
OM	983	992	992	1025	51.6	0.60	0.78
N	34.0	38.4	38.9	41.7	2.30	0.02	0.98
Excretion, g/d							
Fecal DM	48.1	33.1	51.7	56.3	12.11	0.55	0.29
Fecal OM	40.5	27.6	42.3	47.8	11.00	0.59	0.30
Fecal N	3.89	2.84	4.16	4.48	0.791	0.52	0.26
Urinary N	5.82	5.73	7.15	7.50	1.078	0.21	0.60
Total N	9.71	8.57	11.3	12.0	1.46	0.21	0.32
Disappearance, g/d							
DM	1004	1022	1018	1043	8.1	0.66	0.96
OM	942	958	955	978	7.3	0.67	0.95
N	30.1	35.6	34.8	37.3	2.29	0.04	0.67
Digestibility, %							
DM	95.3	96.0	95.6	95.0	0.21	0.72	0.28
OM	95.8	96.3	96.2	95.5	0.19	0.75	0.28
N	88.3	92.6	89.3	89.3	1.94	0.80	0.21
N Retained							
g	24.3	29.9	27.6	29.8	2.26	0.12	0.50
% of N intake	70.8	77.5	71.0	71.6	3.44	0.99	0.26

[1]Standard error of the mean (n = 6 for 0, 0.6, and 0.8 g Leu/kg BW treatments; n = 5 for 0.4 and 0.6 g Leu/kg BW treatments).

Increasing supplemental Leu did not influence mass (g and g/kg BW; **Table 2-4**) of the small intestine, reticulorumen, or abomasum. Mass of omasum (g and g/kg BW) responded quadratically ($P = 0.01$) to supplemental Leu treatment where Leu supplemented at 0.4 g/kg BW decreased tissue mass compared to other treatments. Tissue mass (g and g/kg BW) of the colon, cecum, and liver were not influenced by supplemental Leu treatment. Pancreas mass (g) increased linearly ($P = 0.05$) with increasing Leu supplementation and was affected quadratically ($P = 0.04$) when expressed relative to BW (g/kg BW) as relative mass decreased with Leu supplementation of 0.4 g Leu/kg BW and then increased with increasing amounts of supplemental Leu. Mass of the spleen (g and g/kg BW) responded quadratically ($P = 0.02$) to increasing supplemental Leu as tissue mass decreased at Leu supplementation of 0.4 g Leu/kg BW and then increased with increasing amounts of supplemental Leu. Mass (g and g/kg BW) of

visceral fat, kidney, heart, lung, and semimembranosus muscle were not affected by treatment. Relative mass (g/kg BW) of the semitendinosus muscle tended ($P = 0.07$) to respond quadratically with increasing amounts of supplemental Leu as relative tissue mass increased in calves on the 0.4 g Leu/kg BW treatment, and then decreased with greater amounts of supplemental Leu. Mass of the longissimus dorsi (g and g/kg BW) was not affected by increasing amounts of supplemental Leu.

Table 2-4. Tissue masses of calves fed milk replacer with increasing amounts of supplemental leucine

	Treatment, g Leu/kg BW					Contrasts	
Tissue mass, g	0	0.4	0.6	0.8	SEM[1]	Linear	Quadratic
Small intestine							
g	1456	1554	1449	1574	32.6	0.55	0.90
g/kg BW	24.7	27.0	23.7	26.0	1.24	0.79	0.74
Reticulorumen							
g	381	281	344	340	20.7	0.58	0.31
g/kg BW	6.34	5.04	5.60	5.56	0.62	0.38	0.32
Abomasum							
g	308	298	326	295	6.9	0.94	0.92
g/kg BW	5.32	5.06	5.32	4.91	0.32	0.50	0.84
Omasum							
g	104	72	86	110	8.64	0.91	0.01
g/kg BW	1.77	1.32	1.41	1.84	0.14	0.93	<0.01
Colon							
g	430	451	423	416	7.6	0.83	0.83
g/kg BW	7.23	7.55	6.91	6.81	0.59	0.54	0.58
Cecum							
g	57.4	54.9	62.5	64.4	2.20	0.26	0.40
g/kg BW	0.98	0.94	1.01	1.07	0.07	0.32	0.35
Liver							
g	1486	1357	1600	1606	58.8	0.25	0.32
g/kg BW	25.2	24.0	26.2	26.8	1.11	0.23	0.24
Pancreas							
g	51.3	46.0	58.6	63.3	3.84	0.05	0.14
g/kg BW	0.87	0.82	0.95	1.05	0.05	<0.01	0.04
Spleen							
g	190	154	197	208	11.8	0.20	0.02
g/kg BW	3.21	2.66	3.25	3.45	0.17	0.23	0.01
Fat							
g	486	454	488	479	7.8	0.99	0.78
g/kg BW	8.12	7.50	7.86	7.84	0.67	0.78	0.64
Kidney							
g	305	281	325	332	11.5	0.43	0.48
g/kg BW	5.23	4.97	5.40	5.51	0.53	0.67	0.62
Heart							
g	452	587	486	499	28.7	0.17	0.30
g/kg BW	7.67	9.75	7.96	8.27	0.85	0.76	0.22
Lung							
g	1086	922	957	1038	37.4	0.75	0.33
g/kg BW	19.3	15.0	15.5	17.8	2.90	0.61	0.30
Semimembranosus							
g	755	834	828	784	18.7	0.64	0.42
g/kg BW	13.0	14.2	13.6	13.0	0.84	0.97	0.24
Semitendinosus							
g	256	269	275	262	4.3	0.75	0.52
g/kg BW	4.31	4.71	4.48	4.31	0.16	0.94	0.07
Longissimus dorsi							
g	304	299	311	300	2.7	0.97	0.87
g/kg BW	5.10	5.33	5.07	4.90	0.33	0.65	0.47

[1]Standard error of the mean (n = 6 for 0, 0.6, and 0.8 g Leu/kg BW treatments; n = 5 for 0.4 g Leu/kg BW treatments).

There were no interactions between treatment, day, and time for serum urea N or glucose concentration. Increasing amounts of supplemental Leu had no effect on serum urea N concentration (**Table 2-5**). Serum glucose concentration tended ($P = 0.10$) to be affected quadratically by treatment where glucose concentration was lower in calves supplemented with 0.4 and 0.6 g Leu/kg BW than calves supplemented with the control and 0.8 g Leu/kg BW treatments. Serum urea N concentration decreased ($P = 0.05$) over time (**Table A1**) and serum glucose concentration increased ($P < 0.01$) up to d 22 and then decreased on d 28.

Table 2-5. Serum metabolites of calves fed milk replacer with increasing amounts of supplemental leucine

	Treatment, g Leu/kg BW					Contrasts	
Item, mg/dL	0	0.4	0.6	0.8	SEM	Linear	Quadratic
Urea N[2]	5.07	5.30	5.67	5.46	0.29	0.21	0.69
Glucose[3]	104	96	92	102	4.63	0.54	0.10

[1]Standard error of the mean (n = 6 for 0, 0.6, and 0.8 g Leu/kg BW treatments; n = 5 for 0.4 g Leu/kg BW treatments).
[2]Interaction effects for urea N: day × time ($P = 0.99$), day × treatment ($P = 0.15$), time × treatment ($P = 0.98$), and day × treatment × time ($P = 0.96$).
[3]Interaction effects for glucose: day × time ($P = 0.12$), day × treatment ($P = 0.30$), time × treatment ($P = 0.44$), and day × treatment × time ($P = 0.57$).

A treatment × day × time interaction was observed ($P = 0.002$) for serum Leu concentration (**Fig. 2-1**). Serum Leu concentrations increased after feeding for all treatments and serum Leu increased linearly across treatments post-feeding, with d 18 having the greatest serum Leu concentrations.

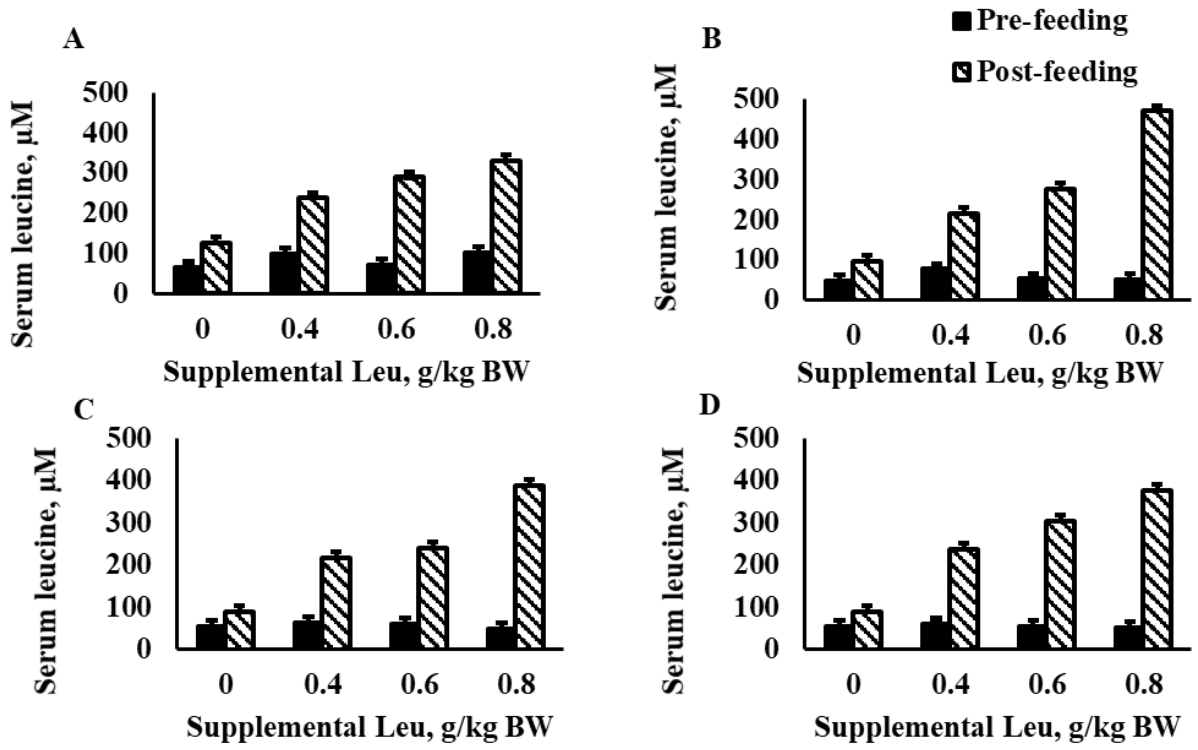

Figure 2-1. Serum leucine concentration of calves fed milk replacer with increasing amounts of supplemental leucine on day 1 (A), 18 (B), 22 (C), and 28 (D). Treatments were 0, 0.4, 0.6, or 0.8 g/kg BW supplemental leucine to milk replacer. Effects for serum leucine were Day × Time × Treatment ($P = 0.002$), Time × Treatment ($P < 0.001$), Day × Treatment ($P = 0.03$), Treatment ($P < 0.001$), Day × Time ($P = 0.22$), Time ($P < 0.001$), and Day ($P = 0.20$).

No interactions were observed for treatment × day × time, treatment × time, or day × time for serum AA concentrations. Day × treatment interactions (**Table A2**) were observed for serum Glu ($P = 0.03$) and Ser ($P = 0.04$) concentrations, but these interactions were largely driven by differences in variability within specific days more so than changes in the order of response to supplemental Leu.

Serum Arg concentration linearly decreased ($P = 0.04$) with increasing amounts of supplemental Leu (**Table 2-6**). Serum His concentration was unaffected by increasing amounts of supplemental Leu. Serum Ile concentration linearly decreased ($P = 0.02$) with increasing amounts of supplemental Leu, and serum Lys concentration was affected quadratically ($P =$

0.03) with Lys concentration decreasing in calves supplemented with 0.4 g Leu/kg BW and then increasing with greater amounts of supplemental Leu. Serum Met concentration linearly decreased ($P < 0.01$) with increasing amounts of supplemental Leu, and serum Phe concentration tended ($P = 0.06$) to be affected quadratically where Phe concentration increased at lower levels of supplemental Leu. There was a tendency ($P = 0.07$) for a quadratic effect for serum Thr concentration, where Thr concentration decreased at lower levels of supplemental Leu. Serum Trp concentration was unaffected by increasing amounts of supplemental Leu. There was a tendency ($P = 0.09$) for a quadratic effect for serum Val concentration where Val concentration increased at lower levels of supplemental Leu. Also, quadratic effects ($P = 0.02$) of increasing supplemental Leu were observed for serum essential AA (EAA) concentrations with EAA concentrations decreasing with lower levels of supplemental Leu and then increasing at greater levels of supplemental Leu.

Serum Ala concentration linearly decreased ($P < 0.01$) with increasing amounts of supplemental Leu, and serum concentration of Asn was unaffected by increasing amounts of supplemental Leu. Serum Asp concentration tended ($P = 0.07$) to linearly decrease with increasing amounts of supplemental Leu. Serum Gly concentration was unaffected by increasing amounts of supplemental Leu, and serum Glu concentration was affected quadratically ($P = 0.04$) with Glu concentration decreasing in calves supplemented with 0.4 g Leu/kg BW and then increasing with greater amounts of supplemental Leu. Increasing amounts of supplemental Leu had no effect on serum Gly concentration. Serum Pro concentration linearly decreased ($P = 0.05$) with increasing amounts of supplemental Leu, and serum Ser concentration was unaffected by increasing amounts of supplemental Leu. There was a tendency ($P = 0.08$) for a linear effect for serum Tyr concentration, where Tyr concentration decreased as supplemental Leu increased.

However, serum total nonessential AA (NEAA) concentration was not influenced by supplemental Leu treatment. Serum total AA concentration was affected quadratically ($P = 0.05$) where total AA concentration decreased at lower levels of supplemental Leu and increased at greater levels of supplemental Leu.

Table 2-6. Serum AA concentrations of calves fed milk replacer with increasing amounts of supplemental leucine[1]

	Treatment, g Leu/kg BW					*P*-value	Contrasts	
AA, µM	0	0.4	0.6	0.8	SEM[2]	Trt	Linear	Quadratic
Arg	225	203	182	207	9.3	0.02	0.04	0.06
His	31.6	31.0	35.0	28.1	2.58	0.32	0.65	0.30
Ile	55.2	49.8	47.8	46.7	2.74	0.14	0.02	0.70
Leu	78.3	151	169	228	10.2	<0.01	<0.01	0.44
Lys	303	234	251	298	26.6	0.19	0.69	0.03
Met	365	192	232	194	39.1	0.01	<0.01	0.14
Phe	48.0	58.7	53.9	47.2	4.86	0.29	0.97	0.06
Thr	141	110	126	134	10.8	0.24	0.61	0.07
Trp	38.1	32.4	36.5	34.8	1.77	0.16	0.29	0.21
Val	103	113	99.2	90.5	6.93	0.17	0.18	0.09
Total EAA[3]	1388	1181	1233	1312	57.7	0.08	0.25	0.02
Ala	280	233	208	213	15.5	0.01	<0.01	0.33
Asn	46.4	39.6	45.6	46.2	3.26	0.40	0.95	0.17
Asp	18.1	14.3	15.7	15.5	0.98	0.06	0.07	0.08
Gln	181	210	194	195	11.6	0.42	0.46	0.22
Glu	119	91.9	101	119	11.0	0.21	0.82	0.04
Gly	443	415	417	442	22.7	0.72	0.84	0.26
Pro	112	93.9	102	95.6	5.37	0.09	0.05	0.30
Ser	113	109	107	110	4.1	0.76	0.51	0.46
Tyr	32.0	32.7	27.5	27.8	2.06	0.18	0.08	0.51
Total NEAA[4]	1351	1250	1222	1270	50.9	0.33	0.16	0.25
Total AA	2739	2427	2455	2581	98.9	0.12	0.16	0.05

[1]Interaction effects of day × time ($P \geq 0.13$), day × treatment ($P \geq 0.12$), time × treatment ($P \geq 0.15$), and day × time × treatment ($P \geq 0.14$).
[2]Standard error of the mean (n = 6 for 0, 0.6, and 0.8 g Leu/kg BW treatments; n = 5 for 0.4 g Leu/kg BW treatments).
[3]Essential AA.
[4]Non-essential AA.

There were also effects of day on serum AA concentrations, where serum concentration of Ala, Arg, Asp, Glu, Gly, His, Ile, Lys, Pro, Ser, Thr, and Val decreased ($P < 0.03$) and Asn, Gln, Met, Phe, Trp, and Tyr increased ($P < 0.02$) over the experimental period (**Table A3**). Serum concentrations post-feeding were elevated ($P < 0.04$) for Ala, Arg, Asn, Asp, Gln, His,

Ile, Lys, Met, Pro, Ser, Thr, Trp, Tyr, and Val concentrations, but serum Gly concentration decreased ($P < 0.01$) after feeding (**Table A4**).

There were no interactions between treatment, day, and time for serum insulin. Serum insulin concentration tended ($P = 0.10$) to be affected linearly by treatment where insulin concentration decreased with increasing amounts of supplemental Leu (**Table 2-7**). Serum insulin tended ($P = 0.10$) to increase over time (**Table 2-8**).

Table 2-7. Serum insulin concentrations of calves fed milk replacer with increasing amounts of supplemental leucine

	g Leu/kg BW					Contrasts	
Item, mg/dL	0	0.4	0.6	0.8	SEM[1]	Linear	Quadratic
Insulin	3044	2933	2728	2559	223	0.10	0.67

[1]Standard error of the mean (n = 6 for 0, 0.6, and 0.8 g Leu/kg BW treatments; n = 5 for 0.4 g Leu/kg BW treatments)

Table 2-8. Serum insulin concentrations of calves fed milk replacer throughout the experimental period

	Day					*P*-value
Item, mg/dL	1	18	22	28	SEM	Day
Insulin	2592	2667	3003	3002	157	0.10

There was no effect of increasing supplemental Leu on protein abundance of p4E-BP1 (**Table 2-9**) or eIF4E (**Table 2-10**) in ST or LD tissue.

Table 2-9. p4E-BP1 immunoreactive bands in ST muscle, normalized to band 3 of pooled control

	g Leu/kg BW					Contrasts	
	0	0.4	0.6	0.8	SEM	Linear	Quad
Band 1, 16 kDa	0.057	0.136	0.042	0.138	0.0578	0.51	0.99
Band 2, 18.5 kDa	0.427	0.905	0.267	0.793	0.2652	0.61	0.86
Band 3, 19 kDa	0.628	0.587	1.36	1.22	0.7179	0.46	0.87
Total	1.11	1.63	1.67	2.15	0.9101	0.44	0.94

Table 2-10. eIF4E immunoreactive bands in semitendinosus (ST) and longissimus dorsi (LD) muscle.

	g Leu/kg BW					Contrasts	
	0	0.4	0.6	0.8	SEM	Linear	Quad
ST, 25 kDa	0.946	0.979	0.948	1.00	0.0601	0.61	0.89
LD, 25 kDa	1.20	1.16	0.980	1.12	0.0986	0.30	0.61

2.5. Discussion

Meeting the AA needs of pre-ruminants may allow for more efficient utilization of milk replacer and increased growth. Leucine has been observed to increase muscle mass and growth performance in non-ruminants (Escobar et al., 2010; Columbus et al., 2015). Because data are limited on Leu supplementation in ruminants and because pre-weaning growth rate in calves has been associated with increased milk production during their first lactation (Soberon et al., 2012), our objectives were to determine how increasing amounts of supplemental Leu affects calf growth, serum AA, and tissue growth and development. Limited research on supplemental Leu in calves fed milk replacer had been conducted at the time this research was designed. We felt that an appropriate starting point for this research was to examine graded amounts of supplemental Leu. Future research should also examine other nutritional factors that could influence the response to supplemental Leu, such as examining different ratios of Leu to other amino acids at similar CP intake and milk replacer differing in nutrient composition or energy density. Similar experimental designs, where Leu was supplemented or provided parenterally without adjustments for dietary CP, have been used in the foundational studies with piglets showing positive effects on muscle protein synthesis (Escobar et al, 2010; Boutry et al., 2015; Columbus et al., 2015).

In our study, supplemental Leu did not influence calf BW or ADG. Our results are similar to results from Cao et al. (2018) who reported that final BW of 8-week-old Holstein calves was not influenced by Leu supplementation at 1.435 g Leu/L milk (approximately 0.2 g Leu/kg BW). However, supplemental Leu has been shown to increase growth and muscle development in pre-weaned pigs when supplied to a restricted protein diet (Columbus et al.,

2015). It is possible that Leu supplementation to pre-weaned pigs on a restricted protein diet was able to compensate for the deficiency in dietary protein.

Currently, data are limited on effects of supplemental Leu on digestibility in calves. Blaxter and Wood (1951) reported DM digestibility of skim milk with lard was 94%, similar to our result of 95%. More recent data is limited on calf nutrient digestibility when fed diets of milk replacer without starter feed. As the digestibility of the milk replacer was high, it may not be surprising that no differences in digestibility were observed with supplemental Leu feeding. Similarly, N retention was not influenced by supplemental Leu which suggests that Leu did not promote an increase in overall N retention by calves. Gerrits (2019) reported that N retention of pre-weaned calves is around 70%, which is similar to our result of 73%. Although it is unknown why increasing amounts of supplemental Leu did not influence ADG, nutrient digestibility, or N balance, it is possible that inadequate supply of other EAA limited the potential effects of Leu or that the sensitivity of approaches used for measuring growth and N balance used in the present experiment were not great enough to measure treatment differences. In support of the concept that alternative measures other than growth or N balance may be more appropriate for examining subtle dietary effects on N utilization, Pencharz and Ball (2003) reported that use of N balance and growth measurements in humans may be a less sensitive measurement than measuring plasma AA levels to assess AA and protein status. However, it should be noted that N retention numerically increased in the 0.4 g/kg BW treatment and then plateaued with increasing supplemental Leu. This may suggest that the effects of supplemental Leu could be observed by utilizing greater experimental units or more sensitive approaches for measuring whole animal N metabolism.

Proportional tissue mass (g/kg BW) will be discussed because of the effect that overall BW has on absolute tissue mass (g). Pancreatic proportional mass was decreased at 0.4 g/kg BW of supplemental Leu and increased with greater amounts of supplemental Leu. These changes may have been because of a shift towards more protein deposition in skeletal muscle at lower amounts of supplemental Leu and increased protein deposition in the pancreas at greater Leu supplementation levels. Differences in pancreatic mass could potentially influence pancreatic digestive enzymes (Swanson et al., 2002) and insulin production (Baumgard et al., 2016). Changes in enzyme activity could alter the ability to degrade and absorb nutrients from the diet, and alterations in insulin production could influence signaling for protein synthesis (Baumgard et al., 2016) and energy utilization (van den Borne et al., 2006). Similar to the pancreas, spleen and omasum masses relative to BW were quadratically affected by increasing amounts of supplemental Leu which supports the suggestion of a shift in site of protein deposition from visceral tissues to skeletal muscle, at least at lower amounts of supplemental Leu.

The tendency for an increase in semitendinosus proportional mass in calves receiving 0.4 g Leu/kg BW could indicate increased protein synthesis relative to other tissues at lower supplementation levels. This observed tendency for an increase in semitendinosus mass could potentially be specific for muscle protein synthesis as other tissues either had reduced relative masses at 0.4 g Leu/kg BW or no observed changes. Similar numerical trends to increasing Leu supplementation were observed for semimembranosus and longissimus mass suggesting that the number of experimental units may not have been adequate to detect differences. However, specific muscles differ in regards to function, cellular and molecular composition, and rates of growth during development (Jury et al., 1977; Schreurs et al., 2008). Specifically, the semitendinosus has a greater proportion of glycolytic cells with larger muscle fibers (Schreurs et

al., 2008) and has a rapid linear growth trajectory, whereas the longissimus has a greater proportion of oxidative cells with smaller muscle fibers and rapid growth early in development followed by a decreasing growth rate in later development (Jury et al., 1977). This could suggest muscle-specific effects which could be associated with differences in function and growth rate. Further research is needed to further study potential muscle-specific effects of supplemental Leu.

Serum glucose concentration tended to be quadratically affected with an observed decrease in serum glucose in calves supplemented with 0.4 and 0.6 g Leu/kg BW. Leucine is a ketogenic AA (Brosnan and Brosnan, 2006; Voet et al., 2008) and does not have a direct role in glucose production. However, the tendency for an increase in semitendinosus proportional mass could be associated with increased muscle protein synthesis and an increased demand for glucose, resulting in increased blood glucose utilization (Santos and Sabatinie, 2017). Others have reported no effects of Leu supplementation on glucose concentrations (Manjarín et al. 2016; Cao et al., 2018) in pre-weaned calves and piglets. The reasons for this may be because only a single sample collected at harvest was analyzed in the calf study (Cao et al., 2018) and the piglet diet was restricted in CP which could have decreased the magnitude of response on protein synthesis (Manjarín et al. 2016).

As expected, serum Leu concentration increased as supplemental Leu increased, with greater increases following feeding. Our results are similar to those of Cao et al. (2018) who reported increasing plasma Leu in calves supplemented with Leu. Increased concentrations of serum Leu has been reported to be associated with decreased plasma concentrations of the other branched-chain AA (BCAA), Val and Ile in piglets (Boutry et al., 2013). Similarly, as Leu supplementation increased in our study, both Val and Ile decreased. The BCAA are catabolized in muscle tissue by branched-chain aminotransferase (Brosnan and Brosnan, 2006). Both Ile and

Val were observed to decrease at greater Leu concentrations, and may indicate they were catabolized in muscle tissue or Leu may have decreased the absorption of Ile and Val resulting in less circulating Ile and Val. Loest et al. (2001) reported that in growing steers fed a soybean hull-based diet and abomasally infused with AA with or without specific BCAA, removal of Leu from the AA infusate increased plasma Ile and Val concentration, and suggested that this may have occurred because of the stimulatory effect of Leu on BCAA oxidation. The physiological effects of the observed decreases in Ile and Val in the current experiment are unknown, but the observed results in ADG, N balance, and tissue masses suggest minimal negative effects.

Serum concentrations of EAA decreased at 0.4 and 0.6 g Leu/kg BW, but increased at 0.8 g Leu/kg BW. This quadratic effect was likely driven by the decreases observed in Arg, Lys, and Met. These results disagree with results of Cao et al. (2018) who reported no effects of Leu on plasma concentrations of Arg or Met. Boutry et al. (2013) reported lower concentrations of serum Met in piglets supplemented Leu compared to control piglets. Zheng et al. (2019) also reported no effects of supplemental Leu on serum concentrations of Lys in calves, but reported an increase in serum Met and a decrease in serum Arg with supplemental Leu. The discrepancy between Zheng et al. (2019) and our results could be because serum collection occurred during a fasted state prior to morning feedings, whereas in the current study, samples pre- and post-feeding were analyzed. Additionally, L-Lys and DL-Met were included in the commercial milk replacer used in the current experiment. Milk replacer and serum AA analyses in the current experiment did not differentiate separate isoforms (D or L) of AA and supplemental Leu could potentially have different effects on the utilization of these isoforms. For example, it has been reported that cattle can utilize D-Met after conversion to L-Met but the conversion of D-Met to L-Met is slow (Lapierre et al., 2011), potentially decreasing its efficiency of utilization.

Regardless, our results may suggest that lower levels of supplemental Leu increased utilization of EAA for muscle protein synthesis.

Altered AA transport, metabolism, or uptake by muscle or other tissues for protein synthesis are possible reasons for the observed changes in serum AA concentrations. Alterations in serum AA concentrations in the current experiment, especially in EAA concentrations, may be because of increased competition for shared AA transporters when greater amounts of Leu were fed. Bröer (2008) reported that the AA transporter, B^0AT1, is used in the intestinal lumen to take up neutral AA into intestinal cells, with the greatest affinity for Met and BCAA. Also, as reviewed by Bröer and Fairweather (2019), Leu shares a common transporter with most of the other neutral AA (system L AA transporter 2, LAT2) in the small intestinal basolateral membrane. The observed quadratic effects of Leu supplementation on total EAA and some other individual EAA suggest that greater inclusion of supplemental Leu did not inhibit uptake of other AA.

Serum concentration of NEAA were unaffected by increasing amounts of supplemental Leu. This is likely because of minimal effects on individual NEAA, including Asp, Glu, Pro, and Tyr. Past research in calves also suggests that supplemental Leu had minimal effects on plasma or serum NEAA concentrations (Cao et al., 2018; Zheng et al., 2019). Taken together, this suggests that supplemental Leu has no effect on NEAA uptake, synthesis, or utilization by milk replacer-fed calves. Protein synthesis requires both NEAA and EAA (D'Mello, 2003). It is possible that differences in NEAA concentrations were not observed because endogenous production of NEAA may have increased when protein synthesis increased.

Besides being important in regulating blood glucose concentrations, insulin is also believed to upregulate mTORC1 activity through the Akt pathway. Furthermore, it has also been

reported that Leu increases insulin secretion from the pancreas through the mTOR pathway (Yang et al., 2010; Yang et al., 2012). Serum insulin concentrations in this study decreased with increasing amounts of supplemental Leu, but increased from d 1 to 28. However, serum glucose concentration was lower in the 0.4 and 0.6 g Leu/kg BW treatments, and increased from d 1 to 22, then decreased on d 28. Other studies have reported no effect of supplemental Leu on serum insulin concentrations in humans or piglets (Nair et al., 1992; Boutry et al., 2013; Manjarín et al., 2016). Reasons for the discrepancies between studies are unknown. It is possible that Leu supplementation may not have been at concentrations needed to elicit an increase in serum insulin concentrations.

The translation initiation factor eIF4E is a key regulator of protein translation (Suryawan and Davis, 2014), and binds with eIF4G to upregulate protein synthesis. When eIF4E is bound with eIF4E-BP1 (4E-BP1), it is unable to bind with eIF4G and protein translation cannot be carried out. When 4E-BP1 is phosphorylated (p4E-BP1), it dissociates from eIF4E, allowing it to bind to eIF4G. Past research evaluating Leu supplementation to neonatal calves showed an increase in phosphorylation of 4E-BP1, mTOR, and S6K1 in the LD of calves receiving supplemental Leu (Zheng et al., 2019). Manjarín et al. (2016) also reported an increase in phosphorylation of 4E-BP1 and S6K1 in protein and energy restricted piglets receiving supplemental Leu compared to piglets receiving a protein and energy restricted diet. These studies suggest that Leu supplementation activates the mTOR pathway, causing the phosphorylation of proteins involved in protein synthesis in muscle tissues. However, in the current study, no effect of supplemental Leu on eIF4E expression in ST or LD was observed. Coupled with the lack of effect of supplemental Leu on p4E-BP1 expression, it is possible that increasing amounts of supplemental Leu did not upregulate protein translation or synthesis. It is

unknown if supplemental Leu down-regulated protein degradation within muscle, though it seems unlikely due to the lack of BW gain and only a tendency for differences in semitendinosus proportional weight.

2.6. Conclusions

Our results showed that supplemental Leu did not influence the gross measurements of calf growth, nutrient digestibility, N balance, or protein abundance of p4E-BP1 or eIF4E. However, supplemental Leu decreased the concentration of some EAA, especially in calves supplemented with the lowest amount of supplemental Leu (0.4 g/kg BW). Furthermore, at lower levels of supplemental Leu, proportional mass of some visceral tissues decreased, proportional mass of semitendinosus tended to increase, and serum glucose concentration tended to decrease, suggesting a shift towards greater protein synthesis in muscle. Future research to examine the effects on protein synthesis and turnover, as well as mechanisms regulating translation in muscle and visceral tissues, is needed to better define the potential for Leu supplementation to increase muscle protein synthesis in the pre-weaned calf, and if these effects are associated with future productivity.

2.7. Acknowledgements

This project was supported by Animal Health and Production and Animal Products Proposal No. 2016-08395 from the USDA National Institute of Food and Agriculture. Authors would like to thank Cargill (Minneapolis, MN) for supplying milk replacer and Ajinomoto (Chicago, IL) for providing Leu used in this study. The authors would like to thank the employees of the NDSU Animal Nutrition and Physiology Center (Fargo, ND), the Nutrition, Physiology, and Muscle Biology Laboratories in the Department of Animal Sciences at North Dakota State University (Fargo, ND), and students who assisted with this project.

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3. EFFECTS OF SUPPLEMENTAL LEUCINE IN MILK REPLACER ON PRE- AND POST-WEANING GROWTH, CARCASS CHARACTERISTICS, SERUM AMINO ACIDS, AND VISCERAL TISSUE MASS IN LAMBS

3.1. Abstract

To determine long-term effects of leucine supplementation in milk replacer on growth performance, 19 commercial Dorset ram lambs (5.07 ± 0.15 kg) were used. Leucine was added to milk replacer at 0 (control) or 2.9% of DM and provided to lambs for ad libitum intake for 42 d using automated milk feeders. Lambs were then fed a corn-based finishing diet and slaughtered in two blocks based on final body weight (BW). Body weight and average daily gain (ADG) during the pre-weaning and finishing phase, tissue mass, serum amino acid (AA) and metabolite concentrations, finishing intake and ADG, and carcass characteristics were determined. Data were analyzed using the MIXED and GLM procedures of SAS. Leucine supplementation increased lamb BW ($P < 0.001$) by d 7 and ADG ($P = 0.007$) during the pre-weaning phase. Final finishing BW, ADG, and total weight gain during the finishing phase were not affected by pre-weaning Leu supplementation. Mass of the reticulorumen tended ($P = 0.09$) to be greater in lambs supplemented with Leu pre-weaning; no other tissue masses were affected by pre-weaning Leu supplementation. Hot carcass weight and 12th rib fat thickness were unaffected by treatment. Thickness at the body wall was greater ($P = 0.05$) in lambs supplemented with Leu pre-weaning. Longissimus area, yield grade, quality grade, and percent boneless, closely trimmed retail cuts were not different between treatments. Results suggest that supplemental Leu during the pre-weaning period increases growth in the pre-weaning period without negatively affecting lamb performance in the post-weaning period.

3.2. Introduction

Leucine is an essential AA and is required in diets to meet physiological needs; however, in typical diets it is not believed to be limiting for production (Wu, 2009). Leucine has numerous effects on metabolism (Wu, 2009; Dodd and Tee, 2012; Millward, 2012) and is the primary AA signal for increasing muscle protein synthesis (Pedroso et al., 2015). Leucine provided to pre-weaned pigs has been shown to increase skeletal muscle protein synthesis (Escobar et al., 2010) and BW and mass of the longissimus dorsi muscle (Columbus et al., 2015). Data are limited on the effects of supplemental Leu in pre-weaned lambs.

Lambs from twin or triplet births and twin lambs with a greater range in birth weights have been reported to have lower survivability during the pre-weaning period (Miller et al., 2010; Juengel et al., 2018). Removing lambs with lower birth weights and rearing with milk replacer may increase lamb survivability and production later in life. Chai et al. (2018) reported that removing a twin lamb from its dam after 10, 20, or 30 days and reared on milk replacer until 60 days of age resulted in greater ADG than their siblings that remained with their dam. Additionally, Soberon et al. (2012) reported that increasing ADG during the pre-weaning period in Holstein heifers increased milk production during the first lactation suggesting pre-weaning programming of productivity later in life.

The objectives of this study were to determine the effects of supplemental Leu to lambs during the pre-weaning period on ADG and serum AA during the pre-weaning period, DMI and ADG during the finishing period, visceral organ masses, and carcass characteristics of lambs. We hypothesized that supplemental Leu in milk replacer fed to lambs would increase pre- and post-weaning ADG, gain:feed in the finishing phase, and the cutability of carcasses.

3.3. Materials and Methods

3.3.1. Animals, Facilities, and Experimental Design

All procedures involving the use of animals were approved by the North Dakota State University (NDSU) Institutional Animal Care and Use Committee. Nineteen neonatal fall-born ram lambs (5.07 ± 0.15 kg; twin-born n = 16, triplet-born n = 3) predominately of Dorset breeding were used. Lambs remained with ewes (2.8 ± 0.4 years old) for 12 h post-birth to ensure adequate colostrum intake and then randomly allotted to either a control milk replacer (Shepherd's Choice, Premier1 Supplies, Washington, IA, USA; n = 10) or milk replacer with 2.9% (DM basis) added Leu (n = 9). Lambs were housed at the NDSU Sheep Unit and trained to consume milk from LAC-TEK Stainless 61450 milk dispensers (1 per treatment; Biotic Industries, Inc., Bell Buckle, TN, USA) which allowed for ad libitum consumption of milk replacer. LAC-TEK machines were calibrated to deliver one part milk replacer (**Table 3-1**) and four parts heated water.

Table 3-1. Dietary composition and nutrient concentrations of milk replacer and milk replacer supplemented with leucine (DM basis).

Ingredient	Milk replacer	Milk replacer + leucine
Milk replacer	100	97.1
Leu	0	2.9
Nutrient Composition		
Ash	6.15	5.98
Fat	16.0	15.5
CP	24.4	26.6
Ca	0.990	0.962
P	0.728	0.707
AA, % of DM		
Glu	3.95	3.84
Leu	2.41	5.25
Asp	2.36	2.29
Lys	2.21	2.15
Thr	1.68	1.63
Pro	1.67	1.62
Val	1.58	1.54
Ile	1.53	1.49
Ser	1.25	1.21
Ala	1.14	1.11
Phe	0.977	0.949
Tyr	0.850	0.826
Arg	0.850	0.826
Met	0.648	0.630
Gly	0.573	0.557
His	0.563	0.547
Cys	0.467	0.454
Trp	0.446	0.433

Lambs were randomly assigned to treatment. Lambs were allowed access to milk replacer for 42 d. Water and starter feed, consisting of a creep feed and chopped alfalfa hay (**Table 3-2**), were provided for ad libitum intake when individual lambs reached 14 d of age. A partition within each pen was used to divide lambs that were less than 14 d of age from older lambs. On d 42, lambs were weaned and removed from pre-weaning pens, comingled with weaned lambs from both treatments, and provided ad libitum access to water, creep feed, and chopped alfalfa hay.

Table 3-2. Nutrient composition of feeds provided to lambs (DM basis)

	Creep		Finishing	
Nutrient composition	Pellet[1]	Hay	Pellet[2]	Hay
Dry matter	89.1	89.7	88.0	87.7
Ash	6.87	8.80	4.31	9.33
CP	21.8	14.0	18.2	16.7
Fat	3.38	0.77	3.18	0.815
NDF	13.5	57.7	11.1	52.4
ADF	7.70	40.3	3.27	37.9
Ca	0.767	1.04	0.686	1.08
P	0.434	0.295	0.330	0.284

[1]Creep pellet consisted of (DM basis) corn (46.6%), soybean meal (30%), beet pulp (19%), limestone (1.5%), urea (0.10%), and trace mineral salt supplement (2.8%).
[2]Finishing pellet consisted of (DM basis) corn (86.1%), soybean meal (9.6%), urea (1.65%), limestone (1.1%), and trace mineral salt supplement (1.58%).

After all lambs were weaned, lambs were moved to the NDSU Animal Nutrition and Physiology Center and penned in groups of five (0.91 × 2.4 m pens) in a temperature-controlled room (14 ºC) on Tenderfoot flooring for 39 ± 3.6 d. Lambs were penned in groups until individual lambs reached approximately 30 kg BW (backgrounding period) or 12 weeks of age, and then were penned individually (0.91 × 1.2 m pens) for the remainder of the experiment (finishing period) to monitor daily feed intake. Creep feed and chopped alfalfa hay were provided for ad libitum intake, and lambs were transitioned to a finishing diet (**Table 3-2**) over 14 d by feeding 75% of the backgrounding diet and 25% of the finishing diet for 5 d, 50% of the backgrounding diet and 50% of the finishing diet for 4 d, and 25% of the backgrounding diet and 75% of the finishing diet for 5 d. The finishing diet consisted of 90% pellet and 10% chopped alfalfa hay, and was formulated to meet or exceed nutritional requirements for growing lambs gaining 300 g/d (NRC, 2007). Lambs were provided the finishing diet at 5% BW to ensure ad libitum intake, and feeding amounts were adjusted every 14 d based on lamb weight.

The five heaviest lambs from each treatment were selected for slaughter after 68 (± 3.4) days of feeding the finishing diet, and all remaining lambs were slaughtered after 96 (± 3.6) days

of feeding the finishing diet. Hot carcass weight (HCW) and dressing percent was determined after slaughter. After a 24 h chill, carcasses were knife-ribbed between the 12th and 13th rib. Carcasses were evaluated for longissimus muscle area (LMA), fat thickness at the 12th rib (BF), body wall thickness (BWT), yield grade, leg score, flank streakings, and quality grade by trained personnel. Standard USDA grading procedures were used to derive a calculated yield grade. Percent of boneless, closely trimmed retail cuts (%BCTRC) was calculated, as described by Savell and Smith (2000).

3.3.2. Sample Collection

Lambs were weighed after birth and every 7 d until weaning. After weaning, lambs were weighed every 14 d through the backgrounding and finishing periods and 2 consecutive days before slaughter. Blood samples were collected via jugular venipuncture on d 1, 21, and 42 of the pre-weaning period at 1200 h for serum metabolite and AA concentration analyses. Blood samples were allowed to clot for 30 min at room temperature before being placed on ice and transferred to the laboratory. Serum was harvested by centrifugation (3,000 × g at 4 ºC) for 20 min, transferred to microcentrifuge tubes, and stored at -20 ºC until subsequent analysis. Samples of milk replacer, creep feed, chopped alfalfa hay, and finishing diet were sampled weekly. At slaughter, contents of the digestive tract were emptied and trimmed from the mesentery, and the mass and length of the small intestine, and mass of the reticulorumen, abomasum, omasum, colon, cecum, liver, pancreas, spleen, visceral fat, and kidneys were recorded.

3.3.3. Sample Analysis

Feed samples were thawed at room temperature and subsequently dried (60 ºC) in a forced-air oven for 48 h before being ground to pass a 1-mm screen. Feed samples were analyzed (AOAC Int., 2006) for DM (method 934.01), OM (method 942.05), N (method 2001.11), and fat

(method 920.39). The techniques of Van Soest et al. (1991) were used to quantify NDF and ADF non-sequentially. Crude protein concentration was calculated as 6.25 × N. Amino acid concentration of milk replacer was analyzed by HPLC (method 982.30, AOAC Int., 2006).

Serum glucose concentration was measured using the hexokinase/glucose-6-phosphate dehydrogenase method (Farrance, 1987) using the Infinity Glucose hexokinase kit (Thermo Trace, Louisville, KY, USA). Serum urea N concentration was measured (Jung et al., 1975) using the QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, USA). Serum free AA concentrations were analyzed by reversed phase UPLC after pre-column derivatization of AA with AQC (Salazar et al., 2012). Chromatography was achieved on a BEH C_{18} column (2.1 × 150 mm; 1.7 µm; Waters Corp., Milford, MA, USA). The combined flow rate of the mobile phase was constant and 0.2 mL/min. The initial mobile phase was composed of ultrapure water containing 30 mmol/L ammonium acetate and acetonitrile (containing 0.15% (v/v) formic acid) at a flow rate of 0.7 mL/min. The column was maintained at 43 ºC and injection volumes were 1µL. Amounts of amino acids were quantified in reference to norvaline and measured at 260 nm (Acquity UPLC; Waters Corp., Milford, MA, USA).

3.3.4. Statistical Analysis

Pre-weaning and backgrounding data were analyzed as a completely randomized design. Lamb pre-weaning weight, serum AA, and serum metabolites were analyzed using the MIXED procedure in SAS (SAS 9.4, SAS Institute Inc., Cary, NC, USA) with repeated measures. The experimental unit was lamb, the repeated effect was day, and the model included effect of day and treatment and their interaction. The REG procedure of SAS was used to examine how Leu supplementation influenced the relationship between birth weight and pre-weaning ADG. Tissue mass, initial and final finishing weight, ADG, and weight gain during the finishing phase were

analyzed using the GLM procedure in SAS. The experimental unit was lamb, and the model included effects of treatment; days on feed during the backgrounding period was used as a covariate for finishing period data. Carcass characteristics data were analyzed as a randomized complete block design (slaughter date; n = 2 blocks). The experimental unit was lamb, and the model included effects of block and treatment. Significance was declared at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$.

3.4. Results

Birth weight did not differ between treatments (**Figure 3-1A**). A treatment × week interaction was observed ($P < 0.01$) for pre-weaning BW, where BW increased at a greater rate in lambs supplemented with Leu pre-weaning. Average daily gain of lambs during the milk-feeding phase (**Figure 3-1B**) was greater in lambs supplemented with Leu pre-weaning than control lambs ($P < 0.01$). In lambs supplemented with Leu pre-weaning, ADG decreased as lamb birth weight increased ($r^2 = 0.56$; $P = 0.02$; **Figure 3-2**). Control lambs were observed to have a weaker relationship ($r^2 = 0.04$; $P = 0.60$) between birth weight and ADG than lambs supplemented with Leu pre-weaning.

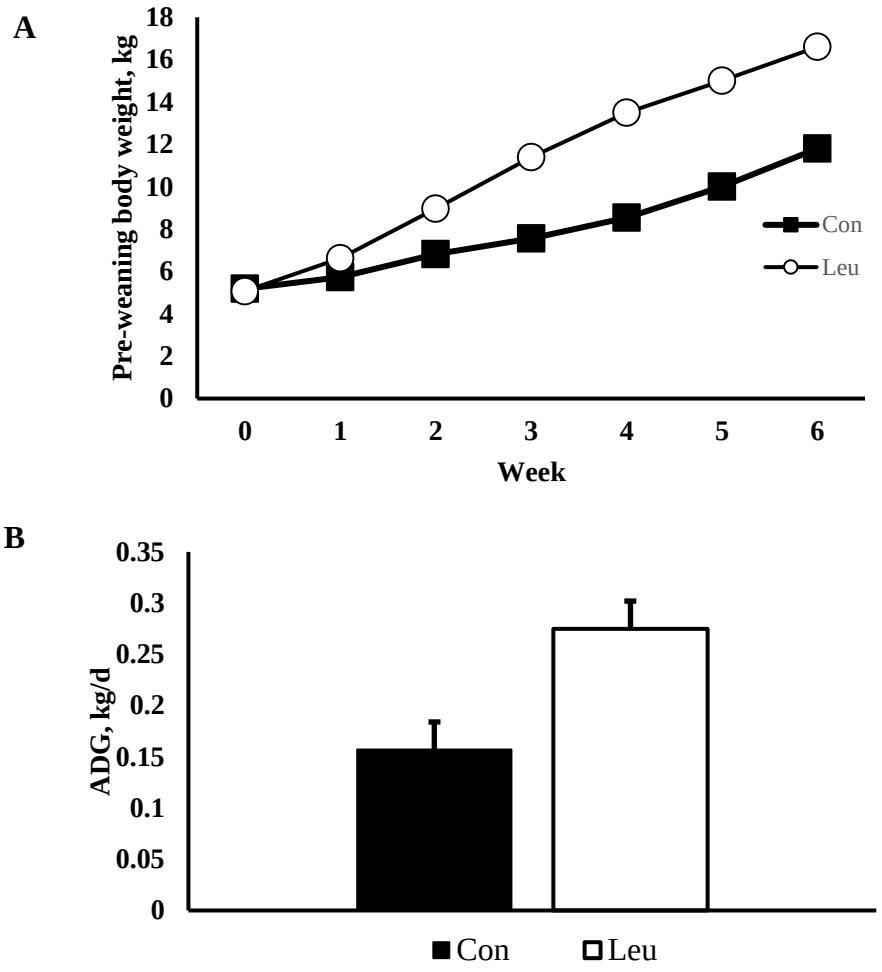

Figure 3-1. Body weight (kg; A) and average daily gain (ADG, kg/d; B) of lambs during the pre-weaning period. Week 0 corresponds to lamb weight at birth. Effects for body weight were treatment × week ($P < 0.01$), treatment ($P < 0.01$), and week ($P < 0.01$), and effect for ADG was treatment ($P < 0.01$).

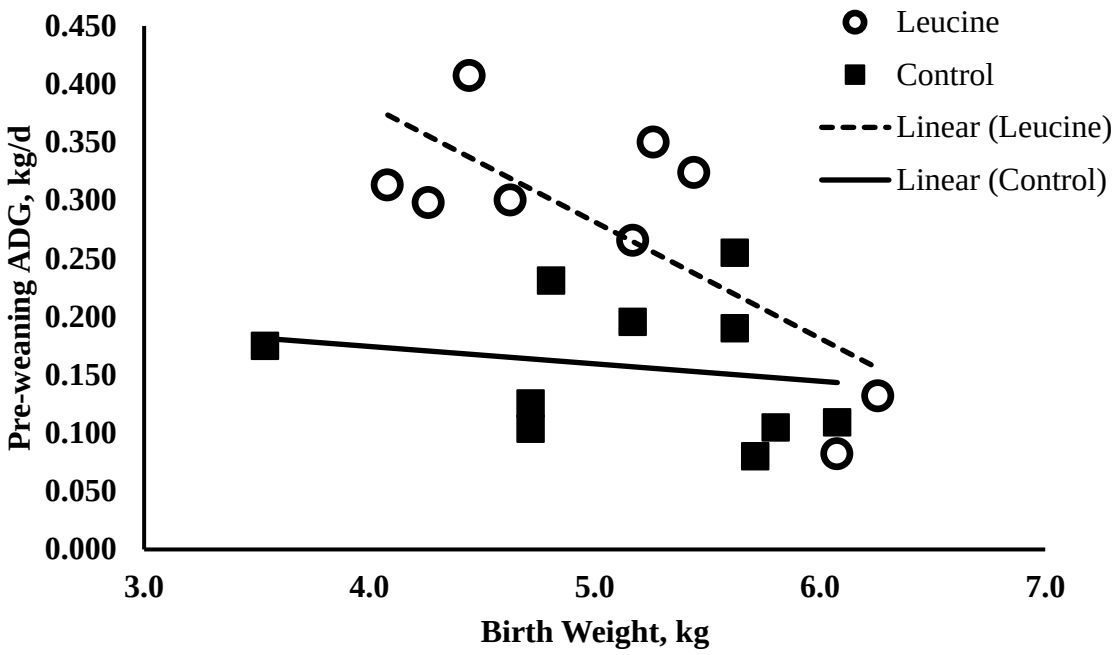

Figure 3-2. Linear regression of birth weight and pre-weaning average daily gain (ADG) for lambs during the pre-weaning period in Leu-supplemented and control lambs. Solid square and solid line are control, open circle and dashed line are leucine supplemented lambs. Linear regression of birth weight and pre-weaning ADG in control lambs: $y = -0.0149x + 0.2343$, $r^2 = 0.04$ ($P = 0.60$). Linear regression of birth weight and pre-weaning ADG in lambs supplemented with Leu pre-weaning: $y = -0.1001x + 0.7818$, $r^2 = 0.56$ ($P = 0.02$).

No day × treatment interactions were observed for serum AA concentrations (**Table A5**). Serum Leu concentrations were greater ($P < 0.01$) in lambs supplemented with Leu pre-weaning (**Table 3-3**). Lambs supplemented with Leu pre-weaning had greater ($P = 0.03$) serum Asp concentrations than control lambs. Serum concentrations of Arg, His, Ile, Lys, Met, Phe, Thr, Val, total EAA, Ala, Asn, Gln, Glu, Gly, Pro, Ser, Tyr, total NEAA and total AA were not affected by treatment. A day effect was observed ($P < 0.01$) for serum concentrations of His, Lys, Met, Phe, Val, total EAA, Asp, Gln, Glu, Pro, and Tyr where serum AA concentrations increased over the experimental period (**Table A5**). Serum concentrations of His, Ile, Thr, Ala, Asn, Gly, Ser, total NEAA, and total AA decreased ($P < 0.01$) from d 1 to 21, then increased on d 42. Serum concentrations of Arg increased ($P < 0.01$) from d 1 to 21, then decreased on d 42.

Table 3-3. Serum AA profile of lambs fed milk replacer with or without supplemental leucine during the pre-weaning period

	Treatment			
Item, μM	Control	Leucine	SEM	*P*-value
Arg	255	255	22.2	0.98
His	144	143	9.97	0.91
Ile	84.7	94.7	10.23	0.50
Leu	167	308	23.6	<0.01
Lys	160	180	10.8	0.22
Met	35.5	44.9	4.60	0.17
Phe	87.1	89.1	5.80	0.81
Thr	369	438	33.0	0.16
Val	294	281	22.4	0.67
Total EAA	1679	1911	101.8	0.13
Ala	233	240	9.7	0.61
Asn	88.6	95.5	5.73	0.40
Asp	13.0	15.2	0.69	0.03
Gln	161	151	12.8	0.60
Glu	240	270	18.4	0.27
Gly	517	476	28.7	0.32
Pro	224	234	15.9	0.67
Ser	135	150	8.1	0.20
Tyr	99.4	104	7.5	0.69
Total NEAA	1737	1732	46.7	0.94
Total AA	3416	3643	137.9	0.26
Metabolites, mg/dL				
Urea N	19.0	21.4	1.09	0.13
Glucose	94.1	107	4.46	0.06

There was no effect of treatment on serum urea N but a day effect was observed (P < 0.01) where serum urea N decreased from d 1 to 21, then increased on d 42 (**Table A5**). Serum glucose concentrations tended to be greater (P = 0.06) in lambs supplemented with Leu pre-weaning than control lambs (**Table 3-3**). A day effect was observed (P = 0.02) for serum glucose, where concentrations increased from d 1 to 21, then decreased on d 42 (**Table A5**).

Days on feed for the backgrounding period was not affected by treatment (**Table 3-4**) although control lambs had numerically 24% greater (43 vs 36 days, respectively, P = 0.11) days on feed than Leu lambs. Control lambs had greater weight gain (P = 0.01) and ADG (P = 0.05) during the backgrounding period compared to lambs supplemented with Leu during the pre-

weaning period. Lambs supplemented with Leu pre-weaning were transitioned to the finishing diet at heavier weights than control lambs ($P = 0.05$). For the finishing period, final BW, ADG, days on feed, average DMI, gain:feed, and age at slaughter were not affected by Leu supplementation.

Table 3-4. Backgrounding and finishing performance of lambs fed milk replacer with or without supplemental leucine during the pre-weaning period

	Treatment			
Backgrounding	Control	Leucine	SEM	*P*-value
Days on feed	43.3	34.8	3.59	0.11
Weight gain, kg	13.7	9.8	0.98	0.01
ADG, g	320	284	12.2	0.05
Finishing				
Initial finishing BW, kg	27.7	30.1	0.78	0.05
Final finishing BW, kg	56.3	59.3	1.50	0.18
ADG, g	374	374	14.1	0.99
Days on feed	81.1	82.5	1.47	0.53
Daily feed intake, kg	1.46	1.40	0.078	0.65
Gain:feed	0.284	0.282	0.0272	0.66
Age at slaughter, d	179	181	1.69	0.39

Supplemental Leu pre-weaning did not affect small intestinal length or mass at slaughter (**Table 3-5**). Mass of the reticulorumen tended ($P = 0.09$) to be greater in lambs supplemented with Leu pre-weaning. Supplemental Leu pre-weaning did not affect mass of the abomasum, omasum, colon, cecum, liver, pancreas, spleen, visceral fat, or kidneys on an absolute or on a g tissue/kg BW basis.

Table 3-5. Tissue mass of lambs fed milk replacer with or without supplemental leucine during the pre-weaning period

	Treatment			
Item	Control	Leucine	SEM	*P*-value
Small intestine				
Length, m	23.6	24.4	0.63	0.40
g	576	595	34.1	0.70
g/kg BW	10.4	9.98	0.628	0.66
Reticulorumen				
g	1052	1169	45.5	0.09
g/kg BW	19.0	19.5	0.67	0.61
Abomasum				
g	151	162	8.9	0.40
g/kg BW	2.71	2.73	0.164	0.92
Omasum				
g	116	127	17.4	0.67
g/kg BW	2.13	2.13	0.309	0.99
Colon				
g	425	449	38.2	0.66
g/kg BW	7.68	7.48	0.621	0.82
Cecum				
g	48.9	57.6	6.90	0.39
g/kg BW	0.880	0.989	0.1310	0.57
Liver				
g	965	1006	49.8	0.57
g/kg BW	17.4	16.9	0.88	0.68
Pancreas				
g	65.0	59.8	5.97	0.55
g/kg BW	1.16	1.01	0.092	0.27
Spleen				
g	90.5	98.0	4.07	0.21
g/kg BW	1.62	1.61	0.072	0.96
Visceral fat				
g	2928	3014	250.0	0.81
g/kg BW	52.2	50.2	3.62	0.71
Kidneys				
g	131	129	7.5	0.84
g/kg BW	2.34	2.18	0.129	0.39

Hot carcass weight, dressing percent, and back fat at the 12th rib was not affected by treatment (**Table 3-6**). Body wall thickness was greater ($P = 0.05$) in lambs supplemented with Leu pre-weaning. Longissimus dorsi area, yield grade, percent of boneless closely trimmed retail cuts, leg score, flank streakings, and quality grade were not affected by treatment.

Table 3-6. Carcass characteristics of lambs fed milk replacer with or without supplemental leucine during the pre-weaning period

	Treatment			
Item	Control	Leucine	SEM	*P*-value
Hot carcass weight, kg	30.1	31.9	0.89	0.31
Dressing percent	54.0	53.3	0.51	0.33
12th rib back fat, mm	9.70	11.1	0.922	0.34
Body wall thickness, cm	2.84	3.16	0.087	0.05
Longissimus area, cm^2	15.8	15.6	0.75	0.20
Yield grade	4.10	4.79	0.315	0.22
% BCTRC	44.7	43.6	0.47	0.12
Leg score[1]	2.20	2.04	0.278	0.27
Flank streaking[2]	2.50	2.07	0.360	0.37
Quality grade[3]	1.90	1.76	0.198	0.45

Percent boneless closely trimmed retail cuts
[1]Leg scores on scale 1-4; 1 = Low Choice, 2 = Average Choice, 3 = High Choice, 4 = Low Prime
[2]Flank scores on scale 1-4; 1 = Slight, 2 = Small, 3 = Modest, 4 = Moderate
[3]Quality grade on scale 1-3; 1 = High Choice, 2 = Low Prime, 3 = Average Prime

3.5. Discussion

Supplemental Leu has been shown to increase muscle mass and growth in pigs (Escobar et al., 2010; Columbus et al., 2015); increasing ADG of Holstein heifers during the pre-weaning period has also been shown to increase milk production up to the third lactation (Soberon et al., 2012). However, data are limited on effects of supplemental Leu during the pre-weaning period on pre- and post-weaning growth in lambs. Therefore, our objectives were to evaluate the effects of Leu supplemented pre-weaning on pre- and post-weaning growth of lambs, serum AA, visceral organ mass, and carcass characteristics.

In the current study, pre-weaning supplemental Leu increased lamb BW and ADG in the pre-weaning period, suggesting that Leu supplementation in pre-weaning lambs increases growth up until weaning at 42 days of age. These results differ from results of Mao et al. (2019) who reported no effects of Leu on BW or ADG in Hu lambs up to 30 d of age. There are several potential reasons for the discrepancy in results, including the fact that lambs from the Mao et al.

(2019) experiment nursed their dams until 5 d of age, Leu supplementation started at 11 d of age which resulted in the supplementation period of only 19 d, and lambs were limit-fed. This may suggest that Leu needs to be supplemented earlier in life and/or for a longer time period to elicit effects on growth during the pre-weaning period. Interestingly, the stronger relationship between birth weight and ADG in light-weight lambs supplemented with Leu in the current study suggests that increasing Leu concentration in milk replacers may be beneficial for lambs with low birth weights. Previous research has shown that low birth weight twin or triplet lambs and twin lambs with a greater range of birth weights had lower incidences of survivability in the pre-weaning period (Miller et al., 2010; Juengel et al., 2018). Removing lambs with low birth weights or lambs from triplet births from ewes and feeding with milk replacer supplemented with Leu could increase ADG and survivability.

As expected, serum Leu concentration was greater in lambs supplemented with Leu pre-weaning, with the greatest concentrations on d 1 and 21. Our results are similar to those of Nair et al. (1992) and Cao et al. (2018) who reported an increase in plasma Leu in humans and calves when supplemented with Leu. Supplemental Leu during the pre-weaning period had minimal effects on serum concentrations of other AA, suggesting that Leu was not inhibiting uptake or utilization of other AA, or that other AA were not limiting for growth in lambs supplemented with Leu pre-weaning. Supplemental Leu did increase serum Asp concentrations in lambs supplemented with Leu pre-weaning. These results contradict those of Zheng et al. (2019) and Mao et al. (2019) who reported increases in serum Met, Thr, His, EAA, Gly, and Ser, increases in plasma concentrations of Ile, and decreases in plasma concentrations of Ala and Met from calves and lambs, respectively, receiving supplemental Leu. These differences between studies could be because of differences in feeding management. Lambs in the current study were

allowed ad libitum access to milk replacer, whereas calves from Zheng et al. (2019) and lambs from Mao et al. (2019) were meal-fed two or three times each day, respectively. El-Kadi et al. (2012) reported that continuous enteral delivery of milk replacer to piglets has limited effects on plasma AA, whereas bolus enteral delivery of milk replacer increased plasma AA concentrations. Boutry et al. (2013) also reported that bolus feedings increase EAA and NEAA concentrations in piglets compared to piglets that had a continuous orogastric infusion of milk replacer. It is possible that the lack of response in serum AA is because of the continuous access to feed for lambs in the current study.

In the current study, lambs supplemented with Leu pre-weaning had a tendency for greater serum glucose concentrations. Leucine is unlikely to directly affect glucose production as it is a ketogenic AA (Voet et al., 2008). These results are surprising as Leu is known to increase insulin sensitivity in humans, which helps regulate circulating glucose levels (Zanchi et al., 2012). Previous research has reported no effects of supplemental Leu on glucose concentrations in pre-weaned piglets, calves, and lambs (Manjarín et al., 2016; Cao et al., 2018; Mao et al., 2019). Jarrett et al. (1964) reported that as lambs develop a functional rumen, glucose concentrations in the blood decreases. Lambs on the control milk replacer could have had greater ruminal development, leading to lower serum glucose concentration than lambs supplemented with Leu pre-weaning. However, lambs supplemented with Leu pre-weaning tended to have greater reticulorumen weights than non-supplemented lambs which would not support this hypothesis.

In this study, an increase in total weight gain and ADG during the backgrounding period was observed in lambs fed the control milk replacer pre-weaning. The lambs likely experienced compensatory growth during this period, as at weaning they were 29% lighter than lambs

supplemented with Leu pre-weaning. Similarly, Greenwood and Café (2007) reported that calves with lower weaning weights had greater ADG during the backgrounding period, when fed until a common age or weight, but ADG during the finishing period was unaffected. However, further research is needed to better understand how pre-weaning Leu-supplementation influences post-weaning growth in different growing and finishing systems.

During the finishing period, initial BW of lambs supplemented with Leu pre-weaning was greater than control lambs; this was likely due to Leu lambs reaching the target weight more rapidly than control lambs. However, final weight of lambs supplemented with Leu pre-weaning was not different than control lambs. Lambs supplemented with Leu pre-weaning in this experiment attained a heavier weight at weaning, and numerically started the finishing period after a shorter backgrounding period (36 vs. 43 d) than control lambs. This resulted in a numerical increase in days on feed for the finishing period for lambs supplemented with Leu pre-weaning as lambs within both treatments were slaughtered after a common time on feed on one of two dates. Gain:feed often decreases as animals mature and deposit more fat than protein (Ferrell, 1988). The greater body wall fat thickness observed in lambs supplemented with Leu pre-weaning could suggest greater fat deposition later in the finishing period. More efficient feed conversion during the finishing phase may have been observed in lambs supplemented with Leu pre-weaning if slaughtered at a common body fatness rather than common days on feed. This could suggest that the resulting increase in ADG during the pre-weaning period in lambs supplemented with Leu pre-weaning may be beneficial in reducing the number of days lambs spend on feed and attain market weight, without negatively affecting carcass yield and quality.

In conclusion, supplemental Leu during the pre-weaning period increased lamb ADG by 75% at weaning and increased weaning weight by 5 kg, but the increased ADG did not persist

through the finishing period. Additionally, Leu supplementation to lambs fed milk replacer may be useful to increase ADG of lighter weight lambs in the pre-weaning period, and produce lambs for harvest at a similar compositional endpoint in fewer days on feed post-weaning.

3.6. Conclusions

Our results indicate that supplemental Leu during the pre-weaning period increased lamb ADG shortly after the onset of supplementation, but this increased ADG did not persist through the finishing period. Additionally, Leu supplementation to lambs fed milk replacer may be useful to increase ADG of lighter weight lambs in the pre-weaning period. However, no effects of supplemental Leu were observed on serum AA, organ weights, BW of lambs in the post-weaning period, or overall cutability of carcasses. Further research to determine the effects on skeletal muscle protein synthesis during the pre-weaning period should be conducted to determine the composition of gain during this period, and whether this increase in mass is through an activation of protein synthetic pathways or a downregulation of protein degradation pathways.

3.7. Acknowledgements

This research was partially funded by the North Dakota State Board of Agricultural Research and Education. Authors would like to thank the employees of the NDSU Sheep Unit, Animal Nutrition and Physiology Center, Meat Laboratory, Nutrition Laboratory, and students who assisted with this project.

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4. EFFECTS OF SUPPLEMENTAL LEUCINE IN MILK REPLACER ON PANCREATIC AND INTESTINAL DIGESTIVE ENZYME ACTIVITY IN HOLSTEIN BULL CALVES AND LAMBS

4.1. Abstract

Two experiments were conducted to determine effects of Leu supplementation in milk replacer on pancreatic and intestinal digestive enzyme activity in neonatal calves and lambs. In experiment one, twenty-three Holstein bull calves (43.3 ± 1.16 kg; 11.3 ± 0.57 days of age) were used in a randomized complete block design. Leucine was supplemented at 0, 0.4, 0.6, or 0.8 g Leu/kg BW per day for 28 d and pancreatic and small intestinal tissue was collected. Pancreatic activity of α-amylase, trypsin, and protein concentration, and small intestinal activity of glucoamylase, isomaltase, lactase, maltase and protein concentration were determined. Activity of enzymes and protein concentration of tissues were analyzed using the GLM procedure of SAS. Leucine supplementation did not affect pancreatic α-amylase or trypsin activity. A quadratic effect of treatment was observed for pancreatic protein concentration ($P = 0.01$), where protein decreased at lower amounts of supplemental Leu. Glucoamylase activity was not affected by Leu supplementation. Isomaltase linearly decreased ($P = 0.03$) in the jejunum with increasing amounts of supplemental Leu. Lactase (U/g intestine) was affected quadratically ($P = 0.02$) in the jejunum by Leu supplementation where lactase activity decreased at lower amounts of Leu supplementation. Maltase linearly decreased ($P = 0.05$) with increasing amounts of supplemental Leu. In experiment two, nineteen Dorset-cross ram lambs (5.07 ± 0.15 kg) were used in a randomized complete block design. Leucine was supplemented at 2.9% DM in milk replacer and lambs were fed via automated feeders for ad libitum intake for 42 d. Lambs were then weaned and transitioned to a finishing diet. Sample and data analyses were similar as for experiment one.

Leucine supplementation did not affect α-amylase or trypsin activity, or protein concentration in the pancreas. Glucoamylase was not affected by Leu supplementation in the duodenum, jejunum, or ileum. Isomaltase tended to decrease ($P = 0.06$) and maltase decreased ($P < 0.01$) in the jejunum of lambs supplemented with Leu. These data indicate that supplemental Leu pre-weaning has a negative effect on small intestinal carbohydrase activity at the neonatal stage and these effects may persist through finishing. More research is warranted to determine potential negative effects on post-ruminal starch digestion.

4.2. Introduction

Starch is a major energy source in diets of finishing cattle and lambs, and dairy cows for production of meat and milk, but greater inclusion levels of starch can lead to ruminal acidosis and decreased production. Changing the site of starch digestion from the rumen to the small intestine decreases the chance for metabolic disorders. Furthermore, small intestinal starch digestion is 42% more efficient than digestion in the rumen (Huntington et al., 2006), which could improve feed efficiency without increasing the risk of metabolic disorders. However, the capacity for small intestinal starch digestion is limited (Harmon, 2009). Small intestinal starch digestion is initiated by α-amylase (EC 3.2.1.1), which is secreted by the pancreas. The α-glycosidases (e.g., glucoamylase [EC 3.2.1.3], isomaltase [EC 3.2.1.10], and maltase [EC 3.2.1.20]) along the intestinal brush border membrane in the small intestine are important in degrading starch breakdown products, and increasing their activity could also increase carbohydrate digestion, potentially increasing animal productivity.

Leucine has been reported to enhance small intestinal development of suckling piglets (Sun et al., 2015), and duodenal infusion of Leu increased pancreatic α-amylase secretion in goats (Yu et al., 2014). However, Cao et al. (2019) observed no effect of Leu on α-amylase

secretion in milk-fed calves. Harmon (1993) reported that pancreatic α-amylase can be influenced by dietary manipulations in ruminants, but concentrations of α-glycosidases in the intestines seem to be influenced less by alterations to diet composition. Data on small intestinal α-glycosidase activity in neonatal ruminants are limited. The objective of this study was to determine effects of increasing amounts of supplemental Leu during the pre-weaning period on pancreatic α-amylase and trypsin activity, and small intestinal activity of glucoamylase, isomaltase, lactase, and maltase both during pre-weaning and the end of the finishing period in calves and lambs, respectively. We hypothesized that supplemental Leu would increase pancreatic enzyme activity, and increasing amounts of supplemental Leu would increase pancreatic enzyme activity, and have no effect on carbohydrate enzymes in the small intestine, and that these effects would persist through the finishing period.

4.3. Materials and Methods

All procedures involving the use of animals were approved by the North Dakota State University (NDSU) Institutional Animal Care and Use Committee.

4.3.1. Experiment 1

Twenty-three Holstein bull calves (43.3 ± 1.16 kg; 11.3 ± 0.57 days of age) were used. Calves were sourced from one commercial dairy. Calves were individually housed (0.91 × 1.2 m pens) in a temperature-controlled room (14ºC) on Tenderfoot flooring with rubber stall mats and no bedding, and fed twice daily (0730 and 1630 h) for 28 d. Calves received milk replacer (Nurture, Cargill Inc., Minneapolis, MN, USA) at 2.5% (DM basis) of their initial BW split evenly between daily feedings. Milk replacer (**Table 4-1**) was reconstituted with 1.5 L warm tap water at each feeding. Water was offered for ad libitum intake, and starter was not provided to calves.

Table 4-1. Nutrient concentration of calf milk replacer

	g Leu/kg BW			
Item, %	0	0.4	0.6	0.8
DM, % of as fed	23.8	23.8	23.4	23.4
OM, % of DM	93.4	93.3	93.3	93.3
CP, % of DM	21.2	22.6	22.9	23.7
Crude fat, % of DM	20.0	18.6	18.3	17.5
AA[1], % of DM				
Glu	3.08	3.02	3.00	2.97
Lys	2.68	2.63	2.61	2.58
Leu	1.89	3.69	6.19	9.83
Asp	1.79	1.76	1.74	1.73
Pro	1.25	1.23	1.22	1.21
Thr	1.13	1.11	1.10	1.09
Val	1.11	1.09	1.08	1.07
Ile	1.10	1.08	1.07	1.06
Ser	0.87	0.85	0.85	0.84
Ala	0.86	0.84	0.84	0.83
Phe	0.73	0.72	0.71	0.70
Arg	0.59	0.58	0.57	0.57
Tyr	0.57	0.56	0.55	0.55
Met	0.52	0.51	0.51	0.50
Gly	0.47	0.46	0.46	0.45
His	0.42	0.41	0.41	0.40

[1]From analysis of milk replacer powder. Amino acid concentrations were calculated for treatments with added Leu from AA concentration in milk replacer and the amount of added Leu for each treatment.

Calves were blocked by arrival date (n = 4 per block except for 1 block with n = 3) into one of four treatment groups: milk replacer with no supplemental Leu or with 0.4, 0.6, or 0.8 g/kg initial BW added Leu (99.7% purity; Ajinomoto, Raleigh, NC, USA). Daily Leu allotments were split evenly between feedings and mixed with milk replacer prior to feeding. Amounts of supplemental Leu were chosen to include values above and below (0.63 g/kg BW) amounts provided parenterally by Boutry et al. (2013) to piglets that resulted in increased skeletal muscle protein synthesis.

Weights of organs were recorded and samples (approximately 5 g) of the pancreas (body) and jejunum (mucosal scrape 5 m from pylorus) were snap-frozen in liquid nitrogen and stored at -80 ºC until analysis.

4.3.2. Experiment 2

Nineteen neonatal fall-born Dorset-cross ram lambs (5.07 ± 0.15 kg; twin-born n = 16, triplet-born n = 3), predominately of Dorset breeding were used. Lambs remained with ewes (2.8 ± 0.4 years old) for 12 h post-birth to ensure adequate colostrum intake and then randomly allotted to either a control milk replacer (Shepherd's Choice, Premier1 Supplies, Washington, IA, USA; n = 10) or milk replacer with 2.9% (DM basis) added Leu (n = 9). Lambs were housed at the NDSU Sheep Unit and trained to consume milk from LAC-TEK Stainless 61450 milk dispensers (1 per treatment; Biotic Industries, Inc., Bell Buckle, TN, USA) which allowed for ad libitum consumption of milk replacer. LAC-TEK machines were calibrated to deliver one part milk replacer (**Table 4-2**) and four parts heated water. Lambs were allowed access to milk replacer for 42 d. Water and starter feed, consisting of a creep feed and chopped alfalfa hay (**Table 4-3**), were provided for ad libitum intake when individual lambs reached 14 d of age. A partition within each pen was used to divide lambs that were less than 14 d of age from older lambs. On d 42, lambs were weaned and removed from pre-weaning pens, comingled with weaned lambs from both treatments, and provided ad libitum access to water, creep feed, and chopped alfalfa hay.

Table 4-2. Dietary composition and nutrient concentrations of milk replacer and milk replacer supplemented with Leu (DM basis).

Ingredient	Milk replacer	Milk replacer with leucine
Milk replacer	100	97.1
Leu	0	2.9
Nutrient Composition		
Ash	6.15	5.98
Fat	16.0	15.5
Crude protein	24.4	26.6
Ca	0.990	0.962
P	0.728	0.707
AA, % of DM		
Glu	3.95	3.84
Leu	2.41	5.25
Asp	2.36	2.29
Lys	2.21	2.15
Thr	1.68	1.63
Pro	1.67	1.62
Val	1.58	1.54
Ile	1.53	1.49
Ser	1.25	1.21
Ala	1.14	1.11
Phe	0.977	0.949
Tyr	0.850	0.826
Arg	0.850	0.826
Met	0.648	0.630
Gly	0.573	0.557
His	0.563	0.547
Cys	0.467	0.454
Trp	0.446	0.433

Table 4-3. Nutrient composition of feeds provided to lambs (DM basis)

	Creep		Finishing	
Nutrient composition	Pellet[1]	Hay	Pellet[2]	Hay
Dry matter	89.1	89.7	88.0	87.7
Ash	6.87	8.80	4.31	9.33
Crude protein	21.8	14.0	18.2	16.7
Fat	3.38	0.77	3.18	0.815
NDF	13.5	57.7	11.1	52.4
ADF	7.70	40.3	3.27	37.9
Ca	0.767	1.04	0.686	1.08
P	0.434	0.295	0.330	0.284

[1]Creep pellet consisted of (DM basis) corn (46.6%), soybean meal (30%), beet pulp (19%), limestone (1.5%), urea (0.10%), and trace mineral salt supplement (2.8%).
[2]Finishing pellet consisted of (DM basis) corn (86.1%), soybean meal (9.6%), urea (1.65%), limestone (1.1%), and trace mineral salt supplement (1.58%).

After all lambs were weaned, lambs were moved to the NDSU Animal Nutrition and Physiology Center and penned in groups of five (0.91 × 2.4 m pens) in a temperature-controlled room (14 ºC) on Tenderfoot flooring for 39 ± 3.6 d. Lambs were penned in groups until individual lambs reached approximately 30 kg BW (backgrounding period) or 12 weeks of age, and then were penned individually (0.91 × 1.2 m pens) for the remainder of the experiment (finishing period) to monitor daily feed intake. Creep feed and chopped alfalfa hay were provided for ad libitum intake, and lambs were transitioned to a finishing diet (Table 3) over 14 d by feeding 75% of the backgrounding diet and 25% of the finishing diet for 5 d, 50% of the backgrounding diet and 50% of the finishing diet for 4 d, and 25% of the backgrounding diet and 75% of the finishing diet for 5 d. The finishing diet consisted of 90% pellet and 10% chopped alfalfa hay, and was formulated to meet or exceed nutritional requirements for growing lambs gaining 300 g/d (NRC, 2007). Lambs were provided the finishing diet at 5% of BW (DM basis) to ensure ad libitum intake, and feeding amounts were adjusted every 14 d based on lamb weight.

The five heaviest lambs from each treatment were selected for slaughter after 68 (± 3.4) days of feeding the finishing diet, and all remaining lambs were slaughtered after 96 (± 3.6) days

of feeding the finishing diet. Weights of organs were recorded and samples (approximately 5 g) of the pancreas (body) and sections from the duodenum, jejunum, and ileum (mucosal scrape) were snap-frozen in liquid nitrogen and stored at -80 ºC until analysis. A section of the duodenum was taken 1 m from the pylorus, the jejunum was taken 5 m from the pylorus, and the ileum was taken 1 m from the ileo-cecal junction.

4.3.3. Sample Analysis

Pancreatic tissue (0.25 g) was homogenized in 0.9% NaCl (2.25 mL) using a polytron (Brinkmann Instruments Inc., Westbury, NY, USA). Concentration of protein was evaluated using the bicinchoninic acid procedure with BSA as the standard (Smith et al., 1985). Activity of α-amylase was evaluated using a kit from Teco Diagnostics (Anaheim, CA, USA). Activity of trypsin was evaluated according to Geiger and Fritz (1986) after activation with 100-U/L enterokinase (Swanson et al., 2008). Analyses were adapted for use on a microplate spectrophotometer (Synergy H1, BioTek, Winsooki, VT, USA). One unit (U) of enzyme activity equals 1 µmol product produced per min. Pancreatic digestive data were expressed as U/g pancreas, U/kg BW, and U/g protein.

Intestinal tissue (0.50 g) was homogenized in 0.9% NaCl (2 mL) using a polytron. Concentration of protein was determined using the bicinchoninic acid procedure with BSA as the standard (Smith et al., 1985). Activity of glucoamylase, isomaltase, maltase, and lactase was evaluated according to Kreikemeier et al. (1990). Analyses were adapted for use on a microplate spectrophotometer (Synergy H1, BioTek). One U of enzyme activity equals 1 µmol product produced per min. Enzyme data are expressed as U/g intestine and U/g protein.

4.3.4. Statistical Analysis

Holstein calf data were analyzed as a randomized complete block (arrival date; n = 6 blocks) design. Pancreatic and intestinal enzymes were analyzed using the GLM procedure in SAS (SAS 9.4, SAS Institute Inc., Cary, NC, USA). The experimental unit was calf and the model included effects of block and treatment. Linear and quadratic effects of treatment were determined by orthogonal contrasts. Because of unequal spacing of supplemental Leu treatments, the IML procedure of SAS was used to develop contrast coefficients. Significance was declared at $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.10$.

Lamb data were analyzed as a randomized complete block (slaughter date, n = 2 blocks) design. Pancreatic and intestinal enzymes were analyzed using the GLM procedure in SAS. The experimental unit was lamb and the model included effects of block and treatment. Significance was declared at $P \leq 0.05$ and a tendency at $0.05 < P \leq 0.10$.

4.4. Results

4.4.1. Experiment 1

As described in **Table 2-4**, pancreas mass (g) increased linearly ($P = 0.05$) with increasing Leu supplementation and was affected quadratically ($P = 0.04$) when expressed relative to BW (g/kg BW) as relative mass decreased with Leu supplementation of 0.4 g Leu/kg BW and then increased with increasing amounts of supplemental Leu (**Table 4-4**). There was a quadratic effect of g protein/pancreas ($P = 0.01$) and mg protein/kg BW ($P < 0.01$) of treatment, where calves receiving 0.4g Leu/kg BW had decreased protein concentration, but protein concentration increased at greater amounts of supplemental Leu. Increasing supplemental Leu did not influence protein concentration when expressed as mg protein/g pancreas or activity of α-amylase and trypsin when expressed as U/g pancreas, U/g protein, U/pancreas, or U/kg BW.

Table 4-4. Pancreatic enzyme activity in calves fed milk replacer with or without supplemental leucine

	g Leu/kg BW					Contrasts	
Item	0	0.4	0.6	0.8	SEM	Linear	Quadratic
Pancreas							
g	51.3	46.0	58.6	63.3	3.84	0.05	0.14
g/kg BW	0.87	0.82	0.95	1.05	0.05	<0.01	0.04
Protein							
g protein/pancreas	6.88	5.61	7.34	8.48	0.52	0.04	0.01
mg protein/g pancreas	134	126	127	134	6.24	0.84	0.24
mg protein/kg BW	116	100	120	140	4.9	<0.01	<0.01
Amylase							
U/g pancreas	685	529	703	665	136	0.97	0.56
kU/g protein	5.21	4.17	5.60	4.82	1.031	0.97	0.76
kU/pancreas	33.9	24.6	41.8	44.9	8.30	0.27	0.28
U/kg BW	576	433	669	710	120.1	0.34	0.28
Trypsin							
U/g pancreas	7.10	8.40	7.31	7.70	0.717	0.67	0.45
U/g protein	52.5	66.4	55.5	63.1	6.62	0.38	0.57
U/pancreas	372	379	431	506	69.7	0.19	0.47
U/kg BW	6.17	6.70	6.96	8.14	0.81	0.11	0.54

There was no effect of supplemental Leu on glucoamylase activity in the jejunum (**Table 4-5**). Isomaltase activity decreased linearly with increasing amounts of supplemental Leu when expressed as U/g intestine ($P = 0.03$) and U/g protein ($P = 0.02$). Lactase activity in the jejunum was quadratically affected by supplemental Leu ($P = 0.02$) where lactase activity decreased at lower levels of supplemental Leu and increased at 0.8 g Leu/kg BW. There was no effect of Leu on lactase activity in the jejunum when expressed as U/g protein. Maltase activity decreased linearly with increasing amounts of supplemental Leu when expressed as U/g intestine ($P = 0.02$) and U/g protein ($P = 0.05$).

Table 4-5. Intestinal carbohydrase activity in jejunum of calves fed milk replacer with or without supplemental leucine

	g Leu/kg BW					Contrasts	
Item	0	0.4	0.6	0.8	SEM	Linear	Quadratic
Glucoamylase							
U/g intestine	0.619	0.532	0.500	0.516	0.102	0.41	0.74
U/g protein	3.64	2.83	2.91	3.07	0.761	0.55	0.58
Isomaltase							
U/g intestine	0.710	0.552	0.599	0.512	0.057	0.03	0.67
U/g protein	4.09	3.71	2.93	3.33	0.266	0.02	0.49
Lactase							
U/g intestine	13.3	9.33	11.3	12.2	1.00	0.41	0.02
U/g protein	72.6	59.0	68.3	72.6	9.97	0.99	0.34
Maltase							
U/g intestine	0.931	0.660	0.643	0.632	0.091	0.02	0.30
U/g protein	5.44	3.41	3.57	3.74	0.612	0.05	0.15

4.4.2. Experiment 2

There was no effect of pre-weaning treatment on protein concentration in the pancreas of finished lambs when expressed as mg protein/g pancreas, g protein/pancreas and mg protein/kg BW, or activity of α-amylase or trypsin when expressed as kU/g pancreas, kU/g protein, kU/pancreas, or kU/kg BW (**Table 4-6**).

Table 4-6. Pancreatic enzyme activity of finished lambs fed milk replacer with or without supplemental leucine pre-weaning

	Treatment			
Item	Control	Leucine	SEM	*P*-value
Protein				
mg protein/g pancreas	11.3	11.1	0.83	0.88
g protein/pancreas	0.751	0.649	0.0908	0.44
mg protein/kg BW	13.9	11.3	1.47	0.23
Amylase				
kU/g pancreas	6.35	5.79	0.487	0.43
kU/g protein	574	542	43.8	0.61
kU/pancreas	419	355	55.5	0.42
kU/kg BW	7.85	6.20	0.958	0.24
Trypsin				
U/g pancreas	0.722	0.748	0.1317	0.89
U/g protein	73.3	75.1	17.2	0.94
U/pancreas	44.7	44.9	8.37	0.99
U/kg BW	0.866	0.801	0.1762	0.80

There was no effect of pre-weaning treatment on duodenal carbohydrase activity in finished lambs (**Table 4-7**). There was no effect of pre-weaning treatment on glucoamylase activity in the jejunum of finished lambs. Isomaltase activity in the jejunum of finished lambs tended to decrease ($P = 0.06$) in lambs supplemented with Leu pre-weaning when expressed as U/g intestine, but no effect of pre-weaning treatment was observed in finished lambs when expressed as U/g protein. Maltase activity in the jejunum of finished lambs decreased ($P < 0.01$) in lambs supplemented with Leu pre-weaning when expressed as U/g intestine and tended to decrease ($P = 0.08$) when expressed as U/g protein. There was no effect of pre-weaning treatment on ileal carbohydrase activity in finished lambs. Lactase activity was undetectable in all sections of the small intestine of lambs.

Table 4-7. Intestinal enzyme activity of finished lambs fed milk replacer with or without supplemental leucine pre-weaning

	Treatment			
Item	Control	Leucine	SEM	*P*-value
Duodenum				
Glucoamylase				
U/g intestine	0.498	0.477	0.0839	0.86
U/g protein	8.38	7.58	0.987	0.57
Isomaltase				
U/g intestine	1.26	1.36	0.076	0.40
U/g protein	22.7	21.7	1.90	0.74
Maltase				
U/g intestine	1.06	1.22	0.149	0.46
U/g protein	18.8	19.4	2.61	0.89
Jejunum				
Glucoamylase				
U/g intestine	1.11	0.945	0.1812	0.52
U/g protein	21.3	21.1	3.35	0.97
Isomaltase				
U/g intestine	2.90	2.03	0.303	0.06
U/g protein	57.0	46.9	7.93	0.38
Maltase				
U/g intestine	4.84	2.37	0.583	0.008
U/g protein	95.0	56.0	14.79	0.08
Ileum				
Glucoamylase				
U/g intestine	0.908	0.994	0.1196	0.62
U/g protein	15.6	15.6	2.87	0.99
Isomaltase				
U/g intestine	2.45	2.71	0.238	0.46
U/g protein	42.2	40.6	6.01	0.85
Maltase				
U/g intestine	3.84	4.74	0.595	0.30
U/g protein	65.1	71.0	11.18	0.71

4.5. Discussion

Pancreatic mass as a proportion of BW in calves was decreased at 0.4 g/kg BW of supplemental Leu and increased with greater amounts of supplemental Leu (Reiners et al., 2018). It was previously reported that no differences were observed in pancreatic mass in finished lambs receiving supplemental Leu pre-weaning (Reiners et al., 2020). Differences in pancreatic mass could potentially influence pancreatic digestive enzymes (Swanson et al., 2002) and insulin

production (Baumgard et al., 2016). Changes in enzyme activity could alter the ability to degrade and absorb nutrients from the diet, and alterations in insulin production could influence signaling for protein synthesis (Baumgard et al., 2016) and energy utilization (van den Borne et al., 2006).

As amounts of supplemental Leu increased in calves, pancreatic protein concentration increased. However, pancreatic protein concentration in finished lambs was not affected by pre-weaning supplementation of Leu. Studies by Cao et al. (2018a,b) reported no differences in pancreatic protein concentration among low and high Leu-supplemented treatments in goats and dairy calves. It is possible that Leu supplementation during Exp 1 allowed for greater protein deposition in the pancreas, whereas in Exp 2 Leu was preferentially utilized for BW gain in lambs.

Pancreatic α-amylase activity was not influenced by pre-weaning Leu supplementation in calves and in finished lambs receiving supplemental Leu pre-weaning. It was reported previously that α-amylase activity in calves under 1 month of age is insignificant (Radostits and Bell, 1968). Guo et al. (2018) and Cao et al. (2018a) reported that increasing dietary Leu increased pancreatic activity of α-amylase in dairy calves and goats. It is possible that delivery method and concentration of Leu affected the results. Calves in this study were supplied Leu on a g/kg BW basis as a bolus delivery, whereas Cao et al. (2018a) supplied Leu on a g/d basis as a continuous duodenal infusion. Additionally, calves in this study were provided milk replacer and no starter feed, which may have resulted in limited α-amylase activity. Cao et al. (2018a) reported that α-amylase activity can be upregulated by supplemental Leu in dairy goats, which could lead to increased small intestinal starch digestion in post-weaning ruminants. However, lambs in this study were allowed ad libitum access to creep feed and hay during the milk-feeding phase, and

no differences in α-amylase activity was observed after finishing. It is likely that the effects of supplemental Leu in milk replacer on pancreatic α-amylase activity are negligible.

Pancreatic trypsin activity in calves and lambs was unaffected by treatment. As previously reported, nutrient digestibility in the calves in this experiment was unaffected by treatment (Reiners et al., 2018). Cao et al. (2018a) reported increases in pancreatic trypsin activity in both Leu-supplemented treatments in goats compared to control goats, but Leu treatments did not differ from each other. However, Cao et al. (2018b) reported no differences in activity of pancreatic trypsin between control calves and Leu-supplemented calves. It is likely that trypsin activity was not limiting digestion of protein flowing to the small intestine, and supplementation of Leu had minimal effects on influencing protein digestion.

Small intestinal glucoamylase activity in pre-weaning calves and finished lambs was unaffected by treatment. Data are limited on nutritional effects on glucoamylase activity in neonatal calves. Galand (1989) reported low glucoamylase activity in rats and mice during the milk-feeding phase. It is possible that glucoamylase activity is low in calves and therefore unlikely that Leu has a regulatory role on glucoamylase activity.

Coombe and Siddons (1973) reported that one protein regulates isomaltase activity in the bovine small intestine, and Galand (1989) reported that isomaltase activity is low during the suckling period of rats. It is not clear as to the physiological effects of the observed linear decrease of isomaltase activity in neonatal calves with increasing Leu supplementation as starch concentration was low in the milk replacer. However, isomaltase activity also tended to decrease at the end of the finishing period in lambs supplemented with Leu pre-weaning, suggesting a potential programming effect that could be detrimental to post-ruminal starch digestion later in

life. Further research is needed to better characterize the roles and importance of these enzymes for small intestinal starch digestion in ruminants.

Lower levels of supplemental Leu decreased lactase activity in Leu-supplemented calves pre-weaning. Also, lactase activity across all treatments in neonatal calves was lower than values reported by Guilloteau et al. (2009). Cao et al (2019) reported that Leu supplemented to milk-fed neonatal calves did not alter lactase activity compared to control calves. Lactase activity was not detected in finished lambs (data not shown). This was expected as Siddons (1968) reported that lactase activity decreases with age, and likely does not affect small intestinal starch hydrolysis (Kreikemeier et al., 1990). It is possible that Leu has a limited regulatory role on lactase activity in pre-weaned calves.

We observed a 33% decrease in maltase activity in Leu-supplemented calves compared to controls. Radostits and Bell (1968) reported that enzymatic activity of maltase is found in insignificant amounts in neonatal calves. Guilloteau et al. (2009) observed that maltase activity was 1-3 U/g protein when calves were 28 days old. On a U/g protein basis, calves receiving supplemental Leu had maltase activity similar to that reported by Guilloteau et al. (2009), which was lower in calves receiving no supplemental Leu. These differences may not be significant during the pre-weaning period because of the relatively insignificant amounts of maltase activity and limited need for maltose digestion. However, Leu supplementation to lambs pre-weaning resulted in a 49% decrease in maltase activity compared to control lambs at the end of the finishing. This suggests that Leu supplemented during the pre-weaning period has a developmental programming effect on maltase activity, and potentially could inhibit the hydrolysis of starch hydrolysis products. In non-ruminants, maltase activity mostly comes from sucrase-isomaltase (80%) and maltase-glucoamylase (20%; Galand, 1989; Van Beers et al.,

1995). Glucoamylase activity was unaffected by Leu supplementation, suggesting that Leu selectively influences the sucrase-isomaltase protein.

In lambs receiving Leu pre-weaning, no differences in duodenal or ileal carbohydrase enzymes were observed in finished lambs, and enzyme activity was low in the duodenum, increased in the jejunum, and remained at higher levels in the ileum. The jejunum has a more optimal pH for enzyme activity than the duodenum and ileum (Merchen, 1988); therefore, decreases in carbohydrase activities in the jejunum could potentially have greater effects on digestive efficiency than in the duodenum or ileum.

4.6. Conclusions

Our data indicate that supplemental Leu pre-weaning decreases isomaltase and maltase activity in the small intestine and this effect persists through the finishing period. Our past research suggests that supplemental Leu during the pre-weaning period has limited effects on ADG and gain:feed (Reiners et al., 2018) but supplemental Leu during the pre-weaning period may have long-term effects on α-glycosidase activity in the small intestine, which could potentially reduce digestive efficiency of starch in the small intestine during the finishing period. Future research to examine effects of supplemental Leu pre-weaning on small intestinal carbohydrase activities are needed to better elucidate its effects on carbohydrate digestion and absorption in finishing ruminants.

4.7. Acknowledgments

This project was supported by Animal Health and Production and Animal Products Accession No. 101206 from the USDA National Institute of Food and Agriculture and the North Dakota State Board of Agriculture Research and Education. Authors would like to thank Cargill for supplying calf milk replacer and Ajinomoto for providing Leu used in this study. The authors

would like to thank the employees of the NDSU Animal Nutrition and Physiology Center and the NDSU Sheep Unit, the Nutrition, and Physiology Laboratories at NDSU, the NDSU Meat Laboratory, and students who assisted with this project.

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5. SUMMARY AND CONCLUSIONS

Leucine has a major role in upregulating protein synthesis via the mTOR pathway, enhancing growth of muscle tissue. Studies where Leu is supplemented to piglets have shown an increase in longissimus muscle and enhancing protein accretion. As feed is a major input cost for producers, improving animal growth efficiency while feeding the same amount of feed and/or decreasing their days on feed would enhance producer productivity. However, supplementation of amino acids to functional ruminants do not always result in increased productivity as amino acids are rapidly deaminated in the rumen. Therefore, determining the effects of an additional source of Leu supplemented to calves and lambs during the preruminant stage is essential to understand the role of this amino acid and its ability to influence growth and productivity.

The experiments performed and discussed in this dissertation have shown how supplying an additional source of Leu to milk replacer fed to calves and lambs affects the growth, digestibility, serum metabolite profiles, and enzymatic activity. It has been shown that there may be a species or age specific effect of Leu on growth of neonates, and both a short- and long-term effect on digestive enzymatic activity in calves and lambs. Supplementation of Leu to milk replacer can have positive effects on growth in lambs, but detrimental effects on their enzyme activity.

Increasing amounts of supplemental Leu in milk replacer fed to neonatal calves has the potential to affect serum metabolites without affecting animal growth and development. In experiment 1, we investigated physiological responses by calves to incremental amounts of supplemental Leu in milk replacer. While increasing supplemental Leu did not affect calf body weight growth, it did affect select organ masses, where low levels of supplementation decreased the mass of the omasum, pancreas, and spleen, and tended to increase mass of the

semitendinosus at lower supplementation levels. Additionally, lower levels of supplemental Leu decreased serum glucose. Numerous amino acids were influenced by supplemental Leu; however, serum insulin was unaffected by treatment. Finally, proteins involved in the mTOR pathway were unaffected by increasing amounts of supplemental Leu. Together, these results suggest that Leu supplementation to calves is unable to elicit a positive effect on growth, and its supplementation at this age would have no benefit for producers to implement in production practices.

In experiment 2, the effect of supplemental Leu fed to neonatal lambs on short- and long-term growth and development was investigated. During this experiment, it was determined that Leu supplementation not only increased weaning weight in lambs, but increased ADG of low-birth weight lambs compared to control lambs with low-birth weights. However, there was no long-term effect on body weight or carcass composition or quality. The results from this study suggest that Leu supplementation to lambs, especially those with low birth weights, would be able to increase producer productivity by increasing the weight of lambs when they are weaned. These lambs may then be able to move to a finishing diet more quickly than their non-supplemented counterparts, and may attain a finishing weight for market more quickly without detrimental effects on finishing performance or carcass characteristics.

Starch has long been used as an energy source for livestock, and it is well established that high rates of starch fermentation in the rumen can lead to metabolic disease. However, shifting the site of starch digestion to the small intestine results in greater digestion efficiency, increasing the amount of starch available to the animal without the concomitant increase in risk of metabolic distress. Supplementing Leu to ruminants has previously shown either no effect or an increase in the pancreatic activity of α-amylase. In experiment 3, it was noted that the

supplementation of Leu had no effect on the activity of the pancreatic enzymes α-amylase or trypsin. However, it was determined that supplementing Leu for 28 days in calves or 6 weeks in lambs will decrease the activity of carbohydrases in the jejunum. This decrease in carbohydrase activity could lead to a decrease in digestive efficiency, and the animal may need to increase intake to compensate for the loss in carbohydrase activity. In lambs, there was no effect of supplemental Leu on carbohydrase activities in the duodenum or ileum; however, these sections of the small intestine are not the major sites of nutrient absorption, and a lack of change in these areas would likely have a lesser impact than changes observed in the jejunum. The results from this experiment show both a short- and long-term detrimental effect of supplementing Leu to neonatal preruminants, and this effect persists well after Leu supplementation has ceased.

The studies contained herein suggest that Leu may have a strong effect on animal growth when supplemented early in life, as supplementation of calves over 10 days of age did not experience alterations in growth compared to lambs supplemented 12 hours post-birth. Additionally, supplemental Leu has minimal effects on organ growth and nutrient digestibility while causing alterations in select amino acids in serum, likely related to increased growth potential. However, any gains seen in body growth during the pre-weaning period did not continue post-weaning, and animals attained a similar finishing weight compared to animals not receiving supplemental Leu. No detrimental effects on carcass composition and quality were observed, suggesting that increasing growth during the pre-weaning period would not compromise the ability to produce meat products at a similar quality as non-supplemented animals. The similarity in final product quality was apparently unaffected by the observed decreases in carbohydrase activity, which could decrease the digestive efficiency when starch-based diets are provided to animals during the finishing period. Further research into the effects

of supplemental Leu on more mTOR pathway proteins, whether their role is in protein synthesis or degradation, is warranted to determine the activation status of these pathways in both calves and lambs.

APPENDIX. SUPPLEMENTARY TABLES

Table A1. Serum metabolites of calves fed milk replacer throughout experimental period pre- and post-feeding

	Day											
	1		18		22		28			*P*-values		
Item, µM	Pre	Post	Pre	Post	Pre	Post	Pre	Post	SEM	Day	Time	Day × Time
Serum urea N	5.57	5.56	5.35	5.33	5.11	5.03	5.57	5.47	0.21	0.05	0.87	0.99
Glucose	79.2	98.4	92.1	113	98.0	111	87.3	109	4.63	<0.01	<0.01	0.12

Table A2. Serum AA concentrations of calves fed milk replacer with increasing amounts of supplemental leucine

	g Leu/kg BW																	
	0				0.4				0.6				0.8					*P*-value
Item, µM	1	18	22	28	1	18	22	28	1	18	22	28	1	18	22	28	SEM[1]	Day × Trt
Glu	168	110	110	90.0	140	71.7	88.9	66.9	171	82.1	84.3	65.5	194	106	103	73.5	11.50	0.03
Ser	124	119	113	95.3	119	106	111	98.5	100	125	100	101	116	123	109	92.4	6.60	0.04

[1]Standard error of the mean (n = 6 for 0, 0.6, and 0.8 g Leu/kg BW treatments; n = 5 for 0.4 g Leu/kg BW treatments)

Table A3. Serum AA concentrations of calves fed milk replacer throughout the experimental period

	Day					*P*-value
Item, µM	1	18	22	28	SEM	Day
Ala	280	235	222	196	10.3	<0.01
Arg	228	205	189	194	8.1	<0.01
Asn	37.8	52.0	44.0	44.1	2.3	<0.01
Asp	19.4	16.3	14.6	13.4	0.56	<0.01
Glu	168	92.3	96.3	74.0	6.2	<0.01
Gln	145	213	198	223	7.5	<0.01
Gly	476	448	420	374	15.5	<0.01
His	40.7	26.4	27.2	31.5	2.0	<0.01
Ile	59.6	49.2	44.1	46.8	2.2	<0.01
Lys	304	294	231	258	17.5	<0.01
Met	136	321	262	263	23.2	<0.01
Phe	49.8	49.6	53.1	55.1	2.7	<0.01
Pro	114	104	95.7	90.3	3.6	<0.01
Ser	115	118	108	96.8	3.3	<0.01
Thr	135	139	116	120	7.5	0.03
Trp	27.1	39.2	37.6	37.9	1.3	<0.01
Tyr	26.7	31.5	30.2	31.7	1.5	0.02
Val	117	98.4	90.8	99.7	4.3	<0.01

Table A4. Serum AA concentrations of calves fed milk replacer pre- and post-feeding

	Time			*P*-value
Item, µM	Pre	Post	SEM	Time
Ala	205	263	10.9	<0.01
Arg	171	238	6.5	<0.01
Asn	34.5	54.4	2.3	<0.01
Asp	14.7	17.1	0.68	0.02
Gln	163	227	8.2	<0.01
Gly	467	392	16.0	<0.01
His	27.5	35.4	1.8	<0.01
Ile	39.4	60.4	1.9	<0.01
Lys	127	416	18.8	<0.01
Met	205	286	27.6	0.04
Pro	91.4	111	3.8	<0.01
Ser	101	118	2.9	<0.01
Thr	107	147	7.6	<0.01
Trp	31.9	39.0	1.5	<0.01
Tyr	24.4	35.6	1.45	<0.01
Val	86.0	117	4.8	<0.01

Table A5. Serum AA and metabolites of lambs fed milk replacer with or without supplemental leucine during the pre-weaning period

	Treatments									
	Con			Leu				*P*-value		
AA, µM	1	21	42	1	21	42	SEM[1]	Day	Trt	Day × Trt
Arg	153	371	240	176	338	252	33.5	<0.01	0.98	0.80
His	295	67.1	71.2	273	73.2	82.1	17.3	<0.01	0.91	0.61
Ile	96.8	77.1	80.2	115	67.5	102	14.8	0.01	0.50	0.26
Leu	226	147	127	334	337	253	37.4	0.13	<0.01	0.31
Lys	142	140	198	158	168	213	20.9	0.08	0.22	0.93
Met	49.5	28.7	28.4	54.4	35.7	44.7	7.21	0.01	0.17	0.70
Phe	136	71.1	54.1	139	70.3	57.6	8.62	<0.01	0.81	0.91
Thr	604	197	306	570	472	272	67.3	<0.01	0.16	0.11
Val	459	222	202	498	175	169	32.4	<0.01	0.67	0.54
Total EAA	2309	1368	1359	2379	1807	1547	176	<0.01	0.13	0.57
Ala	394	141	162	425	133	161	15.8	<0.01	0.61	0.57
Asn	145	49.9	71.2	151	66.5	69.1	10.3	<0.01	0.40	0.68
Asp	17.7	10.0	11.2	24.2	11.0	10.5	1.31	<0.01	0.03	0.06
Gln	62.3	20.9	17.6	22.3	22.0	18.5	20.6	<0.01	0.60	0.28
Glu	471	132	118	542	116	152	25.5	<0.01	0.27	0.10
Gly	537	319	697	471	363	592	52.2	<0.01	0.32	0.36
Pro	439	108	124	433	127	141	20.8	<0.01	0.67	0.94
Ser	241	68.1	95.0	251	85.2	113	11.5	<0.01	0.20	0.96
Tyr	193	54.9	50.4	213	53.0	45.7	10.61	<0.01	0.69	0.72
Total NEAA	2580	1082	1550	2524	1164	1508	96.0	<0.01	0.94	0.80
Total AA	4888	2450	2909	4902	2971	3055	231.4	<0.01	0.26	0.50
Metabolites, mg/dL										
Urea N	24.6	17.9	14.5	27.2	15.9	21.1	1.87	<0.01	0.13	0.08
Glucose	82.6	101	98.5	93.7	126	101	7.43	0.02	0.06	0.14

[1]Standard error of the mean (n = 10 for con treatment; n = 9 for Leu treatment