THE IMPACT OF DIGESTIVE ENZYMES IN THE RUMINANT ANIMAL

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

By

Faithe Elizabeth Keomanivong

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

Major Department:
Animal Sciences

November 2016

Fargo, North Dakota

North Dakota State University
Graduate School

Title

THE IMPACT OF DIGESTIVE ENZYMES IN THE RUMINANT ANIMAL

By

Faithe Elizabeth Keomanivong

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

SUPERVISORY COMMITTEE:

Dr. Kendall Swanson
Chair

Dr. Marc Bauer

Dr. Joel Caton

Dr. Lawrence Reynolds

Dr. Kevin Sedivec

Approved:

November 16, 2016
Date

Dr. Greg Lardy
Department Chair

ABSTRACT

Understanding the enzymatic activity in ruminant digestive systems is essential for securing adequate growth, reproduction and overall metabolism. In order to evaluate the impact of various nutritional sources and dietary strategies on enzymatic activity, five experiments were designed. Experiment 1 examined the influence of nutrient restriction and melatonin supplementation on maternal and fetal pancreatic development. Experiment 2 explored various phases of the reproductive cycle and the impact of intravenous arginine infusion amid differing levels of feed intake. Experiment 3 determined the effectiveness of realimentation during advancing stages of gestation. Experiments 4 and 5 examined the impact of variable rations on ruminal pH, NH_3, VFA, total gas and methane concentration and enzymatic activity in steers consuming rations of fine- vs coarse-rolled corn and 20% vs 40% DDGS (Experiment 4) and corn vs barley based diets with low- vs moderate-oil DDGS (Experiment 5). Overall, nutrient restriction caused reduced BW, pancreatic mass and pancreatic enzyme activity in mature animals. In Experiment 1, the addition of dietary melatonin diminished the impact of nutrient restriction on maternal pancreatic mass and α-amylase activity while reducing the secretion of insulin and size of insulin-containing cell clusters. Fetal pancreatic enzymes were unaffected by treatment, however, pancreatic morphology exhibited greater insulin-containing cell cluster size in fetuses from adequately fed dams. In Experiment 2, arginine infusion did not alter pancreatic exocrine or endocrine function during the various luteal stage phases. In Experiment 3, realimentation during different stages of gestation decreased the impact of reduced feed intake and, in some cases, allowed for compensatory gain of the exocrine pancreas. The maternal and fetal endocrine pancreas was unaffected. Mature animals had greater changes in pancreatic exocrine secretions whereas fetuses differed mainly in endocrine function as a result of improper

nutritional status. Comparison of pancreatic tissue revealed a greater quantity, and larger size, of insulin-containing cell clusters in fetuses which appear to separate as the animal matures. Differences in rumen enzymatic activity was found in Experiments 4 and 5, however, despite changes in lag time of gas production or ruminal degradation rates, the concentration of greenhouse gases (CH_4 or CO_2) produced were unaffected.

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to all who helped make the completion of this dissertation possible. I am forever indebted to my major professor, Dr. Kendall Swanson, for giving me the opportunity to obtain my doctoral degree in Animal Science and for his continual leadership, encouragement, and patience as I worked to complete this goal. I would also like to thank Dr. Marc Bauer, Dr. Joel Caton, Dr. Lawrence Reynolds, and Dr. Kevin Sedivec, my committee members, for their guidance and assistance in learning during my time at NDSU.

Thank you to the other faculty members that I have had the opportunity to work with (Dr. Kim Vonnahme, Dr. Pawel Borowicz, Dr. Carl Dahlen, Dr. Anna Grazul-Bilska, Dr. Dale Redmer, Dr. Casie Bass, Dr. Eric Berg, Dr. Caleb Lemley, Dr. Leticia Camacho, Dr. Alison Meyer, Dr. Md Borhan, and Dr. Shafiqur Rahman). The passion and expertise that these professors show for teaching and research was truly an inspiration to me and I am forever grateful to you for your contribution to my education and future.

Some special thanks go to James Kirsch, Marsha Kapphahn, Wanda Keller, Laurie Geyer, the staff at the Animal Nutrition and Physiology Center, Jennifer Young, and my fellow graduate students for their willingness to assist me in laboratory analysis, animal care and handling, and comfort during the stress of graduate school.

I would also like to thank my parents and siblings (Kristina, David, and Theodore) for always being there to listen, reassuring me and pushing me to continue in my pursuit of an advanced degree. Without their help, none of this would have been possible. Finally, I would like to thank my husband, Jamie Keomanivong, for your ability to make me smile even in the most stressful of situations and your dedication to stand by my side and take hold of my hand through it all.

DEDICATION

To my best friend and loving husband

Jamie Keomanivong

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

˚C.....degrees Celsius

µL.....microliter

µm.....micrometer

ADF.....acid detergent fiber

ADG.....average daily gain

ADQ.....adequate

ARG.....arginine

BCS.....body condition score

BSA.....bovine serum albumin

BW.....body weight

CCDS.....condensed corn distiller's solubles

CH_4.....methane

cm.....centimeter

CON.....control

CO_2.....carbon dioxide

CP.....crude protein

d.....day

DGS.....distiller's grains with solubles

DDGS.....dried distiller's grains with solubles

DM.....dry matter

EE.....ether extract

EDTA.....ethylenediaminetetraacetic acid

hour.....hr

g.....grams

kg.....kilograms

L ..... liter

m .....meters

mm ..... millimeters

MEL .....melatonin

mL .....milliliter

mM.....millimolar

min ..... minute

NaCl .....sodium chloride

NDF.....neutral detergent fiber

NUT .....nutrition

OM ..... organic matter

RES .....restricted

SEM .....Standard error of the mean

TBST.....tris-buffered saline with tween

TEMED.....tetramethylethylenediamine

U..... unit

vs ..... versus

WDGS..... wet distiller's grain with solubles

wk..... week

wt.....weight

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1. The Importance of the Ruminant Animal

The world's population is predicted to increase to 11 billion people by 2100. This accounts for a growth of 53% compared to its current level of 7.2 billion (World Population Prospects, 2012). Unfortunately, it has also been calculated that food insecurity currently affects nearly 795 million people (FAO, 2015). Of these, 140 million are children under the age of 5 (WHO, 2013).

While many are lead to believe that only undeveloped countries are enduring such suffering this has proven to be untrue. Within the United States nearly 18.5% of the population is experiencing poverty (Reynolds et al., 2015) and, therefore, are unable to afford adequate nutrition. The tragedy of undergoing such malnutrition is consequently one of the leading causes of death and disability.

Therefore, the need to increase the food supply is significantly related to future health. This includes not only physical and mental health but also plays a strong role in future academic achievement and financial productivity impacting the nations as a whole (Seligman et al., 2010). Due to such an effect, researchers are quickly working to increase the efficiency of livestock production to provide food and economic growth to the world (Thorton, 2010).

In regards to livestock, some of the greatest nutritional resources obtained by the population are provided by ruminants due to their ability to utilize cellulosic feed sources which would otherwise be indigestible by humans. This can be considered a benefit (Oltjen and Beckett, 1996; Jayathilakan et al., 2012). In order to produce significant meat and milk products,

however, it is important to consider the efficiency of their digestive tracts and determine the value of the products generated and also to factor in the environmental impact of the animal.

1.2. Overview of Digestive Enzymes

The anatomy of the ruminant digestive system includes the mouth, esophagus, four compartment stomach (rumen, reticulum, omasum, and abomasum), pancreas, gall bladder, liver, small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum). The impact of these organs on nutrient utilization is highly variable depending on the feed source provided and maturity of the animal (Church, 1988). Therefore, in order to gain a further understanding, extensive research must be continued. One area of focus that has gained considerable interest is on the subject of enzymatic activity.

An enzyme can be defined as an organic catalyst that facilitates a chemical reaction without being affected. Within the ruminant animal, enzymatic secretions are found throughout the digestive tract. For example, the mouth of the ruminant contains salivary glands which secrete the enzyme known as salivary lipase to begin the breakdown of fat (Van Soest, 1982). These glands are also responsible for the secretion of salivary buffers such as phosphate and bicarbonate in an effort to keep the pH of the rumen and reticulum within 5.5 to 7.5 and maximize ruminal microbial activity (Franzolin and Dehority, 2010).

After ingestion, the feed travels down the esophagus and into the rumen and reticulum. Little separation exists between these organs as food and water easily pass between the two. Due to this, feed is predominantly exposed to similar microorganisms responsible for the synthesis of digestive enzymes such as amylase, β-glucanase, maltase, lipase, cellulase, pectinase, protease, xylanase, and tannase (Jenkins, 1993; Wang and McAllister, 2002). Amylase is predominantly

responsible for the breakdown of starch into the components of glucans and maltose (Swanson et al., 2000). β-glucanase and maltase then further this breakdown into glucose molecules more easily available for microorganisms or absorption by the rumen papillae. The fats ingested by the animal undergo breakdown by lipase (Bauchart, 1993) while the enzymes responsible for digestion of the plant cell wall polymers include cellulase, pectinase, protease and xylanase. Tannase is also an important enzyme as it is responsible for the degradation of specific plant toxins (Wang and McAllister, 2002). While these enzymes are capable of breaking down many nutrients some feed components continue traveling along the digestive tract for further degradation.

In addition to the rumen, fermentation occurs in the omasum at a similar rate. The folds of the omasum begin to absorb fluid contained in the digesta (Prins et al., 1972) whereas the remaining nutrients enter the abomasum. Within the abomasum, protein is further broken down by enzymes such as pepsin and rennin (Guilloteau et al., 1985). Lysozyme is also produced to break down the cell walls of the bacteria passing through the abomasum helping to provide microbial protein (Kisia, 2010). Despite an alkaline secretion by chief cells, the pH in the abomasum is typically 2 to 4 (Jackson and Cockcroft, 2002). The pH is reduced largely as a result of H^+ that is being secreted and used to continue the digestion of feed particles.

Once leaving the abomasum, digesta enters the first compartment of the small intestine known as the duodenum. Although the pH in the abomasum was low it is quickly neutralized in the duodenum as digesta is being subjected to an alkaline bile secretion from the gall bladder and bicarbonate released by the pancreas (Noble, 1978). Enzymes secreted by the pancreas are also important for the breakdown of nutrients. Those most commonly recognized in pancreatic secretions include amylases, lipases and proteases. As discussed before, amylases are responsible

for the breakdown of carbohydrates such as starch into dextrins, maltose and isomaltose. Next the enzymes known as maltase and isomaltase become active and continue to digest the maltose and isomaltose into glucose (Croom et al., 1992; Harmon, 1993). The enzyme lipase works with bile salts and colipase to break down triglycerides, into monoglycerides and finally free fatty acids. Proteins also face further breakdown in this area by proteases such as elastase, gelatinase, chymotrypsin and trypsin. Chymotrypsin and trypsin first appear as inactive proenzymes called chymotrypsinogen and trypsinogen. Once these proenzymes are secreted into the duodenum they are activated by enterokinase (also known as enteropeptidase) secreted from intestinal glands called the crypts of Lieberkühn (Pizauro et al., 2004) and continue the breakdown of the majority of proteins into peptides. In addition to the enzymes discussed here, the pancreas is also responsible for the secretion of ribonuclease and deoxyribonuclease which is responsible for degrading RNA and DNA.

Further activity of these enzymes continues within the brush border of the intestine. Disaccharidases such as maltase, lactase, isomaltase, trehalase etc. continue the breakdown of sugar to allow its absorption through the intestinal wall and into the blood stream. Aminopeptidase and dipeptidase help to further the denaturing of proteins into amino acids. Nuclease is also present and begins the breakdown of nucleotides. Nutrients incapable of further degradation in the small intestine continue to move into the large intestine where microbial fermentation occurs. Finally, undigested components (as well as endogenous components) are excreted from the body through the rectum.

While the enzymes that have been listed are commonly secreted it is important to note that the microbial community within the gastrointestinal tract is highly impacted by the nutrients

ingested and this determines the number and composition of enzymes present (Yanke et al., 1995).

1.3. Carbohydrates

1.3.1. Fiber Digestion in the Rumen

Fiber is a component of carbohydrates which includes cellulose, hemicellulose, and soluble fibers such as fructans, pectans, galactans, and beta-glucans (Parish and Rhinehart, 2008). Fibers, such as cellulose, are commonly composed of crosslinked linear chains of glucose molecules which are connected in a β-1,4 linkage (Sujani and Seresinhe, 2015) and can be found in amorphous or crystalline forms. The bacteria capable of breaking down these cellulolytic forms are predominantly *Bacteroides succinogenes, Ruminococcus albus* (degrading amorphous cellulose) and *Ruminococcus flavefaciences* (hydrolyzing crystalline cellulose; Varga and Kolver, 1997; Morales and Dehority, 2014).

Hemicellulose is degraded by hemicellulase through a non-specific hydrolysis of β-1,4 xylosidic linkages (Kamble and Jadhav, 2012). The enzymes involved in the breakdown of cellulose are C-1 cellulase which breaks up the hydrogen bonds and makes glucose more easily accessible for further hydrolysis by C-x cellulase, which will then degrade the chains of cellobiose to glucose (Otajevwo, 2011). The solubility of hemicellulose occurs much quicker than cellulose.

Once the plant matures lignin has been found to fill spaces between the cell walls in cellulose, hemicellulose and pectin. It is able to make the plant fiber highly indigestible due to the cross links which it forms with the sugar molecules of these components (Moreira et al., 2013). The addition of starch or sugar in the diet of ruminants also has a tendency to reduce fiber

digestibility (Mertens and Loften, 1980; Hoover, 1986). Once leaving the rumen no further digestion occurs in the small intestine as no pancreatic enzymes are capable of breaking down cellulose. Remaining fiber components can be fermented by microbes in the large intestine and what is remaining is expelled through the rectum.

1.3.2. Starch Digestion in the Rumen

Cattle saliva contains no amylolytic enzymes but secretions from the nasal labial glands in the nose do contain amylolytic activity. Regardless, this amount is not sufficient enough to contribute to breakdown of starch. Instead, degradation is primarily conducted by the microorganisms found in the rumen producing extracellular amylase. Some examples of these microorganisms include *Bacteroides amylophilus, Streptococcus bovis, Succinimonas amylolytica* and *Succinovibrio dextrinosolvens* (McAllister et al., 1990). These bacteria are responsible for using the amylase to cause fragmentation of the interior glycosidic linkages of the starch chain which leads to the reduction in its molecular size and forms oligosaccharides such as maltose, maltotriose and sometimes small amounts of free glucose (Harmon, 1993). Typically, these oligosaccharides are unaffected by the further action of α- and β-amylases, unless large quantities of enzymes are added.

Due to the inability to be broken down, amylopectin products are formed containing maltose, maltotriose, small amounts of glucose and a mixture of α-limit dextrins. The α-limit dextrins consist of 4-8 glucose moieties and contain α-(1,6) linkages which can't be hydrolyzed by amylases. Instead they require debranching enzymes such as R-enzyme, pullulanase, iso-amylase or α-limit dextrinase (Cerrilla and Martínez, 2003) while the starch that has been degraded to maltose or glucose undergoes fermentation by saccharolytic microbes such as

Bacteroides ruminicola, *Butyrivibrio fibrosolvens* and *Selenomonas ruminantium* (Walker and Hope, 1964).

1.3.3. Starch Digestion in the Small Intestine

Although typically 70% or more of starch is digested in the rumen, it is possible that these nutrients have escaped fermentation and will now be subjected to breakdown by the pancreas and small intestine (Nocek and Tamminga, 1991; Ørskov, 1986; Owens et al., 1986; Harmon et al., 2004). Upon entering the small intestine, the starch is exposed to pancreatic amylase. Again, this initiates the hydrolysis of amylose and amylopectin into branched chain products and oligomers of 2 or 3 glucose units. Brush border disaccharidases, such as maltase and isomaltase, then hydrolyze the oligomers. Once glucose has been formed it is transferred from the lumen into the bloodstream through either active transport. The active transport of intestinal glucose is mediated by sodium-dependent glucose transporter 1 (SGLT1). It is found in the brush border membrane of the enterocytes (Swanson et al., 2000; Wood et al., 2000; Harmon, 2009). Transport is driven by an electrochemical gradient maintained by Na^+/K^+ ATPase and GLUT2 located in the basolateral membrane. The glucose travels from the intestine through the hepatic portal blood to the liver. GLUT2 is responsible for providing basolateral exit as it helps to facilitate glucose transport across the cell membrane from the liver to the veins throughout the body (Lohrenz et al., 2011; Röder et al., 2014). Although the starch escaping ruminal digestion was then subjected to breakdown in the small intestine, only 35 to 60% is fully degraded while the rest continues in the digestive tract to the large intestine to undergo further fermentation (Harmon, et al., 2004). The cause for the escape includes insufficient amylase or maltase activity, inadequate glucose absorption, the inability to degrade the structural effects, or an increase in the passage rate of the starch (Russell et al., 1981; Owens et al., 1986).

1.4. Fat

1.4.1. Fat Digestion in the Rumen

Fats are commonly added to ruminant diets to increase the energy density provided in a ration and improve feed efficiency. These may be included in either a ruminal protected or non-protected form. Although the non-protected form is broken down in the rumen, the protected forms remain stable until reaching the abomasum and provides direct absorption and digestibility in the intestine. Regardless of their form, fats are typically insoluble in water and may be referred to as lipids and classified into two categories including glycolipids and triglycerides. Glycolipids are those found in the stems and leaves of forages and are composed of two fatty acids linked to glycerol. Triglycerides are the predominant lipid found in cereal grains and distiller's coproducts (Bauman et al., 2003). These are composed of three fatty acids linked to glycerol making it more efficient at storing energy.

In order to undergo breakdown in the rumen, lipids are exposed to lipolysis and biohydrogenation (Hawke and Silcock, 1969). Lipolysis is carried out by anaerobic rumen bacteria such as *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens*, *Clostridium* and *Propionicbacterium* (Jenkins, 1993; Jenkins et al., 2008; Jarvis and Moore, 2010) which secrete the hydrolytic enzymes esterase and lipase. These enzymes initiate degradation of ester bonds holding the fatty acids to their glycerol backbones (Privé et al., 2015). Once released, these fatty acids are either polyunsaturated or monounsaturated and undergo further degradation via microbial biohydrogenation of the double bonds.

1.4.2. Fat Digestion in the Small Intestine

Due to non-protected lipids being hydrolyzed in the rumen, the majority of those reaching the small intestine are in the form of saturated nonesterified fatty acids, however, some may

remain as triglycerides or enter the small intestine as bacterial phospholipids (Arienti et al., 1974; Bauchart, 1993). Upon reaching the small intestine the esterified lipids undergo emulsification. This includes the cleavage of the ester bonds on the surface of the fat globule again resulting in the formation of fatty acids and glycerol. However, because these globules are hydrophobic the lipase is only capable of cleaving the ester bonds on the outside of the fat globules. In order to mitigate this, the liver produces bile to be stored in the gall bladder. This bile contains phospholipids which help to break down the fat globules and create emulsion droplets (Bauer et al., 2005). After this, lipase is able to attach to the surface of the emulsion droplets by connecting to an amphopathic molecule called colipase (Bauchart, 1993). This breakdown then forms fatty acids capable of being absorbed into the small intestine through enterocytes.

1.5. Protein

1.5.1. Protein Digestion in the Rumen

Ruminants consume sources of protein and non-protein nitrogen. The protein consumed is classified as rumen degradable or rumen undegradable. Bacteria known as *Prevotella* (formerly *Bacteroides*) *ruminicola* and *Butyrivibrio fibrisolvens* are found within the rumen and produce proteolytic enzymes such as proteases, peptidases, and deaminases to help break down the rumen degradable protein (Selinger et al., 1996; Wallace, 1996) while the undegradable protein maintains its structure. The enzymatic action on degradable protein produces amino acids which are rapidly deaminated into keto-acids and ammonia (NH_3). The non-protein nitrogen is also rapidly degraded to produce NH_3. Regardless of its source, ruminal bacteria then utilize this NH_3 for the synthesis of microbial crude protein to be passed to the intestine (van der Walt and Meyer, 1988). It has been proposed that 60 to 90% of the nitrogen received through intake in the

ruminant diet on a daily basis is converted to NH_3 accounting for approximately 50-70% of the bacterial nitrogen derived (Leng and Nolan, 1984). While these numbers seem large it is important to remember that some microbes also utilize amino acids or peptides rather than ammonia (Russell et al., 1992).

The abundant amount of NH_3 produced can also be very beneficial to the ruminal environment as it helps to keep the pH near a neutral zone to allow greater activity of microbial species. While some NH_3 leaves the rumen via absorption through the epithelium and into the portal vein the rest continues to be flushed into the small intestine.

1.5.2. Protein Digestion in the Small Intestine

The proteins supplied to the small intestine are the result of ruminally undegradable dietary protein, microbial cells, and endogenous secretions (Harmon, 1993) and require a greater amount of time to be broken down. As the concentration of bicarbonate secreted by the pancreas is lower in ruminants than it is in monogastrics, proteolysis along the small intestine is prolonged. This allows trypsin, chymotrypsin, and carboxipeptidase more time to become active and help to break down some of the remaining protein components into peptides amino acids. Amino acids and small peptides are absorbed through the small intestine. The remaining undegradable protein and microbial crude protein that have not been absorbed will transfer to the large intestine and undergo fermentation where they will be absorbed or recycled. The microbial proteolytic enzymes present in the cecum are made up of protease, deaminase and urease activities (van der Walt and Meyer, 1988). Here, the products of fermentation are ammonia and microbial protein. Any urea that has made it to this point of the large intestine is likely to be converted to NH_3 by urease and absorbed into the blood stream. Amino acids that are not stored

for the synthesis of protein can also be converted to NH_3 when the amino-N is removed and urea is formed while the carbon skeleton is then oxidized to CO_2 (Bach et al., 2005).

Ammonia that is transported from the digestive tract (primarily the rumen) is typically sent to the liver for synthesis of urea. From here it can be recycled to the saliva or rumen where it passes through the rumen wall and becomes a source of N to the microbes. Urea can also be transported post-ruminally to the small or large intestine which occurs predominantly under the control of plasma urea nitrogen. Urea not recycled to the gastrointestinal tract is excreted in urine.

1.6. Production of VFA

While rumen microbes are responsible for providing enzymes and acting as nutrients, they also produce volatile fatty acids (VFA) including acetate, isobutyrate, butyrate, propionate, isovalerate, and valerate (Church, 1988; Dijkstra, 1993). Those most commonly investigated, however, include only acetate, butyrate, and propionate (Jha and Berrocoso, 2016) and therefore will be the focus of the following sections.

In general, the production of these VFA occurs in the rumen and large intestine as the dietary carbohydrates, such as cellulose, hemicellulose, pectin, starch, and soluble sugars are fermented into glucose. This glucose is then used to produce VFA when converted into pyruvate via the Embden-Meyerhof glycolytic pathway (France and Dijkstra, 2005).

Once reaching the stage of pyruvate formation, acetate may be obtained in one of two ways (**Figure 1-1**).

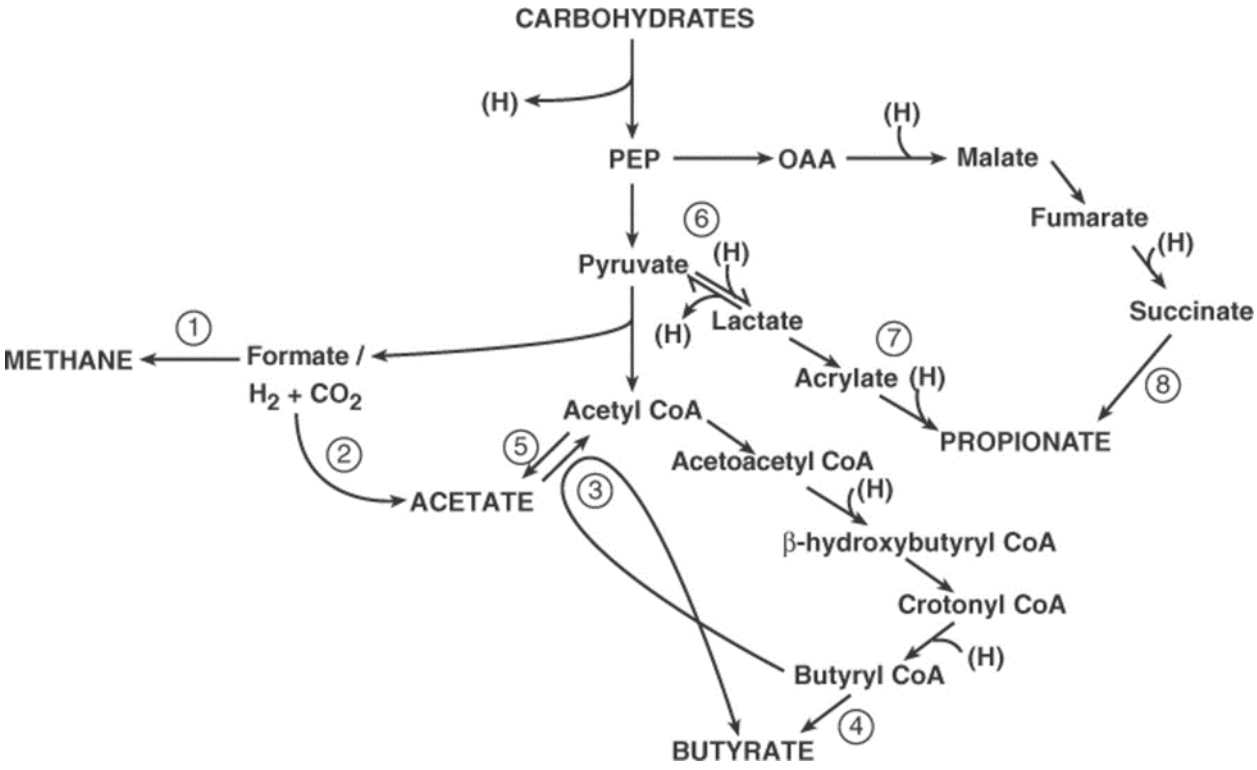

Figure 1-1. Schematic representation of the production of VFA in the large intestine (from Pryde et al., 2002). 1 = Methanogenesis, 2 = reductive acetogenesis, 3 = butyryl CoA:acetate CoA transferase, 4 = phosphotransbutyrylase/butyrate kinase, 5 = phosphotransacetylase/acetate kinase, 6 = lactate dehydrogenase, 7 = acrylate pathway, 8 = succinate decarboxylation.

The first involves the conversion via the pyruvate-formate lyase system outlined as follows: pyruvate + CoA → acetyl-CoA + formate → acetate. The second pathway used to create acetate is known as the pyruvate-ferredoxin oxidoreductase pathway. This pathway is conducted through pyruvate → reduced ferredoxin → acetyl-CoA → acetyl phosphate → acetate and occurs through oxidation and the release of hydrogen (Hungate, 1966; Baldwin and Allison, 1983).

The synthesis of butyrate also involves two differing pathways. The first includes pyruvate → acetyl-CoA → acetoacetyl-CoA → β-hydroxybutyryl-CoA → crotonyl-CoA → butyryl-CoA → butyrate while the second requires the reversal of β-oxidation: pyruvate →

acetyl-CoA → acetoacetyl-CoA → β-hydroxybutyryl-CoA → crotonyl-CoA → butyryl-CoA → butyryl-phosphate → butyrate (Leng, 1970; Yarlett et al., 1985; Nafikov and Beitz, 2007). While butyrate is formed in the rumen it is predominantly converted to ketone bodies during absorption through the epithelium. In fact, 3 times more ketone bodies are found than butyrate in the portal vein (Lomax and Baird, 1983; Lozano et al., 2000). Once reaching the liver, the remaining butyrate is also converted into ketone bodies.

The level of propionate produced rises when the ruminant animal is consuming high-grain diets. Its leading cause of production is through the dicarboxylic acid pathway: pyruvate → oxaloacetate → malate → fumarate → succinate → methylmalonyl CoA → propionate. However, propionate may also be produced through the acrylate pathway: pyruvate → lactate → acrylate → propionate (Baldwin et al., 1965; Cheeke and Dierenfeld, 2010). After absorption through the rumen epithelium, propionate is transported to the liver through the portal vein system where it either undergoes a conversion to glucose via gluconeogenesis or is utilized as an energy source throughout the body via oxidation (Chow and Jesse, 1992).

Glucose that is not converted to VFA may also be used as energy for bacterial growth or converted into gases such as CO_2 or CH_4.

1.7. Impact of Nutrition on the Pancreatic Endocrine System

Insulin is an important hormone within the body as it is the primary hormone involved in glycogenesis and regulating the glucose supply to cells. Its secretion from the pancreas is responsible for the proper metabolism of carbohydrates, proteins, and lipids. Insulin also acts as a major anabolic hormone which functions to preserve nutrients and serve as a regulator of feed intake. This is accomplished by insulin signaling the liver, muscle and fat cells to take in the

excess glucose from the blood and either store it as glycogen or use it as an energy source primarily for protein synthesis (Dimitriadis et al., 2011; Oh et al., 2015). These processes then work to provide adequate energy for the body's growth and functionality which, consequently, change drastically depending on what stage of life the animal is in. For example, the body's energy requirements are high during periods of late gestation or early lactation and more nutrients will typically need to be consumed during these times (Bell and Bauman, 1997).

The amount of feed consumed is not the only area which deserves consideration. The functions of the nutrients provided by the feed are also important. While acetate has not been shown to cause significant effects on insulin secretion, both butyrate and propionate have. Excessive butyrate production may indicate a diabetic concern as it has been found that, during periods of inadequate nutrient consumption, as is typically seen during early lactation, an increased number of free fatty acids are passed to the liver and initiate increased production of β-hydroxybutyrate and ketone bodies resulting in ketosis and initiating hypoglycemia. During this time, it has also been shown that insulin secretion is lowered resulting in diabetes mellitus (Brockman, 1979).

Propionate has been shown to contribute 32% to 73% of the hepatic glucose synthesis in ruminants (Oh et al., 2015). This is due to propionate acting as the major substrate for gluconeogenesis. It enters the system via propionyl-CoA carboxylase, methylmalonyl-CoA mutase, and the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK). Propionate shows a high metabolic priority and will continue its impact on gluconeogenesis even if glucose is adequately supplied (Aschenbach et al., 2010). The production of excess levels of propionate, however, may cause a reduction in feed intake when cattle are fed diets composed of highly fermentable feedstuffs (Bradford et al., 2006). This has been demonstrated in cattle undergoing

infusion of propionate into the rumen and blood stream via the mesenteric vein and portal vein (Allen et al., 2009). Such infusion has been shown to initiate hypophagic responses.

In the past, researchers have provided supplemental propionate intravenously in an attempt to counteract the occurrence of growth retardation in Japanese black cattle. While the concentration of insulin in the plasma increased, the concentration of glucagon decreased (Takasu et al., 2007). In another study conducted by Sano et al. (1999), it was found that the intravenous injection of propionate resulted in an increased plasma glucose concentration in growing calves but no changes in the glucose concentrations in lactating animals. Regardless of the lack of change in glucose concentration, the concentration of insulin in the blood by both the growing and lactating cattle increased.

Although diabetic conditions are the most well-known harmful effects surrounding insulin resistance, there are many more areas that are impacted such as female fertility through its action on gonadotropin-releasing hormone and the stimulation of the uptake of amino acids (Demigné et al., 1988).

1.8. Development of the Ruminant Digestive System

1.8.1 Fetal Stage

During development, the fetus is dependent on its mother for providing adequate nutrition. The nutrients it receives have already undergone significant breakdown in order to allow it to be absorbed into the blood stream and transferred to the embryo (Dunlap et al., 2015). Despite previous nutrient degradation, Guilloteau et al. (1984) evaluated the fetal digestive system of bovines and found the abomasal and pancreatic glands to contain a large number of digestive enzymes. The enzymes discovered included chymosin, colipase, lipase, chymotrypsin,

and trypsin, while pepsin and amylase were not measurable. It has also been found that the weight of the pancreas relative to body weight changed very little throughout the majority of gestation; however, during the last three months in utero the pancreatic weight was shown to increase at a slower rate than whole body weight (Guilloteau et al., 2009).

Although the mother is responsible for providing nutritional resources to the fetus, there are times throughout the production year where meeting these needs can be challenging. When faced with this issue, changes in the functionality and growth of the fetal digestive system may occur regardless of genetics (Godfrey and Barker, 2000; Reynolds et al., 2010). These changes are known as developmental programming and relate to the impact that the intrauterine environment has on the health and well-being of the fetus as it advances in maturity (Barker, 2004; Langley-Evans, 2006; Reynolds and Caton, 2012).

The mechanisms controlling developmental programming according to Reynolds and Caton (2012) include "(1) irreversible alterations in tissue and organ structure and (2) permanent changes in tissue function." These changes may occur throughout all stages of fetal development and cause permanent changes regarding the growth and function of its digestive system (Barker, 2004; Reynolds et al., 2010; Reynolds and Caton, 2012; Oberbauer, 2015). Unfortunately, not only is the fetus affected but these issues have been shown to induce multigenerational consequences transferred via modifications of the epigenetic genome (Pribyl et al., 2008).

Although there is much research to be done, there have been some advances in determining how to mitigate the downfalls associated with poor developmental periods of the fetus. One thought, for example, is to provide the mother with an adequate level of nutrients before parturition. Much of the development of the fetus will occur during the third trimester and, therefore, realimentation has been implicated to "rescue" fetal development during this

time. As seen in several species, realimentation has been able to increase the body weight of restricted fetuses to a mass that matches those provided adequate nutrition (Gonzalez et al., 2013). The same results were seen regarding organ weights and functionality of the fetal digestive system (Wiecek et al., 2011).

Gaining an understanding of fetal development when faced with malnutrition is extremely important as well. Although offspring may have been negatively influenced during fetal development, they were still able to survive. Therefore, there are several aspects to consider. If a breed of animals is likely to endure the issue of nutrient restriction for a prolonged period of time, the fact that they are still able to conceive could be extremely beneficial. This, however, depends on the adaptive ability of the neonates to utilize ingested nutrients in a more efficient way through adaptation of physiological processes such as exocrine and endocrine functions. More work is needed in this research area.

1.8.2. Post-natal Growth

Upon birth, cattle and sheep are technically non-ruminants because of the closure of the reticular groove during nursing. This closure ensures the nutrients found in the milk are transported directly to the omasum bypassing reticulorumen degradation. While the microbial environment of the reticulorumen may not be well developed following parturition, the animal is quickly exposed to bacteria, protozoa, and fungi through nursing and environmental exposure (Morgavi et al., 2015). The mass of the digestive organs also quickly changes as the animal matures and the diet transitions from milk to solid feed (Jami et al., 2013).

Within the first 2 days of extra-uterine life of a bovine, the weight of the pancreas varies little from that present at birth. In sheep, however, the weight is expected to increase by 18%. Once a week has passed, measurements have shown that the pancreatic weight of both species is

increased by 30% of that at birth, and from this point on appears to remain relatively stable (Guilloteau et al., 2009).

While the abomasum is the largest compartment of the stomach at birth, it has been shown to decrease in size relative to BW as the animal continues to develop and to ingest greater amounts of dry matter. At this point the reticulorumen begins to expand as it includes the growth of the rumen papillae to enhance nutrient absorption and increased muscular development to improve the movement of feed (Harrison et al., 1960).

In relation to maternal realimentation, many producers are apt to implement compensatory gain strategies after an animal is born and subjected to nutrient restriction. Compensatory gain is defined as a period of accelerated growth following a lengthy time of restricted development. Meyer and Clawson (1964) suggest that the increased gain is caused by increased energy utilization in feed. Coleman and Evans (1986) investigated compensatory gain and its relation to breed, age, and previous rate of gain and found that older-control steers gained faster than younger-control steers while younger-restricted steers had higher rates of gain than older-restricted steers. These results suggest that the animal's previous weight, age, and rate of growth can influence how great the compensatory gain will be.

1.9. Improving Nutrient Absorption

1.9.1. Distiller's Inclusion Rate and Feed Processing Methods to Enhance Enzymatic Activity and Improve Nutrient Utilization

Grain-based co-products like distiller's grains are a common feedstuff in finishing diets in North America because of their accessibility, nutritional value, and cost per unit of energy. They have proven to be extremely beneficial in improving feed efficiency of cattle. In a finishing

trial, Ham and colleagues (1994) found cattle consuming either wet distiller's grain with solubles (WDGS) or dried distiller's grain with solubles (DDGS) fed at 40% DM showed faster and more efficient rates of gain than those fed dried rolled corn alone. Additionally, Klopfenstein et al. (2008) discovered similar beneficial results across all experiments in his meta-analysis of nine studies where DGS were fed to feedlot cattle. These studies showed that animals consuming the distiller's grain with solubles (DGS) had both higher average daily gain (ADG) and gain to feed (G:F) ratios than those that were fed only corn-based diets without DGS.

With the positive results of increased ADG and gain efficiency seen in feedlot trials, many producers have chosen to incorporate condensed corn distiller's solubles (CCDS) supplementation to offset the negative impact of grazing inadequate forages (Lardy, 2007). Microorganisms within the rumen slowly degrade the protein found in the low-quality forage and this low level of degradation causes a deficiency in metabolizable protein which adversely impacts the animal's growth (Klopfenstein et al., 2001). By supplementing cattle with an increasing energy source, linear increases in weight gain can occur, as was seen by Lake et al. (1974). Morris et al. (2005) and MacDonald et al. (2007) revealed supplementing forage-fed steers with DDGS has been shown to improve ADG and G:F ratios while decreasing forage intake as well.

In both cases of either feedlot or pasture supplementation, the increase in feeding value is thought to be partially due to the rumen undegradable protein (RUP) in DGS in conjunction with the idea that the fat may also be slightly protected from ruminal degradation. This protection permits more unsaturated fat to make its way into the duodenum and allows for a higher total tract fat digestibility (Klopfenstein et al., 2008). Higher concentrations of RUP also allow for a larger number of available amino acids (Belyea et al., 2010) and is fundamental in the optimal

development and productivity of growing ruminants (Mercer and Annison, 1976). Larson et al. (1993) stated that the undegradable protein and fat in WDGS had the potential to raise the feeding value almost 20% over whole corn while studies by Aines et al. (1985) show that DDGS have a bypass value almost 160% greater than that of soybean meal. When consumed through drinking, over 50% of thin stillage bypassed ruminal fermentation (Dehaan et al., 1982).

The lower cost of DGs and their potential for increasing cattle performance make the use of these coproducts an attractive option for feeding livestock. However, when including DDGS in cattle rations, it is important to consider not only the nutrient variability but also the inclusion level. Buckner et al. (2007) conducted a feedlot finishing trial in order to determine the effects of feeding varying levels of DDGS in corn-based diets on cattle performance. In this study, Buckner assigned 250 steers to six different treatments with five pens per treatment and eight steers per pen. Treatments consisted of cattle being fed with 0%, 10%, 20%, 30%, 40% and 50% DDGS replacing dry-rolled corn on a DM basis. All diets also contained 10% corn silage and 2.5% ground alfalfa hay to obtain a roughage level of 7.5%. At the conclusion of the 167-day trial, Buckner and colleagues observed quadratic trends in ADG and final BW with increasing levels of DDGS from 10-40%. They also noticed, however, an increased incidence of polioencephalomalacia when cattle were fed DDGS at 50% DDGS on a DM basis. Because of these findings, Buckner suggests that optimal gains occur when DDGS are included at 20% dietary DM.

Additionally, in an experiment conducted by Gunn et al. (2009), steers with similar body weight were fed corn-based diets supplemented with either 25 or 50% of dietary DM as DDGS. The researchers found that increasing the inclusion level of DDGS to 50% negatively impacted the live animal performance and was believed to be partially due to the negative effect of dietary

fat on rumen fermentation implying that 50% is too great of an inclusion level to be beneficial.

While provision of distiller's grains has many beneficial attributes, it is also important to account for the reduction of starch that occurs during its production (Klopfenstein et al., 2008; Subramaniam et al., 2016). One approach to improve performance in cattle fed DDGS is to change the level of the corn grain processing. Many different methods of processing feeds have been used to improve starch availability. One method commonly employed by feed mills is to utilize mechanical processing of the whole grain. In addition to increasing starch digestibility, this has also been shown to improve animal performance (Galyean, et al, 1979 and Galyean, 1996). Some of these processing methods include steam flaking, dry rolling, grinding, pelleting, etc. The benefit of this is allowing enzymes greater access to the nutrients and helps to increase ruminal fermentation and post-ruminal digestibility (Xiong et al., 1991). When examining starch digestion, steam-rolling caused a greater retention period within the rumen than dry rolling (Zinn, 1994). The longer retention time within the rumen allowed for a greater production and absorption of VFA (Ortega and Mendoza, 2003).

While it has proven to be beneficial to provide ruminants with adequate starch digestibility the source of the starch was shown to impact nutrient utilization. Consumption of the diet has also been shown to decrease with increasing starch intake (Moharrery et al., 2014). Although a great deal of care is taken in formulating rations, it is impossible to obtain a perfectly consistent product and ensure the animal is eating and using the diet in an effective manner. Therefore, the research studying variable rations must continue to be explored.

1.9.2. Addition of Enzymes to Improve Nutrient Utilization

In order to increase livestock performance and the efficiency of nutrient utilization by ruminants, supplemental enzymes have been developed (Beauchemin et al., 1997). While several

studies have shown beneficial results for ADG, others have found little or negative outcomes (Kercher, 1960; Perry et al., 1960; Beauchemin et al., 2004). This is likely the result of the variable feed components included in the diet. For example, when working to determine the impact of a mixture of xylanase (Xylanase B, Biovance Technologies Inc., Omaha, NE) and cellulase products (Spezyme CP®, 17 Genencor, Rochester, NY) on high grain diets composed of either 95% corn or barley, the only increase in feed efficiency with barley diets while feed efficiency of cattle fed corn diets remained unaffected (Beauchemin et al., 1995). Other studies have shown that the inclusion of exogenous amylase tended to reduce digestibility lag time as well as increase the rate of fermentation (Gallo et al., 2016). While it may seem extremely beneficial to increase the breakdown of such nutrients it has been determined that increases in some enzymatic activity may hinder the response of others. For example, Harmon (1992) stated that increases in pancreatic protease content may be associated with decreased amylase content. Increased pancreatic amylase, however, may be associated with decreased protease and lipase secretion indicating that there is a significant interaction between the activity of these enzymes which needs to be considered during ration formulation. Another concern may be related to the idea that the ruminal microorganisms will adapt to the enzymes being supplemented and, therefore, stop producing as many enzymes themselves or opportunistic organisms without extracellular enzymes will benefit.

A reason these enzymes have been shown to be beneficial is reflected on their ability to break down cellulose before entering the rumen. As stated before, when high grain diets are fed, the pH of the rumen has been shown to drop significantly. This drop then impacts the growth and functionality of the anaerobic microbes responsible for the synthesis of fibrolytic enzymes. The supplemental enzymes however, have the ability to counteract this reduction and remain active

upon entering the rumen (Muzakhar et al., 1998). Approximately 40 to70% of the dry matter found in a forage-based ration is composed of plant cell walls and, when undergoing digestion, typically only 65% of these cell walls are able to be degraded (Beauchemin et al., 2004). This too, needs to be taken into consideration.

Although the addition of enzymes to ruminant diets may currently be an expensive process, and further research is needed, it is quickly gaining interest as would not only influence feed degradation and nutrient utilization, but may also help reduce nutrient excretion and the generation of greenhouse gases (Li et al., 2009).

In Asia, rice is a primary crop. While the rice itself is not a viable feed source for ruminants, the rice straw is commonly fed. Unfortunately, the straw is composed largely of lignin which is hard to digest by the microbes in the rumen. Therefore, feed additives are typically utilized during the ensiling process to make the nutrients more available for ruminant digestion (Oladosu et al., 2016). When not being provided to the ruminant the crop straws are typically burned. The burning of crop straws causes the loss of nutrients with calculations showing a reduction of 30 to 35% of phosphorus, 40% of nitrogen, 40 to 50% of sulfur, and 80 to 85% of potassium which may otherwise have proven to be beneficial to the animal (Dobermann and Fairhurst, 2002).

1.10. Environmental Sustainability

By 2050, the global number of cattle is predicted to grow to 2.6 billion whereas goats and sheep will increase to 2.7 billion (Thorton, 2010). Despite the advantage of providing foods for human consumption, the environmental impact of livestock production also needs to be considered (Steinfeld, 2006). One area of concern is the increase in land needed to provide an

adequate feed for ruminants. In 2014 it was calculated that livestock encompassed 26% of the terrestrial surface on the planet (Ripple et al., 2014). While 40% of grasslands remain, leaving room for livestock growth, in order to keep up with the demands predicted for 2050, it is believed that some countries will require a 30 to 50% increase in the amount of land used for animal production (Machovina et al., 2015). In addition to the utilization of the land, the level of greenhouse gases emitted from ruminants must also be taken into consideration. Researchers have calculated that agriculture contributes up to 18% of greenhouse gas emission (Steinfeld, 2006) while livestock are predicted to be responsible for 3% (Pitesky et al., 2009). Much of this difference is accounted for by the variables included in each calculation such as feed production and overall transportation. Regardless, there is no doubt that ruminants are capable of producing greenhouse gases through enteric fermentation.

1.10.1. Methane

The rumen environment is anaerobic and as fermentation continues, gases such as carbon dioxide (CO_2), methane (CH_4), and hydrogen sulfide (H_2SO_4) are produced and rise above the liquid fraction of the rumen and are expelled during eructation. While the concentration of CO_2 may be more abundant, there has always been greater concern over the global warming potential of CH_4. Over a 100-year period, CH_4 is considered to have a global warming potential 23 times higher than that of CO_2 (IPCC, 2000).

Many feeding strategies have been implicated to start helping to reduce methane. One of the most common methods is centered on the inclusion of ionophores into the diet. Once ionophores are included, ruminants have been shown to have reduced enteric methane emissions by 30% (L/ kg of DMI) during the first 2 weeks or 27% during the first 4 weeks (Guan et al., 2006). It is also important to consider not only the concentrate level of the diet, but also the

length of time the diet is to be provided (Hook et al., 2011). This is an important consideration as the ruminal pH is a major indicator of the carbon and hydrogen concentrations available for the production of CH_4. As stated, when ruminants are fed high-concentrate diets, their rumen pH will begin to drop. This then impacts the ruminal bacteria responsible for regulating the acetate to propionate ratio. As the ratio begins to decrease, CH_4 production also will decline (Hatew et al., 2015; Russell, 1998).

While the production of VFA are essential to the growth of the animal, they also have an environmental impact through the production of CO_2 and H_2. When examining the stoichiometry of VFA production, it can be seen that acetate is responsible for producing two moles of CO_2 and four moles of H_2 per mole of glucose fermented. Methanogens will then shift these compounds and form one mole of CO_2 and one mole of CH_4 per mole of glucose fermented. Butyrate is also responsible for generating greenhouse gases through the production of 1.5 moles of CO_2 and 0.5 moles of CH_4 per mole of glucose fermented. Propionate however, will not result in the formation of CO_2 and requires the input of reducing equivalents leading to a decrease in CH_4 production (Sejrsen et al., 2006).

1.11. Literature Summary

Digestive enzymes play a critical role in the breakdown and utilization of nutrients. They also have a profound impact on the production of volatile fatty acids which in turn effects the endocrine function of the pancreas as well as influence the concentration of greenhouse gases expelled by the ruminant. Therefore, the objectives of these studies were to 1) obtain a further understanding of enzymatic activity relating to the impact of dietary components and level of maturity (Chapter 2), 2) investigate the effects of arginine infusion during different stages of the

estrus cycle (Chapter 3), 3) explore the impact of realimentation on fetal pancreatic development during various stages of gestation (Chapter 4), 4) and determine the beneficial aspects of rumen enzymatic function when feeding various levels of distiller's grain with corn grain of different sizes (Chapter 5) and 5) examine enzymatic activity when feeding a corn vs. barley based ration with variable levels of fat in distiller's grain (Chapter 6). The impact of the enzymes on the animal's endocrine system, VFA production, and greenhouse gas production is also important and has been additionally included in these trials.

1.12. Literature Cited

Aines, G., T. J. Klopfenstein, and R. A. Britton. 1985. Thin stillage: Potential use in ruminant diets. NE Beef Cattle Rpt. MP46:62.

Allen, M.S., B. J. Bradford, and M. Oba. 2009. Board-invited review: The hepatic oxidation theory of the control of feed intake and its application to ruminants. J. Anim. Sci. 87:3317-3334.

Arienti, G., F. A. Harrison, and W. M. F. Leat. 1974 The lipase activity of sheep pancreatic juice. Q. J. Exp. Physiol. 60:15–24.

Aschenbach, J. R., N. B. Kristensen, S. S. Donkin, H. M. Hammon, and G. B. Penner. 2010. Gluconeogenesis in dairy cows: The secret making of sweet milk from sour dough. Life 12:869-877.

Bach, A., S. Calsamiglia, and M. D. Stern. 2005. Nitrogen metabolism in the rumen. J. Dairy Sci. 88:E9-E21

Baldwin, R. L. and M. J. Allison. 1983. Rumen metabolism. J. Anim. Sci. 57:461-477

Baldwin, R. L., W. A. Wood and R. S. Emery. 1965. Lactate metabolism by *Peptostreptococcus els denii*: Evidence for lactyl coenzyme A dehydrase. Biochim. Biophys. Acta 97: 202

Barker D. J. P. 2004. Developmental origins of well-being. Philos. Trans. Royal Soc. London. 359:1359–1366.

Bauchart, D. 1993. Lipid absorption and transport in ruminants. J. Dairy Sci. 76:3864-3881.

Bauer, E., S. Jakob, and R. Mosenthin. 2005. Principles of physiology of lipid digestion. Asian-Aust. J. Anim. Sci. 18:282-295.

Bauman, D. E., J. W. Perfield, M. J. de Veth, and A. L. Lock. 2003. New perspectives on lipid digestion and metabolism in ruminants. In 'Proceedings of the Cornell nutrition Conference'. pp. 175–189.

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, W. Z. Yang, and L. M. Rode. 2004. Mode of action of exogenous cell wall degrading enzymes for ruminants. Can. J. Anim. Sci. 84:13-22.

Beauchemin, K. A., S. D. M. Jones, L. M. Rode, and V. J. H. Sewalt. 1997. Effects of fibrolytic enzyme in corn or barley diets on performance and carcass characteristics of feedlot cattle. Can. J. Anim. Sci. 77:645-653.

Beauchemin, K. A., L. M. Rode, and V. J. H. Sewalt. 1995. Fibrolytic enzymes increase fiber digestibility and growth rate of steers fed dry forages. Can. J. Anim. Sci. 75:641-644.

Bell, A. W. and D. E. Bauman. 1997. Adaptations of glucose metabolism during pregnancy and lactation. J. Mammary Gland Biol. Neoplasia. 2:265-278.

Belyea, R. L., K. D. Rausch, T. E. Clevenger, V. Singh, D. B. Johnston, and M. E. Tumbleson. 2010. Sources of variation in composition of DDGS. Anim. Feed Sci. and Tech. 159:122-130.

Bradford, B. J., A. D. Gour, A. S. Nash, and M. S. Allen. 2006. Propionate challenge tests have limited value for investigating bovine metabolism. J. Nutr. 7:1915-1920.

Brockman, R. P. 1979. Roles for insulin and glucagon in the development of ruminant ketosis -a review. Can. Vet. J. 20;121-126.

Buckner, C. D., T. L. Mader, G. E. Erickson, S. L. Colgan, K. K. Karges, and M. L. Gibson. 2007. Optimum levels of dry distillers grains with solubles for finishing beef steers. NE Beef Cattle Rpt. MP90:36–38.

Cerrilla, M. E. O. and G. M. Martínez. 2003. Starch digestion and glucose metabolism in the ruminant: a review. Interciencia 7:380-386. ISSN 0378-1844.

Cheeke, P. R., and E. S. Dierenfeld. 2010. Comparative animal nutrition and metabolism. Wallingford, Oxfordshire, UK: CABI.

Church, D. C. (ed.). 1988. The ruminant animal: digestive physiology and nutrition. Prentice-Hall, Englewood Cliffs, N.J. 564 p.

Chow, J. C., and E. W. Jesse. 1992. Interactions between gluconeogenesis and fatty acid oxidation in isolated sheep hepatocytes. J. Dairy Sci. 75:2142-2148.

Coleman, S. W., and B. C. Evans. 1986. Effect of nutrition, age, and size on compensatory growth in two breeds of steers. J. Anim. Sci. 63:1968-1982.

Croom, W. J. -Jr., L. S. Bull and I. L. Taylor. 1992. Regulation of pancreatic exocrine secretion in ruminants: a review. J. Nutr. 122:191-202.

DeHaan, K., T. Klopfenstein, R. Stock. S. Abrams and R. Britton, 1982. Wet distillers byproducts for growing ruminants. NE Beef Cattle Rpt. MP43:33.

Demigné, C., C, Yacoub, C. Morand, and C. Rémésy. 1988. Findings on intermediate metabolism in ruminants. Reprod. Nutr. Dev. 28:1-17.

Dijkstra, J. 1993. Production and absorption of volatile fatty acids in the rumen. Livestock Production Science 39:61-69.

Dimitriadis, G., P. Mitrou, V. Lambadiari, E. Maratou, and S. A. Raptis. 2011. Insulin effects in muscle and adipose tissue. Diabetes Res. Clin. Pract. 93:S52-59.

Dobermann A. and T. H. Fairhurst. 2002. Rice straw management. Better Crops International. 16:7–11.

Dunlap, K. A., J. D. Brown, A. B. Keith and M. C. Satterfield. 2015. Factors controlling nutrient availability to the developing fetus in ruminants. J. Anim. Sci. and Biotech. 6:16.

FAO. 2015. The state of food security in the world: Meeting the 2015 international hunger targets: taking stock of uneven progress.

France, J., and J. Dijkstra. 2005. Volatile fatty acid production. in: J. Dijkstra, J.M. Forbes, J. France (Eds.) Quantitative Aspects of Ruminant Digestion and Metabolism. 2nd. CABI Publishing, Wallingford, UK:157–176.

Franzolin, R. and Dehority, B. A. 2010. The role of pH on the survival of rumen protozoa in steers. 2010. R. Bras. Zootec. 39:2262-2267.

Gallo, A., G. Giuberti, S. Duval, M. Moschini, and F. Masoero. 2016. Short communication: The effect of an exogenous enzyme with amylolytic activity on gas production and in vitro rumen starch degradability of small and large particles of corn or barley meals. J. Dairy Sci. 99:3602–3606.

Galyean, M. L. 1996. Protein levels in beef cattle finishing diets: industry application, university research, and systems results. J. Anim. Sci. 74:2860–2870.

Galyean, M. L., D. G. Wagner, and R. R. Johnson. 1976. Site and extent of starch digestion in steers fed processed corn rations. J. Anim. Sci. 43:1088–1094

Godfrey, K. M. and D. J. Barker. 2000. Fetal nutrition and adult disease. Am. J. Clin. Nutr. 71(Suppl):1344S-1352S.

Gonzalez, J. M., L. E. Camacho, S. M. Ebarb, K. C. Swanson, K. A. Vonnahme, A. M. Stelzleni, and S. E. Johnson. 2013. Realimentation of nutrient restricted pregnant beef cows supports compensatory fetal muscle growth. J. Anim. Sci. 91:4797-4806

Guan, H., K. M. Wittenberg, K. H. Ominski, and D. O. Krause. Efficiency of ionophores in cattle diets for mitigation of enteric methane. J. Anim. Sci. 84:1896-1906

Guilloteau, P., T. Corring, R. Toullec, and R. Guilhermet. 1985. Enzyme potentialities of the abomasum and pancreas of the calf. II. Effects of weaning and feeding a liquid supplement to ruminant animals. Reprod. Nutr. Dev. 25:481-493.

Guilloteau, P., T. Corring, R. Toullec, and J. Robelin. 1984. Enzyme potentialities of the abomasum and pancreas of the calf. I.--Effect of age in the preruminant. Reprod. Nutr. Dev. 24:315-25.

Guilloteau, P., R. Zabielski, and J. W. Blum. 2009. Gastrointestinal tract and digestion in the young ruminant: ontogenesis, adaptations, consequences and manipulations. J. Physiol. Pharm. 60:37-46

Gunn, P. J., A. D. Weaver, R. P. Lemenager, D. E. Gerrard, M. C. Claeys, and S. L. Lake. 2009. Effects of dietary fat and crude protein on feedlot performance, carcass characteristics, and meat quality in finishing steers fed differing levels of dried distillers grains with solubles. J. Anim Sci. 87:2882-2890.

Harrison, H. N., R. G. Warner, E. G. Sander, and J. K. Loosli. 1960. Changes in the tissue and volume of the stomachs of calves following the removal of dry feed or consumption of inert bulk. J. Dairy Sci. 43:1301-1312

Harmon, D. L. 1992. Impact of nutrition on pancreatic exocrine and endocrine secretion in ruminants: a review. 'J. Anim. Sci. 70:1290-1301.

Harmon, D. L. 1993. Nutritional regulation of postruminal digestive enzymes in ruminants. J. Dairy Sci. 76:2102-2111.

Harmon, D. L. 2009. Understanding starch utilization in the small intestine of cattle. Asian-Aust. J. Anim. Sci. 22:915-922.

Harmon, D. L., R. M. Yamka, and N. A. Elam. 2004. Factors affecting intestinal starch digestion in ruminants: a review. Can. J. Anim. Sci. 84:309-318.

Hatew, B., S. C. Podesta, H. Van Laar, W. F. Pellikaan, J. L. Ellis, J. Dijkstra, and A. Bannink. 2015. Effects of dietary starch content and rate of fermentation on methane production in lactating dairy cows. J. Dairy Sci. 98: 486-499.

Hawke, J. C. and W. R. Silcock. 1969. Lipolysis and hydrogenation in the rumen. Biochem. J. 112,131

Hook, S. E., M. A. Steele, K. S. Northwood, A. D. Wright, and B. W. McBride. 2011. Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. Microb. Ecol. 62:94-105

Hoover, W. H. 1986. Chemical factors involved in ruminal fiber digestion. J. Dairy Sci. 69:2755-2766

Hungate, R. E. 1966. The Rumen and its Microbes. Academic Press, New York.

IPCC. 2000. Good practice guidance and uncertainty management in national greenhouse gas inventories. Intergovernmental panel on climate change. ICPP/OECD/IEA/IGES, Hayama, Japan.

Jackson, P. G. G., P. D. Cockcroft, and Wiley InterScience (Online service). 2002. Clinical examination of farm animals. Oxford, UK: Blackwell Science.

Jami, E., A. Israel, A. Kotser, and I. Mizrahi. 2013. Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7:1069-1079

Jarvis, G. N. and E. R. B. Moore. 2010. Lipid metabolism and the rumen microbial ecosystem. In: K. N. Timmis (ed) Handbook of hydrocarbon and lipid microbiology. Springer, Berlin Heidelberg, p 2246–2257.

Jayathilakan, K., K. Sultana, K. Radhakrishna, and A. S. Bawa. 2012. Utilization of byproducts and waste materials from meat, poultry and fish processing industries: a review. Journal of Food Science and Technology. 49:278-293.

Jenkins, T. C. 1993. Symposium: Advances in ruminant lipid metabolism. J. Dairy Sci. 76:3851-3863

Jenkins, T. C., R. J. Wallace, P. J. Moate, and E. E. Mosley. 2008. Recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J Anim Sci. 86: 397–412.

Jha, R., and J. F. D. Berrocoso. 2016. Dietary fiber and protein fermentation in the intestine of swine and their interactive effects on gut health and on the environment: A review. Animal Feed Science and Technology. 212: 18-26.

Kamble, R. D. and A. R. Jadhav, 2012. Isolation, identification and screening of potential cellulase-free xylanase producing fungi and its production. Afr. J. Biotechnol., 11:14175-14181.

Kercher, J. 1960. Value of diallylstilbesterol and Zymo-pabst enzyme preparation for fattening yearling steers. J. Anim. Sci. 19, 966.

Kisia, S. M. 2010. Vertebrates: Structures and functions. Boca Raton, FL: CRC Press.

Klopfenstein, T. J., G. E. Erickson, and V. R. Bremer. 2008. Board invited review: Use of distillers by-products in the beef cattle feeding industry. J. Anim. Sci. 86:1223-1231.

Klopfenstein, T. J., R. A. Mass, K. W. Creighton, and H. H. Patterson. 2001. Estimating forage protein degradation in the rumen. J. Anim. Sci. 79(E Suppl.):E208–E217.

Lake, R. P., R. L. Hildebrand, and D. C. Clanton. 1974. Limited energy supplementation of yearling steers grazing irrigated pasture and subsequent feedlot performance. J. Anim. Sci. 39:827–833.

Langley-Evans, S. C. 2006. Developmental programming of health and disease. Proc. Nutr. Soc. 65:97-105.

Lardy, G. 2007. Feeding co products of the ethanol industry to beef cattle. AS-1242 http://www.ag.ndsu.edu/pubs/ansci/beef/as1242.pdf Accessed Jan. 14, 2011.

Larson, E. M., R. A. Stock, T. J. Klopfenstein, M. H. Sindt, and R. P. Huffman. 1993. Feeding value of wet distillers byproducts for finishing ruminants. J. Anim. Sci. 71:2228–2236.

Leng, R. A. 1970. Biochemistry I (Nitrogen and carbohydrate metabolism). In Phillipson AD (Ed.) Physiology of Digestion in the Ruminant. Oriel Press. Newcastle Upon Tyne, UK. pp. 406-421.

Leng, R. A. and J. V. Nolan. 1984. Nitrogen metabolism in the rumen. J. Dairy Sci. 67:1072-1089

Li, X. Z., C. G. Yan, S. H. Choi, R. J. Long, G. K. Jin, and M. K. Song. 2009. Effects of addition level and chemical type of propionate precursors in dicarboxylic acid pathway on fermentation characteristics and methane production by rumen microbes in vitro. Asian-Aust. J. Anim. Sci. 22; 82-89

Lohrenz, A. K., K. Duske, U. Schönhusen, B. Losand, H. M. Seyfert, C. C. Metges, and H. M. Hammon. 2011. Glucose transporters and enzymes related to glucose synthesis in small intestinal mucosa of mid-lactation dairy cows fed 2 levels of starch. J. Dairy Sci. 94:4546-4555.

Lomax, M. A. and G. D. Baird. 1983. Blood flow and nutrient exchange across the liver and gut of the dairy cow: Effects of lactation and fasting. British J. Nutr. 49:481-496.

Lozano, O., C. B. Theurer, A. Alio, J. T. Huber, A. Delgado-Elorduy, P. Cuneo, D. DeYoung, M. Sadik, and R. S. Swingle. 2000. Net absorption and hepatic metabolism of glucose, L-lactate, and volatile fatty acids by steers fed diets containing sorghum grain processed as dry-rolled or steam-flaked at different densities. J. Anim. Sci. 78:1364-1371.

MacDonald, J. C., T. J. Klopfenstein, G. E. Erickson, and W. A. Griffin. 2007. Effects of dried distillers grains and equivalent undegradable intake protein or ether extract on performance and forage intake of heifers grazing smooth brome grass pastures. J. Anim. Sci. 85:2614–2624.

Machovina, B., K. J. Feeley, and W. J. Ripple. 2015. Biodiversity conservation: The key is reducing red meat consumption. Sci. Total Environ. 536:419-431.

McAllister, T. A., K. –Jr. Cheng, L. M. Rode, and C. W. Forsberg. 1990. Digestion of barley, maize, and wheat by selected species of ruminal bacteria. Appl. Environ. Microbiol. 56:3146-3153.

Mercer, J. R. and E. F. Annison. 1976. Utilization of nitrogen in ruminants. Protein Metabolism and Nutrition, EAAP Pub. No. 16. Butterworths, London

Meyer, J. H. and W. J. Clawson. 1964. Undernutrition and subsequent realimentation in rats and sheep. J. Anim. Sci. 23:214.

Mertens D. R. and J. R. Loften. 1980. The effect of starch on forage fiber digestion kinetics in vitro. J. Dairy Sci. 63:1437-1446.

Moharrery, A., M. Larsen, and M. R. Weisbjerg. 2014. Starch digestion in the rumen, small intestine, and hind gut of dairy cows- A meta-analysis. Anim. Feed Sci. Tech. 192:1-14.

Morales, M. S. and B. A. Dehority. 2014. Magnesium requirement of some of the principal rumen cellulolytic bacteria. Animal. 8:1427-1432.

Moreira, L. M., F. de Paula Leonel, R. A. M. Vieira, and J. C. Pereira. 2013. A new approach about the digestion of fibers by ruminants. RBSPA. 382-395

Morgavi, D. P., E. Rathahao-Paris, M. Popova, J. Boccard, K. F. Nielsen, and H. Boudra. 2015. Rumen microbial communities influence metabolic phenotypes in lambs. Front Microbiol. 6:1060.

Morris, S., T. J. Klopfenstein, and D. Adams. 2005. Effects of dried distillers grains on heifer consumption of low or high quality forage. J. Anim. Sci. 82 (Suppl. 2):52. (Abstr.)

Muzakhar, K., H. Hayashii, T. Kawaguchi, J. Sumitani, and M. Arai. 1998. Purification and properties of α-L-arabinofuranosidase and endo-β-D-1,4-galactanase from Aspergillus niger KF-267 which liquefied the okara. MIE Bioforum, Genetics, Biochemistry and Ecology of Cellulose Degradation. Suzuka, Japan, p. 133.

Nafikov, R. A. and D. C. Beitz. 2007. Carbohydrate and lipid metabolism in farm animals. J. Nutr. 137:702-705.

Noble, R. C. 1978. Digestion, absorption and transport of lipids in ruminant animals. Prog. Lipid Res. 17:55-91.

Nocek, J. E. and S. Tamminga. 1991 Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. J. Dairy. Sci. 74:3598-3629.

Oberbauer, A. M. 2015. Developmental programming: the role of growth hormone. J. Anim. Sci. 6:8.

Oh, Y. K., J. S. Eun. S. C. Lee, G, M. Chu, S. S. Lee, and Y. H. Moon. 2015. Responses of blood glucose, insulin, glucagon, and fatty acids to intraruminal infusion of propionate in hanwoo. Asian-Australas Journal of Animal Science. 2:200-206.

Oladosu, Y., M. Y. Rafii, N. Abdullah, U. Magaji, G. Hussin, A. Ramli, and G. Miah. 2016. Fermentation quality and additives: A case of rice straw silage. Biomed. Res. Int. 2016:7985167.

Oltjen, J. W. and J. L Beckett. 1996. Role of ruminant livestock in sustainable agricultural systems. J. Anim. Sci. 7:1406-1409.

Ørskov, E. R. 1986. Starch digestion and utilization in ruminants. J. Anim. Sci. 63:1624-1633.

Ortega, C. M. E. and G. D. Mendoza. 2003. Starch digestion and glucose metabolism in the ruminant: A review. Interciencia, 28:380-386.

Otajevwo, F. D. 2011. Cultural conditions necessary for optimal cellulase yield by cellulolyticbacterial organisms as they relate to residual sugars released in broth medium. Mod. Appl. Sci. 5:141-151.

Owens, F. N., R. A. Zinn, and Y. K. Kim. 1986. Limits to starch digestion in the ruminant small intestine. J. Anim. Sci. 63:1634-1648.

Parish, J. A., and J. D. Rhinehart. 2008. Fiber in beef cattle diets. Mississippi State University Extension Service publication 2489.

Perry, T. W., D. D. Cope, and W. M. Beeson. 1960. Low vs high moisture shelled corn with and without enzymes and stilbestrol for fattening steers. J. Anim. Sci. 19, 1284.

Pitesky, M. E., K. R. Stackhouse, and F. M. Mitloehner. 2009. Clearing the air: Livestock's contribution to climate change. Adv. Agron. 103:1-40.

Pizauro, J. M. -Jr., J. A. Ferro, A. C. F. de Lima, K. S. Routman, and M. C. Portella. 2004. The zymogen-enteropeptidase system: A practical approach to study the regulation of enzyme activity by proteolytic cleavage. Biochem. Mol. Biol. Educ. 32:45-48.

Prins, R. A., R. E. Hungate, and E. R. Prast. 1972. Function of the omasum in several ruminant species. Comparative biochemistry and physiology part A. Physiology 43:155-163.

Pribyl, J., H. Krejcova, J. Pribylova, N. Mielenz, J. Kucera, and M. Ondrakova. 2008. Estimation of genetic potential of bulls in a case of environmental limitation and compensation of growth. J. of Agrobiology Vol.: 25 Iss.1:5-7.

Privé, F., J. C. Newbold, N. N. Kaderbhai, S. G. Girdwood, O. V. Golyshina, P. N. Golyshin, N. D. Scollan, and S. A. Huws. 2015. Isolation and characterization of novel lipases/esterases from a bovine rumen metagenome. Appl. Microbiol. Biotechnol. 99:5475-5485.

Pryde, S. E., S. H. Duncan, G. L. Hold, C. S. Stewart and H. J. Flint. 2002. The microbiology of butyrate formation in the human colon. FEMS Microbiology Letters/ 217:133-139.

Reynolds, L. P. and J. S. Caton. 2012. Role of the pre- and post-natal environment in developmental programming of health and productivity. Mol. Cell Endocrinol. 354:54-59

Reynolds, L. P., M. C. Wulster-Radcliffe, D. K. Aaron, and T. A. Davis. 2015. Importance of animals in agricultural sustainability and food security. J. Nutr. 145:1377-1379

Reynolds, L. P., P. P. Borowicz, J. S. Caton, K. A. Vonnahme, J. S. Luther, C. J. Hammer, K. R. Maddock Carlin, A. T. Grazul-Bilska, and D. A. Redmer. 2010. Developmental

programming: The concept, large animal models, and the key role of uteroplacental vascular development. J. Anim. Sci. 88:E61-E72

Ripple, W. J., P. Smith, H. Haberl, S. A. Montzka, C. McAlpine and D. H. Boucher. 2014. Ruminants, climate change and climate policy. Nature Climate Change. Volume 4

Röder P. V., K. E. Geillinger, T. S. Zietek, B. Thorens, H. Koepsell, and H. Daniel. 2014. The role of SGLT1 and GLUT2 in intestinal glucose transport and sensing. PLoS One. 2:e89977

Russell, J. B. 1998. The importance of pH in the regulation of ruminant acetate to propionate ratio and methane production in vitro. J. Dairy Sci. 81:3222-3230.

Russell, J. B., J. D. O'Connor, D. G. Fox, P. J. Van Soest, and C. J. Sniffen. 1992. A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. J. Anim. Sci. 70:3551-3561

Russell, J. R., A. W. Young, and N. A. Jorgensen. 1981. Effect of dietary corn starch intake on pancreatic amylase and intestinal maltase and pH in cattle. J. Anim. Sci. 52:1177-1182.

Sano, H., H. Arai, A. Takahashi, H. Takahashi, and Y. Terashima. 1999. Insulin and glucagon responses to intravenous injections of glucose, arginine and propionate in lactating cows and growing calves. Can. J. Anim. Sci. 309-314.

Seligman, H. K., B. A. Laraia, and M. B. Kushel. 2010. Food insecurity is associated with chronic disease among low-income NHANES participants. Journal of Nutrition. 140:304-310.

Sejrsen, K., T. Hvelplund, and M. O. Nielsen. 2006. Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress. Wageningen: Wageningen Academic Publishers. 600

Selinger, L. B., C. W. Forsberg, and K. -Jr. Cheng. 1996. The rumen: a unique source of enzymes for enhancing livestock production. Anaerobe. 2:263-284

Steinfeld, H. 2006. Livestock's long shadow: Environmental issues and options. Rome: Food and Agriculture Organization of the United Nations

Subramaniam, E., M. G. Colazo, M. Gobikrushanth, Y. Q. Sun, A. L. Ruiz-Sanchez, P. Ponce-Barajas, M. Oba, D. J. Ambrose. 2016. Effects of reducing dietary starch content by replacing barley grain with wheat dried distillers grains plus solubles in dairy cow rations on ovarian function. J. Dairy Sci. 99:2762-2774

Sujani, S. and R. T. Seresinhe. 2015. Exogenous enzymes in ruminant nutrition: A review. Asian J. Anim. Sci. 9:85-99.

Swanson, K. C., J. C. Matthews, A. D. Matthews, J. A. Howell, C. J. Richards, and D. L. Harmon. 2000. Dietary carbohydrate source and energy intake influence the expression of pancreatic amylase in lambs. J. Nutr. 130:2157-2165.

Takasu, M., Y, Kishima, N. Nishii, Y. Ohba, S. Maeda, K. Miyazawa, T. Murase and H. Kitagawa. 2007. Insulin and glucagon secretory patterns during propionate and arginine tolerance tests in Japanese black cattle with growth retardation. J. Vet. Med. Sci. 1:69-72.

Thorton, P. K. 2010. Livestock production: recent trends, future prospects. Phil. Trans. Soc. B. 365:2853-2867.

van der Walt, J. G. and J. H. F. Meyer. 1988. Protein digestion in ruminants. S. Afr. J. Anim. Sci. 18:30-41.

Van Soest, P. J. 1982. Nutritional ecology of the ruminant. Ithaca: Comstock Pub.

Varga, G. A. and E. S. Kolver. 1997. Microbial and animal limitations to fiber digestion and utilization. J. Nutr. 127:819S-823S.

Walker, G. J. and P. M. Hope. 1964. Degradation of starch granules by some amylolytic bacteria from the rumen of sheep. Biochem. J. 90:398.

Wallace, R. J. 1996. The proteolytic systems of ruminal microorganisms. Ann. Zootech. 45:301-308.

Wang, Y. and McAllister, T. A. 2002. Rumen microbes, enzymes and feed digestion- a review. Asian-Aust. J. Anim. Sci. 15:1659-1676.

Wiecek, J., A. Rekiel, and J. Skomial. 2011. Effect of restricted feeding and realimentation periods on compensatory growth performance and physiological characteristics of pigs. Arch. Anim. Nutr. 65:34-35.

WHO, 2013. http://www.unscn.org/layout/modules/resources/files/rwns5.pdf

Wood, I. S., J. Dyer, R. R. Hoffman, S. P. Shirazi-Beechey. 2000. Expression of the Na+/glucose co-transporter (SGLT1) in the intestine of domestic and wild ruminants. Pflugers Arch. 441:155-162.

World Population Prospects. The 2012 revision of the world population prospects, population estimates and projections section [Internet]. New York; Population Division, Department of Economic and Social Affairs, UN; 2012 [cited 2016 November 16]. Available from: http://esa.un.org/wpp.

Xiong Y., S. J. Bartle, and R. L. Preston. 1991. Density of steam flaked sorghum grain, roughage level and feeding regime for feedlot steers. J. Anim. Sci. 69: 1707-1718.

Yanke, L. J., L. B. Selinger and K.-J. Cheng. 1995. Comparison of cellulolytic and xylanolytic activities of anaerobic rumen fungi. Proceedings, 23rd Biennial Conf Rumen Function, Chicago, IL. p. 32.

Yarlett, N., D. Lloyd, and A. G. Williams. 1985. Butyrate formation from glucose by the rumen protozoon *Dasytricha ruminantium*. Biochem. J. 228:187-192

Zinn, R. A. 1994. Influence of flake thickness on the feeding value of steam rolled wheat for feedlot cattle. J. Anim. Sci. *72*: 21-28.

CHAPTER 2. INFLUENCE OF NUTRIENT RESTRICTION AND MELATONIN SUPPLEMENTATION OF PREGNANT EWES ON MATERNAL AND FETAL PANCREATIC DIGESTIVE ENZYMES AND INSULIN-CONTAINING CLUSTERS[1]

2.1. Abstract

Primiparous ewes (n = 32) were assigned to dietary treatments in a 2 × 2 factorial arrangement to determine effects of nutrient restriction and melatonin supplementation on maternal and fetal pancreatic weight, digestive enzyme activity, concentration of insulin-containing clusters and plasma insulin concentrations. Treatments consisted of nutrient intake with 60% (RES) or 100% (ADQ) of requirements and melatonin supplementation at 0 (CON) or 5 mg/d (MEL). Treatments began on day 50 of gestation and continued until day 130. On day 130, blood was collected while ewes were under general anesthesia from the uterine artery, uterine vein, umbilical artery and umbilical vein for plasma insulin analysis. Ewes were then euthanized and the pancreas removed from the ewe and fetus, trimmed of mesentery and fat, weighed, and snap-frozen until enzyme analysis. Additionally, samples of pancreatic tissue were fixed in 10% formalin solution for histological examination including quantitative characterization of size and distribution of insulin-containing cell clusters. Nutrient restriction decreased ($P \leq 0.001$) maternal pancreatic mass (g) and α-amylase activity (U/g, kU/pancreas,

[1] The material in this chapter was co-authored by F. E. Keomanivong, C. O. Lemley, L. E. Camacho, R. Yunusova, P. P. Borowicz, J. S. Caton, A. M. Meyer, K. A. Vonnahme, and K. C. Swanson. It has been accepted for publication by Animal, 10(3):440-448. F. E. Keomanivong had primary responsibility for collecting and analyzing samples in the field. F. E. Keomanivong was the primary developer of the conclusions that are advanced here. F. E. Keomanivong also drafted and revised all versions of this chapter. K. C. Swanson served as proofreader and checked the math in the statistical analysis conducted by F. E. Keomanivong.

U/kg BW). Ewes supplemented with melatonin had increased pancreatic mass ($P = 0.03$) and α-amylase content (kU/pancreas and U/kg BW). Melatonin supplementation decreased ($P = 0.002$) maternal pancreatic insulin-positive tissue area (relative to section of tissue), and size of the largest insulin-containing cell cluster ($P = 0.04$). Nutrient restriction decreased pancreatic insulin-positive tissue area ($P = 0.03$) and percent of large (32,001 to 512,000 μm^2) and giant (≥ 512,001 μm^2) insulin-containing cell clusters ($P = 0.04$) in the fetus. Insulin concentrations in plasma from the uterine vein, umbilical artery and umbilical vein were greater ($P \leq 0.01$) in ewes receiving 100% of requirements. When comparing ewes to fetuses, ewes had a greater percentage of medium insulin-containing cell clusters (2001 to 32,000 μm^2) while fetuses had more ($P < 0.001$) pancreatic insulin-positive area (relative to section of tissue) and a greater ($P \leq 0.02$) percentage of small, large and giant insulin-containing cell clusters. Larger ($P < 0.001$) insulin-containing clusters were observed in fetuses compared to ewes. In summary, the maternal pancreas responded to nutrient restriction by decreasing pancreatic weight and activity of digestive enzymes while melatonin supplementation increased α-amylase content. Nutrient restriction decreased the number of pancreatic insulin-containing clusters in fetuses while melatonin supplementation did not influence insulin concentration. This indicated using melatonin as a therapeutic agent to mitigate reduced pancreatic function in the fetus due to maternal nutrient restriction may not be beneficial.

2.2. Implications

Melatonin is a potential therapeutic to overcome negative effects of nutrient restriction during pregnancy because of its roles in regulating blood flow and gastrointestinal and pancreatic

function. We hypothesized that melatonin supplementation would positively impact fetal pancreatic function in nutrient restricted ewes. Results indicate nutrient restriction during gestation had greater impacts on digestive enzyme concentrations in ewes than fetuses and that nutrient restriction decreased pancreatic insulin concentrations. Melatonin supplementation did not influence fetal pancreatic function suggesting that its role as a therapeutic for overcoming negative effects of undernutrition during pregnancy on fetal pancreatic development is limited.

2.3. Introduction

Although nutrient restriction can result in reduced performance of both the dam and fetus, the development of proper intervention techniques may mitigate harmful consequences. Understanding the influence of maternal nutrition on fetal development provides the potential to design supplementation strategies which may help overcome these negative impacts. Melatonin, most widely known for its role in regulating circadian rhythms, has been shown to increase utero-placental blood flow when supplemented to pregnant ewes which has the potential to reduce or offset the negative effects of nutrient restriction on maternal blood flow and return utero-placental nutrient transfer to normal levels (Lemley et al., 2012). Melatonin is synthesized by many tissues of the body with the largest source being produced in the gastrointestinal tract (GIT; Jaworek et al., 2007 and 2012). Melatonin secretion increases with the ingestion of food and can influence GIT function by causing a reduction in the speed of intestinal peristalsis thereby slowing food transit time and allowing for greater nutrient absorption (Bubenik, 2008). Melatonin receptors have also been found on the pancreas and can influence pancreatic insulin secretion, glucose homeostasis, and provide protective mechanisms against oxidative stress which can selectively destroy β-cells (Sartori et al., 2009; Li et al., 2011). Because of its

potential influence on nutrient supply and pancreatic function, melatonin supplementation may mitigate negative impacts of maternal nutrient restriction on fetal growth and pancreatic development. We hypothesized that melatonin supplementation may have positive effects on the functional morphology of the pancreas in nutrient-restricted pregnant ewes and their fetuses. Therefore, the objectives were to determine the effect of dietary melatonin supplementation on pancreatic digestive enzyme activity, morphology, and plasma insulin levels in undernourished pregnant ewes and their fetuses.

2.4. Materials and Methods

This experiment was approved by the North Dakota State University Institutional Animal Care and Use Committee.

2.4.1. Animals and Dietary Treatments

Animal care and dietary treatments were described previously (Lemley et al., 2012). Briefly, 32 nulliparous ewes carrying singletons were selected for the study; however, one ewe was removed due to a perforated esophagus, which existed before dietary treatment. Ewes were transported on day 28 of gestation to a facility that provided a temperature controlled environment at 14˚C and a 12:12 light-dark cycle with lights on at 07:00 and off at 19:00 each day. On day 50 of gestation, the ewes began their dietary treatments. Ewes were individually-penned and provided 100% (ADQ; adequate diet) or 60% (RES; restricted diet) of nutrient recommendations for a pregnant ewe lamb in mid- to late-gestation (NRC, 2007) and diets were supplemented with either 5 mg of melatonin (MEL; Spectrum Chemical Mfg. Corp., Gardena, CA) or no melatonin (CON) in a 2 × 2 factorial arrangement of treatments. The four treatment groups consisted of CON:ADQ (n = 7), MEL:ADQ (n = 8), CON:RES (n = 8), or MEL:RES (n

= 8). The diet was pelleted and contained beet pulp, alfalfa meal, corn, soybean meal, and wheat middlings. All melatonin supplemented ewes were fed melatonin-enriched pellets at 14:00 (5 hr before the start of the dark cycle at 19:00). The pellets were prepared using melatonin powder dissolved in 95% ethanol at a concentration of 5 mg/ml. The day prior to feeding, 100 g of the control pellet was placed into a small plastic bag and top dressed with 1 ml of melatonin solution. The ethanol was allowed to evaporate overnight at room temperature in complete darkness before feeding (Lemley et al., 2012). The amount of feed offered to individual ewes was adjusted weekly based on BW and change in BW to ensure the desired average daily gains were achieved (Reed et al., 2007). Ewes were maintained on their given dietary treatments until day 130 of gestation.

2.4.2. Sample Collection

On day 130 of gestation, dams were weighed. Maternal and fetal blood plasma was collected as described by Lemley et al. (2013). Briefly, ewes were anesthetized with 3 mg/kg of BW sodium pentobarbital (Fort Dodge Animal Health, Overland Park, KS, USA) and a catheter was placed into the maternal saphenous artery. The uterus was exposed via a midventral laparotomy and simultaneous blood samples were collected from the catheterized maternal saphenous artery and the gravid uterine vein. Immediately following collection of maternal blood, the gravid uterine horn was dissected. The umbilical cord was located and simultaneous blood samples were collected from the umbilical artery and umbilical vein. Blood samples were placed into tubes containing EDTA, placed on ice, and plasma collected after centrifugation at 2000 × g for 20 min. Plasma samples were stored at -20° C until further analysis. Following surgery, ewes were euthanized with sodium pentobarbital as described by Lemley et al. (2013). Pancreases were immediately removed from ewes and fetuses and trimmed of mesentery and fat.

Pancreases were then weighed and a sample of tissue was flash-frozen in liquid nitrogen super-cooled with isopentane (Swanson et al., 2008) until analyses for protein concentration and α-amylase, trypsin, and lipase activities. A subsample of pancreatic tissue was also fixed in 10% formalin and later embedded in paraffin blocks to be used in immunohistochemistry analyses.

2.4.3. Pancreatic Protein, α-Amylase, Trypsin, and Lipase Activity Analyses

Pancreatic tissue (0.25 g) was homogenized in 0.9% NaCl (2.25 ml) using a polytron (Brinkmann Instruments Inc., Westbury, NY). Protein concentration was measured using the bicinchoninic acid procedure with bovine serum albumin as the standard (Smith et al., 1985). Activity of α-amylase was determined using the procedure of Wallenfels et al. (1978) utilizing a kit from Teco Diagnostics (Anaheim, CA). Trypsin activity was assayed using the methods described by Geiger and Fritz (1986) after activation with 100 U/L enterokinase (Swanson et al., 2008). Lipase activity was determined using a colorimetric reagent kit from Point Scientific Inc. (Canton, MI) and the methods of Imamura (1984). Analyses were adapted for use on a microplate spectrophotometer (SpectraMax 340, Molecular Devices, Sunnyvale, CA). One unit (U) of enzyme activity equals 1 μmole product produced per min. Enzyme activity data are expressed as U/g wet tissue, U/g protein, kU/pancreas, and U/kg BW.

2.4.4. Determination of Insulin-Positive Tissue Area in Pancreas

Maternal and fetal pancreatic tissue collected from a representative area of the pancreas was immersion-fixed in formalin and embedded in paraffin blocks with proper and persistent orientation as described previously (Borowicz et al., 2007). From each sample, 5-μm tissue sections were obtained from the blocks and mounted on slides. Tissue sections on the slides were later deparaffinized and rehydrated. Slides were incubated in staining enhancer citrate buffer heated to 95˚C for 5 min then cooled to room temperature. Slides were covered in a solution of

10% donkey serum and 1% bovine serum albumin diluted in tris-buffered saline for a period of 20 min then incubated with 1:100 mouse anti-pig insulin (Santa Cruz Biotech) primary antibody in 1% BSA and tris-buffered saline at 4˚C on a plate rocker for 24 hrs. Next, the slides were washed with tris-buffered saline with added tween 20 (TBST) and incubated with 1:100 CF™633 Goat Anti-Mouse IgG secondary antibody for one hr in complete darkness. A final wash with TBST was performed and coverslips were applied using mounting medium containing 4',6- diamidino-2-phenylindole in order to visualize all pancreatic cell nuclei. Negative control slides were prepared by omitting primary antibody.

2.4.5. Histological Analysis

Photomicrographs were taken with a Zeiss Imager.M2 epifluorescence microscope using a 10x objective and AxioCam HR camera with a Zeiss piezo automated stage. To describe morphology of the pancreas, the mosaic image of a large tissue area of approximately 144 pictures (12 × 12 pictures) on the slide was taken using the MosaiX module of Zeiss AxioVision software. This method allowed creation of a single image covering all the pancreatic tissue present on the histological slide. The MosaiX images were then analyzed using the ImagePro Premier software (ImagePro Premier 9.0, Media Cybernetics, Silver Spring, MD) for the insulin-positive tissue area per section of tissue and size of insulin-containing clusters. The images were analyzed by creating regions of interest corresponding to the whole area of pancreatic tissue visible on the picture. Within the regions of interest, images were segmented based on positive insulin staining and measurements of total positive insulin staining within pancreatic tissue expressed as percent as well as of individual insulin-containing clusters. Insulin-containing clusters were further classified based on size in order to perform population density measurements of small clusters (1 to 2,000 μm^2), medium clusters (2,001 to 32,000 μm^2), large

clusters (32,001 to 512,000 μm^2), and giant clusters ($\geq$ 512,000 μm^2). Insulin positive cell clusters do not correspond to islets of Langerhans because not every cell in the islet is insulin positive.

2.4.6. Insulin Analysis of Blood Plasma

Plasma insulin concentrations were measured using an insulin RIA kit (MP Biomedicals, LLC, Santa Ana, CA). The intra-assay coefficient of variation was 6.99%.

2.4.7. Statistical Analysis

Data were analyzed as a completely randomized design with a 2 × 2 factorial arrangement of treatments using the MIXED procedure of SAS (SAS software version 9.2, SAS Institute Inc., Cary, NC). The model statement included: melatonin treatment, plane of nutrition, and their interaction. The model statement also initially included fetal sex and breeding date which ranged from early September to late December but were later removed from the model as they were found not to influence ($P > 0.25$) the measured variables. To determine if there were differences in enzyme activity and islet morphology between ewes and fetuses, data were analyzed using the MIXED procedure of SAS and physiological stage (maternal or fetal), melatonin treatment, plane of nutrition and the interactions between all factors were included in the model. Statistical significance was declared at $P < 0.05$. Tendencies were declared when $0.05 \geq P \leq 0.10$.

2.5. Results

There were no nutrient restriction × melatonin supplementation interactions in maternal or fetal data ($P \geq 0.05$). Consequently, the main effects of nutrient restriction and melatonin supplementation are discussed.

As expected, and previously reported (Lemley et al., 2012), nutrient restriction decreased ($P < 0.001$) maternal BW (**Table 2-1**).

Table 2-1. Influence of nutrient restriction and melatonin supplementation on maternal BW, pancreas weight and pancreatic enzymes at 130 days of gestation

	Dietary Treatments							
	CON		MEL			*P*-Value		
Item	ADQ	RES	ADQ	RES	SEM	NUT	MEL	NUT × MEL
BW, kg	53.4	41.7	51.9	41.6	1.34	<0.001	0.52	0.62
Pancreas weight, g	60.8	48.0	66.1	54.1	2.64	<0.001	0.03	0.88
Pancreas g/kg of BW	1.17	1.15	1.20	1.31	0.072	0.97	0.05	0.74
Protein								
mg/g tissue	93.9	103	103	103	8.70	0.55	0.60	0.59
total g/pancreas	5.71	4.97	6.80	5.39	0.486	0.03	0.11	0.47
mg/kg of BW	108	120	131	131	11.3	0.58	0.13	0.57
α- Amylase								
U/g	168	101	222	103	17.7	<0.001	0.11	0.14
kU/pancreas	10.2	4.91	14.7	5.95	1.25	<0.001	0.03	0.16
U/kg of BW	194	116	286	145	28.4	<0.001	0.03	0.25
U/g protein	1,814	1,008	2,284	1,200	268.4	0.001	0.21	0.59
Trypsin								
U/g	10.9	11.4	12.8	10.3	0.92	0.29	0.62	0.10
U/pancreas	655	549	838	563	57.1	0.002	0.08	0.13
U/kg of BW	12.4	13.2	16.2	13.8	1.38	0.56	0.10	0.24
U/g protein	116	117	127	105	11.0	0.32	0.96	0.27
Lipase								
U/g	599	720	659	510	73.5	0.84	0.29	0.07
kU/pancreas	36.6	33.9	43.9	28.1	4.72	0.05	0.87	0.16
U/kg of BW	688	819	856	683	104.5	0.83	0.87	0.14
U/g protein	6,469	7,075	6,613	5,420	856.6	0.72	0.36	0.28

*Data are presented as least square means per treatment ± SEM, n = 15 or 16. Control, CON; Melatonin, MEL;Adequate, ADQ; Restricted, RES; Nutrition, NUT.

Nutrient restriction decreased ($P \leq 0.001$) maternal pancreas weight (g) and melatonin supplementation increased (P =0.03) maternal pancreas weight (g) with a tendency (P = 0.05) for an increase in pancreas weight when expressed as g/kg BW). Total protein content (g) in the maternal pancreas was decreased ($P \leq 0.03$) by nutrient restriction and unaltered by melatonin. Maternal pancreatic α-amylase activity (U/g, U/g protein, kU/pancreas, and U/kg of BW) was decreased ($P \leq 0.001$) by nutrient restriction and increased (kU/pancreas and U/kg BW; $P \leq$

0.03) by melatonin supplementation. Nutrient restriction decreased ($P = 0.002$) maternal pancreatic trypsin activity (U/pancreas), while melatonin tended ($P \leq 0.10$) to increase maternal pancreatic trypsin activity when expressed as U/pancreas and U/kg BW. No differences were observed in maternal pancreatic lipase activity in response to nutrient restriction or melatonin supplementation; however, nutrient restriction tended ($P = 0.05$) to decrease lipase activity when expressed as kU/pancreas. Melatonin supplementation decreased ($P \leq 0.04$) pancreatic insulin-positive tissue area (relative to section of tissue). Ewes consuming 60% of requirements had an increased percentage of small insulin-containing cell clusters (1 to 2,000 μm^2; **Table 2-2**) while those receiving adequate nutrition had a greater percentage medium insulin-containing cell clusters (2,000 to 32,000 μm^2). The percentage of large insulin-containing cell clusters was reduced in ewes supplemented with melatonin. Restricted ewes had a tendency for the largest insulin-containing cell cluster ($P = 0.06$) while supplementation with melatonin reduced the size of the largest insulin-containing cell cluster (μm^2; $P = 0.04$). There was a tendency ($P = 0.09$) for an interaction between intake and melatonin supplementation.

As previously described, fetal BW decreased ($P < 0.01$) with nutrient restriction but was not influenced by melatonin supplementation (Lemley et al., 2012; **Table 2-3**). Fetal pancreatic weight, protein concentration, and α-amylase, trypsin, and lipase activity were not influenced by nutrient restriction or melatonin supplementation.

In reference to **Table 2-4**, nutrient restriction decreased ($P \leq 0.04$) fetal pancreatic insulin-positive area (relative to section of tissue), along with the percentage of large and giant insulin-containing clusters. The average size of the cell clusters was reduced ($P \leq 0.04$) in restricted animals and the size of the largest insulin-containing cell cluster was smaller.

Table 2-2. Influence of nutrient restriction and melatonin supplementation on insulin concentration and measurements of insulin containing clusters in maternal pancreas at 130 days of gestation

	Dietary Treatments							
	CON		MEL			*P*-Value		
	ADQ	RES	ADQ	RES	SEM	NUT	MEL	NUT × MEL
Pancreatic insulin-positive area (relative to section of tissue), %	1.62	2.58	0.897	0.773	0.3822	0.25	0.002	0.14
Insulin-containing clusters within the following ranges (μm²), %								
1 to 2,000	70.9	79.9	77.4	82.2	3.44	0.04	0.17	0.51
2,001 to 32,000	28.96	19.82	22.52	17.76	3.429	0.04	0.19	0.49
32,001 to 512,000	0.153	0.320	0.043	0.023	0.0954	0.41	0.03	0.30
Average size of insulin-containing clusters (μm²)	1,870	1,818	1,664	1,341	297.0	0.49	0.22	0.62
Smallest insulin-containing cluster (μm²)	224	254	249	250	17.7	0.35	0.49	0.37
Largest insulin-containing cluster (μm²)	25,492	77,717	20,302	22,514	15,462	0.06	0.04	0.09

*Data are presented as least square means per treatment ± SEM, n = 15 or 16. Control, CON; Melatonin, MEL; Adequate, ADQ; Restricted, RES; Nutrition, NUT.

Table 2-3. Influence of nutrient restriction and melatonin supplementation on fetal BW, pancreas weight and pancreatic enzymes at 130 days of gestation

	Dietary Treatment							
	CON		MEL			*P*-Value		
Item	ADQ	RES	ADQ	RES	SEM	NUT	MEL	NUT × MEL
BW, kg	3.38	3.21	3.61	3.14	0.123	0.01	0.49	0.21
Pancreas weight, g	3.35	3.20	3.71	3.18	0.234	0.14	0.46	0.40
Pancreas g/kg of BW	1.03	0.995	1.02	1.01	0.070	0.73	0.92	0.85
Protein								
mg/g tissue	77.7	83.3	90.2	85.5	4.98	0.92	0.13	0.29
Total g/pancreas	0.256	0.272	0.333	0.273	0.273	0.38	0.12	0.13
mg/kg of BW	76.8	83.7	92.0	87.1	6.92	0.88	0.17	0.38
α-Amylase								
U/g	34.0	39.0	34.5	38.4	6.44	0.47	0.99	0.93
kU/pancreas	0.105	0.131	0.124	0.124	0.0236	0.56	0.79	0.58
U/kg of BW	29.8	39.8	34.5	40.1	7.42	0.28	0.72	0.76
U/g protein	430	458	390	437	73.6	0.60	0.67	0.89
Trypsin								
U/g	0.316	0.246	0.260	0.247	0.0441	0.34	0.52	0.50
U/pancreas	1.03	0.857	0.942	0.805	0.172	0.35	0.67	0.91
U/kg of BW	0.307	0.256	0.263	0.259	0.0487	0.55	0.66	0.62
U/g protein	3.98	2.81	2.99	2.92	0.478	0.19	0.34	0.24
Lipase								
U/g	269	336	291	341	82.0	0.47	0.86	0.91
kU/pancreas	0.842	1.14	1.05	1.10	0.2918	0.52	0.77	0.66
U/kg of BW	238	342	289	360	88.4	0.31	0.69	0.85
U/g protein	3,373	3,987	3,324	3,800	942.7	0.55	0.90	0.94

*Data are presented as least square means per treatment ± SEM, n = 15 or 16. Control, CON; Melatonin, MEL; Adequate, ADQ; Restricted, RES; Nutrition, NUT.

Table 2-4. Influence of nutrient restriction and melatonin supplementation on insulin concentration and measurements of insulin containing clusters in fetal pancreas at 130 days of gestation

	Dietary Treatments							
	CON		MEL			*P*-Value		
Item	ADQ	RES	ADQ	RES	SEM	NUT	MEL	NUT × MEL
Pancreatic insulin-positive area (relative to section of tissue), %	6.38	3.56	7.90	3.19	1.764	0.03	0.73	0.57
Insulin-containing clusters within the following ranges (μm²), %								
1 to 2,000	81.2	83.8	79.6	86.8	3.06	0.10	0.81	0.43
2,001 to 32,000	17.7	15.6	19.4	12.7	2.87	0.11	0.81	0.39
32,001 to 512,000	1.04	0.527	1.01	0.528	0.2458	0.04	0.96	0.95
> 512,001	0.060	0.000	0.022	0.000	0.0204	0.04	0.32	0.32
Average size of insulin-containing clusters (μm²)	2,887	1,782	2,650	1,684	509.8	0.04	0.73	0.88
Smallest insulin-containing cluster (μm²)	257	258	342	280	48.3	0.50	0.25	0.49
Largest insulin-containing cluster (μm²)	390,841	107,067	291,253	90,411	92,209	0.01	0.50	0.63

[*]Data are presented as least square means per treatment ± SEM, n = 15 or 16. Control, CON; Melatonin, MEL; Adequate, ADQ; Restricted, RES; Nutrition, NUT.

Insulin concentrations in the maternal artery at day 130 of gestation tended ($P = 0.09$) to decrease with nutrient restriction and increased ($P = 0.07$) with melatonin supplementation (uIU/mL; **Table 2-5**). Plasma insulin concentration decreased ($P = 0.01$) in the uterine vein of nutrient restricted ewes. Likewise, insulin concentrations in the umbilical artery and vein also decreased ($P < 0.001$) with nutrient restriction and was unaltered by melatonin supplementation.

Table 2-5. Influence of nutrient restriction and melatonin supplementation on plasma insulin concentrations (uIU/mL) at 130 days of gestation

	Dietary Treatments							
	CON		MEL			*P*-Values		
Vessel	ADQ	RES	ADQ	RES	SEM	NUT	MEL	NUT × MEL
Maternal artery	13.4	10.7	14.1	13.5	0.999	0.09	0.07	0.28
Uterine vein	13.8	11.0	15.4	11.7	1.30	0.01	0.34	0.68
Umbilical artery	23.9	13.1	20.4	13.4	2.32	<0.001	0.45	0.39
Umbilical vein	21.5	13.2	19.8	12.6	2.07	<0.001	0.55	0.77

*Data are presented as least square means per treatment ± SEM, n = 15 or 16. Control, CON; Melatonin, MEL; Adequate, ADQ; Restricted, RES; Nutrition, NUT.

Interactions existed between physiological stage (maternal vs. fetal) and intake ($P < 0.05$) for pancreas weight, protein content (total g/pancreas), α-amylase concentration (U/g and U/g protein), α-amylase content (kU/pancreas and U/kg BW), and trypsin content (U/pancreas). Physiological stage × melatonin interactions were also observed ($P < 0.05$) for pancreas weight, and α-amylase concentration (U/g) and content (kU/pancreas). However, interactions occurred primarily because of differences in magnitude between maternal and fetal pancreas with intake or melatonin group and not because of re-ranking between maternal and fetal measurements. Consequently, only the main effects associated with maternal and fetal pancreatic outcomes are presented (**Table 2-6)**.

Table 2-6. Influence of physiological stage (maternal vs. fetal) on digestive enzyme activity and ratio of insulin-positive staining and islet size at 130 days of gestation

	Physiological Stage			
Item	Maternal	Fetal	SEM	*P*-Value
BW, kg	47.2	3.3	0.49	<0.001
Pancreas weight, g[a,b]	57.2	3.4	0.95	<0.001
Pancreas, g/kg of BW	1.22	1.02	0.036	<0.001
Protein				
mg/g tissue	101	84	3.6	<0.002
Total g/pancreas[a]	5.72	0.28	0.176	<0.001
mg/kg of BW	123	84	4.8	<0.001
α-Amylase				
U/g[a,b]	149	36	6.8	<0.001
kU/pancreas[a,b]	8.95	0.12	0.451	<0.001
U/kg of BW[a]	185	36	10.5	<0.001
U/g protein[a]	1,577	429	100.4	<0.001
Trypsin				
U/g	11.3	0.3	0.33	<0.001
U/pancreas[a]	651	1	20.6	<0.001
U/kg of BW	13.9	0.3	0.50	<0.001
U/g protein	116	3	3.9	<0.001
Lipase				
U/g	622	309	39.7	<0.001
kU/pancreas	35.6	1.0	1.70	<0.001
U/kg of BW	761	307	49.4	<0.001
U/g protein	6394	3621	459.4	<0.001
Pancreatic insulin-positive area (relative to section of tissue), %[a]	1.47	5.26	0.667	0.0001
Insulin-containing clusters within the following ranges (μm²), %				
1 to 2,000	77.6	82.9	1.56	0.02
2,001 to 32,000	22.3	16.3	1.51	0.01
32,001 to 512,000[a]	0.135	0.777	0.0961	<0.001
> 512,001[a]	0	0.020	0.0076	0.05
Average size of insulin-containing clusters (μm²)	1673	2251	205.7	0.05
Smallest insulin-containing cluster (μm²)	251	284	18.0	0.18
Largest insulin-containing cluster (μm²)[a]	36505	219893	33647	0.0003

[*]Data are presented as least square means ± SEM, n = 31.

P–Values are representative of significance due to physiologic stage.

[a]Physiological stage × intake ($P < 0.05$).

[b]Physiological stage × melatonin ($P < 0.05$).

Pancreatic mass and all measurements of enzyme activity were greater ($P < 0.02$) in maternal than fetal pancreas at day 130 of gestation. Fetal pancreas had a larger ($P < 0.001$) insulin-positive area (relative to section of tissue) and a greater percentage of small insulin-containing cell clusters ($P = 0.02$). Maternal pancreas had a greater number of medium insulin-containing clusters ($P = 0.01$) while the fetal pancreas had a greater percent of large insulin-containing cell clusters ($P < 0.001$). A tendency ($P = 0.05$) was observed for fetal pancreas to have a higher percent of giant insulin-containing cell clusters and for the average size of cell clusters to be larger. However, due to the high number of small cell clusters, the average size among fetal and maternal pancreases was not significantly different despite the fetal pancreas exhibiting the largest insulin containing cell clusters (μm^2; $P < 0.001$).

2.6. Discussion

Nutrient restriction from mid- to late-pregnancy decreased both maternal and fetal BW as reported previously (Lemley et al., 2012) as well as maternal pancreatic mass (g). Fetal pancreatic mass, however, was not influenced by nutrient restriction, contradicting past research (Reed et al., 2007) which reported that fetal pancreatic mass from undernourished ewes was decreased compared to those from ewes adequately fed. Moreover, the melatonin supplementation strategy did not rescue fetal growth restriction brought about by maternal nutrient restriction from mid- to late-pregnancy (Lemley et al., 2012).

In the growing ruminant, an increase in nutrient intake generally increases pancreatic content of or secretion of exocrine digestive enzymes (Corring, 1980; Harmon, 1992; Wang et al., 1998; Swanson and Harmon, 2002). The ewes in this study on the ADQ treatment had increased pancreatic α-amylase and trypsin activities compared to ewes on the RES treatment

indicating that changes in feed intake also impact pancreatic exocrine function in mature pregnant ewes.

A study conducted by Jaworek et al. (2004) reported that, in anaesthetized rats, exogenous melatonin or its precursor L-tryptophan resulted in a dose-dependent increase in pancreatic α-amylase secretion. These increases were believed to be manifested through the stimulation of cholecystokinin and the triggering of vagal sensory nerves (Jaworek et al., 2004). We also observed this effect of melatonin on maternal pancreatic α-amylase content relative to BW but did not observe effects of melatonin on fetal pancreatic weight or enzyme activity. This, therefore, disagrees with our hypothesis as melatonin supplementation had limited effects on the fetal development of the exocrine pancreas in our study.

Past work has suggested that diets containing lower protein concentration than those with adequate nutrition resulted in decreased pancreatic insulin concentration (Dahri et al., 1995). Other studies have suggested that the β-cell proportional area (relative to total cellular area) and islet number was decreased in rats undergoing nutrient restriction for a period of 4 weeks when compared to a control group fed for ad libitum intake (Chen et al., 2010). Our results suggested no effect of global nutrient restriction on insulin-positive area (relative to section of tissue) in ewes. As expected, however, the insulin-positive area and size of insulin-containing clusters were reduced in fetuses from restricted ewes. Developmental changes in islet size and number potentially could have effects on development in regards to energetics and function of β-cells. When investigating islet size of the endocrine pancreas, pups borne from mothers fed low protein diets were found to have islet cell proliferation reduced by 12% (Dahri et al., 1995).

Plasma insulin concentrations were greater in ADQ ewes and fetuses. This was not surprising as insulin is released in response to nutrient intake (Takahashi et al., 2006). Because

these animals were not subjected to nutrient restriction they would have been secreting insulin at a higher rate to accommodate the rise in blood glucose and to promote protein synthesis. Although no significant differences were observed, there were numerically greater insulin concentrations in the maternal artery in ewes supplemented with melatonin. This trend is unexpected as melatonin supplementation resulted in a decrease in pancreatic insulin-positive area (relative to section of tissue). Interestingly, Lemley et al. (2012) reported an interaction between nutrient restriction and melatonin supplementation on umbilical artery glucose concentrations suggesting that glucose concentrations were influenced differently compared to plasma insulin concentration and islet size measurements. Melatonin, on the other hand, did not influence the insulin-positive area (relative to section of tissue) or insulin-containing cluster morphology in the fetal pancreas, suggesting that supplemental melatonin at the dose and timing relative to the light-dark cycle that we provided had minimal effects on pancreatic fetal endocrine function.

The concentration of pancreatic digestive enzymes was much lower in the fetal than the maternal pancreas. However, when observing the size of insulin-containing clusters, fetuses had a greater number of large islets compared with those of their respective dams amongst all treatments.

Similar results have also been observed when comparing calves to adult cattle (Merkwitz et al., 2012). In the examination of the pancreatic tissue of calves, islets of two differing sizes were discovered. The larger islets (perilobular giant islets) were found in a much smaller quantity while smaller islets (interlobular islets) appeared more frequently. These interlobular islets were shown to persist into adulthood while the perilobular giant islets underwent regression.

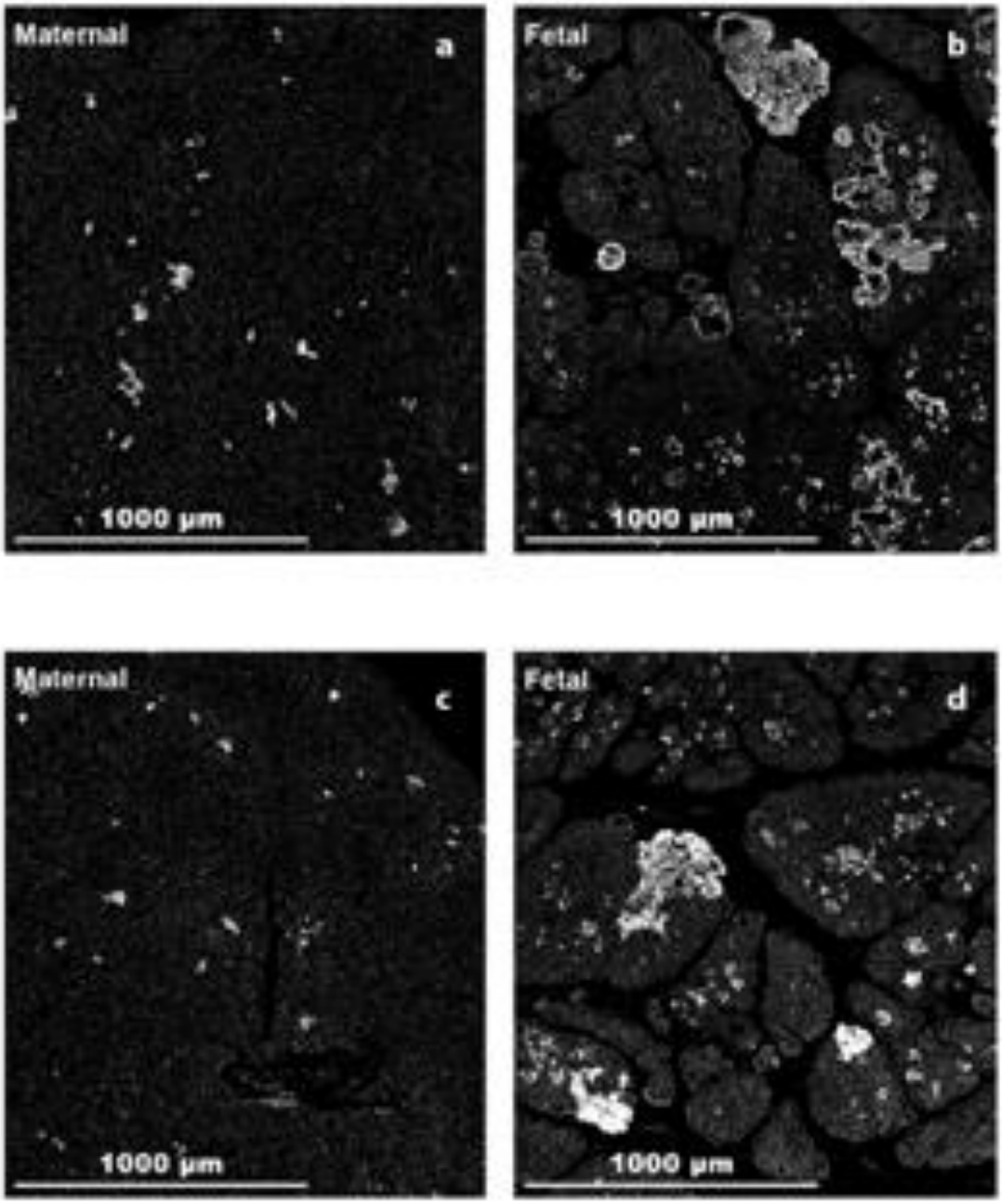

Figure 2-1. Influence of nutrient restriction and melatonin supplementation on insulin-positive staining. Sections represent the maternal and corresponding fetal pancreatic tissues of representative ewes fed 5 mg/d melatonin and receiving 100% of nutrient requirements (a and b) and 60% of nutrient requirements (c and d). The white arrows indicate insulin-containing clusters. Magnification 10x.

It has also been shown that mass related metabolic rate tended to decrease with increasing body size of the organism (Singer, 2006). Therefore, these differences in fetal islet size and number may be associated with differences in function of the endocrine pancreas during development and later in life. Further investigation is needed in this area.

In conclusion, nutrient restriction during gestation has more of an effect on pancreatic protein and digestive enzyme concentrations in the dam than in the fetus. This may be because the dam is adapting to protect the fetus from changes in nutritional status or because of the much lower concentrations of enzymes produced in the fetal compared with the maternal pancreas. In contrast to our hypothesis, melatonin supplementation did not impact the effect of nutrient restriction on maternal and fetal pancreatic function and, therefore, may not be an appropriate supplementation option to help therapeutically mitigate the effects of maternal nutrient restriction on pancreatic development.

2.7. Literature Cited

Borowicz, P. P., D. R. Arnold, M. L. Johnson, A. T. Grazul-Bilska, D. A. Redmer, and L. P. Reynolds. 2007. Placental growth throughout the last two-thirds of pregnancy in sheep: vascular development and angiogenic factor expression. Biol. Reprod. 76:259-267.

Bubenik, G. A. 2008. Thirty-four years since the discovery of gastrointestinal melatonin. J. Physiol. Pharmacol. 58:33-51.

Chen, L., W. Yang, J. Zheng, X. Hu, W. Kong, and H. Zhang. 2010. Effect of catch-up growth after food restriction on the entero-insular axis in rats. Nutr. Metab. (Lond) 7:45.

Corring, T. 1980. The adaptation of digestive enzymes to the diet: its physiological significance. Reprod. Nutr. Dev. 20:1217-1235.

Dahri, S., B. Reusen, C. Remacle, and J. J. Hoet. 1995. Nutritional influences on pancreatic development and potential links with non-insulin-dependent diabetes. Proc. Nutr. Soc. 54:345-356.

Geiger, R. and H. Fritz. 1986. Trypsin. In Methods of Enzymatic Analysis (ed. Bergmeyer H) pp. 119-128. Elsevier, New York, USA.

Harmon, D. L 1992. Impact of nutrition on pancreatic exocrine and endocrine secretion in ruminants: a review. J. Anim. Sci. 70:1290-1301.

Imamura, S. and H. Misaki. 1984. A sensitive method for assay of lipase activity by coupling with β-oxidation enzymes of fatty acids. Selected Topics in Clinical Enzymology. Clin. Chem. 2:73.

Jaworek, J., N. Katarzyna, S. J. Konturek, A. Leja-Szpak, P. Thor, and W. W. Pawlik. 2004. Melatonin and its precursor, L-tryptophan: influence on pancreatic amylase secretion in vivo and in vitro. J. Pineal. Res. 36:155-164.

Jaworek, J., K. Nawrot-Porabka, A. Leja-Szpak, J. Bonior, J. Szklarczyk, M. Kot, S. J. Konturek, and W. W. Pawlik. 2007. Melatonin as modulator of pancreatic enzyme secretion and pancreatoprotector. J. Physiol. Pharmacol. 58:65-80.

Jaworek, J., J. Szklarczyk, A. K. Jaworek, K. Nawrot-Porąbka, A. Leja-Szpak, J. Bonior, and M. Kot. 2012. Protective effect of melatonin on acute pancreatitis. Int. J. Inflam. 2012:173675.

Lemley, C. O., L. E. Camacho, A. M. Meyer, M. Kapphahn, J. S. Caton, and K. A. Vonnahme. 2013. Dietary melatonin supplementation alters uteroplacental amino acid flux during intrauterine growth restriction in ewes. Animal 7:1500-1507.

Lemley C. O., A. M. Meyer, L. E. Camacho, T. L. Neville, D. J. Newman, J. S. Caton, and K. A. Vonnahme. 2012. Melatonin supplementation alters uteroplacental hemodynamics and fetal development in an ovine model of intrauterine growth restriction (IUGR). Am. J. Physiol. Regul. Integr. Comp. Physiol. 302:R454-R467.

Li, G., G. Hou, W. Lu, and J. Kang. 2011. Melatonin protects mice with intermittent hypoxia from oxidative stress-induced pancreatic injury. Sleep Biol. Rhythms 9:78-85.

Merkwitz, C., P. Lochhead, J. Böttger, M. Matz-Soja, M. Sakurai, R. Gebhardt, and A. M. Ricken. 2012. Dual origin, development, and fate of bovine pancreatic islets. J. Anat. 222:358-371.

NRC. 2007. Nutrient Requirements of Small Ruminants. National Academies Press. Washington, DC.

Reed, J. J., M. A. Ward, K. A. Vonnahme, T. L. Neville, S. L. Julius, P. P. Borowicz, J. B. Taylor, D. A. Redmer, A. T. Grazul-Bilska, L. P. Reynolds, and J. S. Caton. 2007. Effects of selenium supply and dietary restriction on maternal and fetal BW, visceral organ mass and cellularity estimates, and jejunal vascularity in pregnant ewe lambs. J. Anim. Sci. 85:2721-2733.

Sartori, C., P. Dessen, C. Mathieu, A. Monney, J. Bloch, P. Nicod, U. Scherrer, and H. Duplain. 2009. Melatonin improves glucose homeostasis and endothelial vascular function in high-fat diet-fed insulin-resistant mice. Endocr. 150:5311-5317.

Singer, D. 2006. Size relationship of metabolic rate: Oxygen availability as the “missing link” between structure and function? Thermochim. Acta. 446:20-28.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.

Swanson, K. C. and D. L. Harmon. 2002. Dietary influences on pancreatic α-amylase expression and secretion in ruminants. In Biology of the Intestine in Growing Animals (ed R Zabielski, V Lesniewska, PC Gregory, and B. Westrom), pp. 515-537. Elsevier, Boston, USA.

Swanson, K. C., N. Kelly, H. Salim, Y. J. Wang, S. Holligan, M. Z. Fan, and B. W. McBride. 2008. Pancreatic mass, cellularity and α-amylase and trypsin activity in feedlot steers fed diets differing in crude protein concentration. J. Anim. Sci. 86:909-915.

Takahashi, H., Y. Kurose, S. Kobayashi, T. Sugino, M. Kojima, K. Kangawa, Y. Hasegawa, and Y. Terashima. 2006. Ghrelin enhances glucose-induced insulin secretion in scheduled meal-fed sheep. J. Endocrinol. 189:67-75.

Wallenfels, K., P. Fold, H. Niermann, H. Bender, and D. Linder. 1978. The enzymatic synthesis, by transglucosylation of a homologous series of glycosidically substituted malto-oligosaccharides, and their use as amylase substrates. Carbohydr. Res. 61:359-368.

Wang, W. B., T. Ogawa, S. Suda, K. Taniguchi, H. Uike, H. Kumagai, and K. Mitani. 1998. Effects of nutrition level on digestive enzyme activities in the pancreas and small intestine of calves slaughtered at the same body weight. Asian Australas. J. Anim. Sci. 11:375-380.

CHAPTER 3. THE IMPACT OF DIET AND ARGININE SUPPLEMENTATION ON PANCREATIC MASS, DIGESTIVE ENZYME ACTIVITY AND INSULIN-CONTAINING CELL CLUSTER MORPHOLOGY DURING THE ESTROUS CYCLE IN SHEEP[2]

3.1. Abstract

To determine the effect of feed intake and arginine treatment during different stages of the estrous cycle on pancreatic mass, digestive enzyme activity, and histological measurements, ewes (n = 120) were randomly allocated to one of three dietary groups; control (CON; 2.14 Mcal metabolizable energy/kg), underfed (UF; 0.6 x CON) or overfed (OF; 2 x CON) over 2 yr. Estrus was synchronized using a controlled internal drug release (CIDR) device for 14 d. At CIDR withdrawal, ewes from each dietary group were assigned to one of two treatments; Arg (L-Arg HCl, 155 µmol/kg BW) or Sal (approximately 10 mL Saline). Treatments were administered 3 times daily via jugular catheter and continued until slaughter on d 5 and 10 of the second estrus cycle (early luteal phase, n = 41 and mid-luteal phase, n = 39; year 1) and d 15 of the first estrus cycle (late luteal phase, n = 40; year 2. A blood sample collected from jugular catheters for serum insulin analysis before slaughter. The pancreas was then removed, trimmed of mesentery and fat, weighed, and a sample snap-frozen until enzyme analysis. Additional pancreatic samples

[2]The material in this chapter was co-authored by F. E. Keomanivong, A. T. Grazul-Bilska, D. A. Redmer, C. S. Bass, S. L. Kaminski, P. P. Borowicz, J. D. Kirsch and K. C. Swanson. It has been accepted for publication by Domestic Animal Endocrinology, dio: 10.1016/j.domaniend.2016.10.001. F. E. Keomanivong had primary responsibility for collecting and analyzing samples in the field. F. E. Keomanivong was the primary developer of the conclusions that are advanced here. F. E. Keomanivong also drafted and revised all versions of this chapter. K. C. Swanson served as proofreader and checked the math in the statistical analysis conducted by F. E. Keomanivong.

were fixed in 10% formalin solution for histological examination of size and distribution of insulin-containing cell clusters. Data were analyzed as a completely randomized design with a factorial arrangement of treatments. Diet, treatment, and diet × treatment was blocked by year and included in the model with initial BW used as a covariate. Day of the estrous cycle was initially included in the model but later removed as no effects ($P > 0.10$) were observed for any pancreatic variables tested. Overfed ewes had the greatest ($P < 0.001$) change in BW, final BW, change in BCS, and final BCS. A diet × treatment interaction was observed for change in BW and final BW ($P \leq 0.004$). Overfed and CON had increased ($P < 0.001$) pancreas weight (g) compared to UF ewes. Protein concentration (g/pancreas) was lowest ($P < 0.001$) in UF ewes while protein content (mg/kg BW) was greater ($P = 0.03$) in UF than OF ewes. Activity of α-amylase (U/g, kU/pancreas, U/kg of BW, and U/g protein) and trypsin (U/pancreas) was greater ($P \leq 0.003$) in OF than UF ewes. Serum insulin was greatest ($P < 0.001$) in OF ewes. No effects were observed for pancreatic insulin-containing cell clusters. This study demonstrated that plane of nutrition affected several measurements of pancreatic function however, the dosage of Arg used did not influence pancreatic function.

3.2. Introduction

One of the biggest challenges in animal production is providing adequate feed with proper nutrient profiles during each stage of life. Sheep breeders are often faced with this dilemma as grazing flocks can at certain times of the year receive less than 50% of NRC recommendations (Wu et al., 2006). Periods of nutrient restriction impact production and secretion of pancreatic digestive enzymes (Corring, 1980). A decrease in the secretion of pancreatic digestive enzyme activity due to reduced feed intake influences the breakdown and

absorption of nutrients in the small intestine (Swanson and Harmon, 2002; Keomanivong et al., 2015a).

Arginine is known to play a role in tissue function and enzyme activity (Wu and Morris, 1998). These functions are impacted by the ability of arginine to stimulate secretion of hormones such as insulin, growth hormone, glucagon and prolactin, and to act as a precursor of proteins, polyamines and nitric oxide (NO) which becomes a powerful vasodilator and biological regulator of tissues (Wu and Morris, 1998; Wu et al., 2006; Arciszewski, 2007). Based on these understandings, arginine supplementation has been indicated as a possible rescue factor for detrimental effects caused by nutrient restriction on organ function (Lassala et al., 2010).

During different stages of life, the animal endures reduced or excess intake and understanding the effect of diet on the pancreas is crucial. The concentration of pancreatic insulin varies throughout the estrus cycle and depends on plane of nutrition (Morimoto et al., 2001; Sartoti et al., 2013; Kaminski et al., 2015). In addition, it has been demonstrated that pancreatic α-amylase has been influenced by nutritional factors capable of modification by steroid sex hormones (Khayambashi et al., 1971; Karsenti et al., 2001). Therefore, sex hormones exert further effects beyond the targeted measurements of reproductive function (Boland et al., 2001; Armstrong et al., 2003; Kaminski et al., 2015). The interaction between day of estrus and variable feed intake or supplemental arginine on pancreatic function is unclear.

We hypothesized that Arg-treatment will affect selected pancreatic functions in non-pregnant ewes fed inadequate diets compared to ewes fed a maintenance control diet. Therefore, the objectives were to determine the effects of different levels of feed intake and arginine supplementation on pancreatic mass and protein concentration, activity of α-amylase and trypsin,

insulin-containing cell cluster measurements, and serum insulin concentration at the early-, mid-, and late-luteal phases of the estrous cycle.

3.3. Materials and Methods

This experiment was approved by the North Dakota State University Institutional Animal Care and Use Committee.

3.3.1. Animal Dietary Groups

Animal use and experimental design have been previously described (Kaminski et al., 2015). Non-pregnant, non-lactating, individually-fed Rambouillet ewes (n = 120) between 2 and 5 yr of age and of similar genetic background were studied over 2 yr. Ewes initially weighed 60.2 kg (± a standard deviation of 5.79 kg) with an initial body condition score of 2.7 (± a standard deviation of 0.24) on a 1 to 5 scale. Ewes were housed in a temperature-controlled environment at 14°C and a 12:12 light:dark cycle with lights on at 07:00 and off at 19:00 each day. Sixty days prior to the beginning of the experiment ewes were stratified by BW and randomly allocated to one of three dietary groups; control (CON; 100% National Research Council (National Research Council, 1996) requirements; 2.4 Mcal metabolizable energy/kg BW), underfed (UF; 0.6 x CON) or overfed (OF; 2 x C; **Table 3-1**).

These respective diets were provided twice daily at 08:00 and 15:00 for the duration of the study and were adjusted weekly based on ewe BW to ensure that proper body condition was achieved. The jugular vein of each ewe was cannulated prior to the initiation of the estrous cycle (Kaminski et al., 2015). Estrus was synchronized using a controlled internal drug release (CIDR) device for 14 d.

Table 3-1. Dietary Composition and Nutrient Analysis

Ingredient (% of Diet DM)	Diet DM %
Beet pulp	36.5
Alfalfa meal	22.3
Corn	18.2
Soy hulls	20.0
Soybean meal	3.0
Nutrient Composition	
DM, %	91.8
OM, % of DM	93.7
CP, % of DM	11.6
NDF, % of DM	37.4
ADF, % of DM	25.3
Calcium, % of DM	0.87
Phosphorus, % of DM	0.27

3.3.2. Saline and Arg Treatments

Approximately 36 hr after CIDR withdrawal, ewes were considered in estrus and were randomly allocated to one of two treatments; Arg (L-Arg HCl, 155 µmol/kg BW) or Sal (~10 mL Saline; Kaminski et al., 2015). Ewes receiving Sal were given 5 to 10 mL of sterile saline solution via the jugular catheter, whereas ewes receiving Arg were given 5 to 10 mL of L-Arg-HCl solution in a dose of 155 µmol/kg BW (Sigma St. Louis, MO, USA). Sal or Arg treatments were initiated on d 0 of the first estrous cycle and occurred three times daily (07:00, 14:00, and 21:00) until the end of the experiment. The dose of Arg was selected based on studies performed by Lassala et al (2010; 2011) to ensure an increased concentration of Arg in maternal serum. After treatment injection, 1 mL of heparin solution was placed into the catheter to prevent clotting.

3.3.3. Blood and Tissue Collection

At the early- (d 5; n = 41) or mid- (d 10; n = 39) luteal phase of the second estrous cycle (Year 1) and at the late-luteal phase (d 15; n = 40) of the first estrus cycle (Year 2), final BW and BCS were recorded and blood was collected from the jugular catheter using 9-mL Luer Monovette blood collection tubes (Sarstedt, Newton, NC, USA). After collection, blood samples were allowed to clot for 15 to 20 min at room temperature and centrifuged (Allegra 6R Centrifuge; Beckman Coulter Inc., Indianapolis, IN, USA) for 20 min at 3500 × g. Serum was then aliquoted into 2-mL serum tubes and immediately stored at −20 °C until further analysis.

Ewes were stunned via captive bolt and exsanguinated. The pancreas was removed, trimmed of mesentery and fat, and a total weight recorded. A sample from the body of the pancreas was snap-frozen in isopentane super-cooled in liquid nitrogen until analyses for protein concentration, and α-amylase and trypsin activities. Additional samples of pancreatic tissue were fixed in 10% formalin solution and later embedded in paraffin blocks to be used in immunohistochemistry analyses including quantitative characterization of size and distribution of insulin-containing cell clusters.

3.3.4. Serum Insulin Concentration

Insulin concentrations in 100 µL of serum were measured with a chemiluminescent immunoassay on an Immulite 1000 analyzer (Siemens Healthcare, Tarrytown, NY, USA), as previously described and validated (including specificity) by Vonnahme et al. (2010). Briefly, the Immulite 1000 test units contain assay-specific antibody-coated polystyrene beads. Once the protein of interest is bound to the antibodies on the bead, it is detected by the binding of an alkaline phosphatase-labeled, assay-specific antibody. Dioxetane is the chemiluminescent alkaline phosphatase substrate, and the amount of emitted light is proportional to the amount of

analyte in the sample. Biorad Lymphochek standards were used to validate the assay with levels 1, 2 and 3 control pools containing low, intermediate and high levels of insulin respectively (Biorad, Hercules, CA, USA). Samples were assayed in duplicate using Immulite 1000 Insulin kits (catalog # LKIN1), and the intra-assay CV was 13.2%. The reportable range for the assay is 2 to 300 µlU/mL, and the analytical sensitivity is 2 µlU/mL.

3.3.5. Pancreatic Protein, α-Amylase and Trypsin Activity Analyses

Pancreatic tissue (0.25 g) was homogenized in 0.9% NaCl (2.25 µl) using a polytron (Brinkmann Instruments Inc., Westbury, NY, USA). Protein concentration was determined using the bicinchoninic acid (BCA) procedure with bovine serum albumin (BSA) used as the standard (Smith et al., 1985). Activity of α-amylase was determined using the procedure of Wallenfels et al. (1978) utilizing a kit from Teco Diagnostics (Anaheim, CA, USA). Trypsin activity was determined using the methods described by Geiger and Fritz (1986) after activation with 100 U/L enterokinase (Swanson et al., 2008a). Analyses were adapted for use on a microplate spectrophotometer (SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA). One unit (U) of enzyme activity equals 1 µmole product produced per min. Enzyme activity data are expressed as U/g wet tissue, U/g protein, kU/pancreas, and U/kg BW.

3.3.6. Immunohistochemistry

A representative sample of the body of the pancreas was immersion-fixed in formalin and embedded in paraffin blocks with proper and persistent orientation, and immunohistochemistry was performed as described previously (Keomanivong et al., 2015a; Keomanivong et al., 2015b). Tissues were cut (5-µm sections), mounted on slides, deparaffinized and rehydrated. Slides were incubated in staining enhancer citrate buffer heated to 95˚C for 5 min then cooled to room temperature. Slides were covered in a solution of 10% donkey serum and 1% bovine serum

albumin diluted in tris-buffered saline for 20 min then incubated with 1:100 mouse anti-pig insulin (Santa Cruz Biotech, Dallas, TX, USA) primary antibody in 1% BSA and tris-buffered saline at 4˚C on a plate rocker for 24 hr. Next, the slides were washed with tris-buffered saline with added tween 20 (TBST) and incubated with 1:100 CF™633 Goat Anti-Mouse IgG secondary antibody (Santa Cruz Biotech, Dallas, TX, USA) for one hr in complete darkness. A final wash with TBST was performed and coverslips were applied using mounting medium containing 4',6- diamidino-2-phenylindole in order to visualize all pancreatic cell nuclei. Negative control slides were prepared with omitting primary antibody.

3.3.7. Histological Analysis

Photomicrographs were taken with a Zeiss Imager.M2 epifluorescence microscope (Carl Zeiss Microscopy, Jena, Germany) using a 10x objective and AxioCam HR camera with a Zeiss piezo automated stage. To describe morphology of the pancreas, the mosaic image of a large tissue area of approximately 144 pictures (12 × 12 pictures) on the slide was taken using the MosaiX module of Zeiss AxioVision software. This method allowed creation of a single image of the pancreatic tissue present on the slide (Keomanivong et al., 2015a). The MosaiX images were then analyzed using the ImagePro Premier software (ImagePro Premier 9.0, Media Cybernetics, Silver Spring, MD, USA) to determine the insulin-positive tissue area per section of tissue, and size of insulin-containing cell clusters. The images were analyzed by creating regions of interest corresponding to the whole area of pancreatic tissue visible on the picture (Keomanivong et al., 2015b). Within the regions of interest, images were segmented based on positive insulin staining and measurements of total positive insulin staining within pancreatic tissue expressed as percent as well as of individual insulin-containing cell clusters. Insulin-containing cell clusters were further classified based on size to perform population density

measurements of small clusters (1 to 2,000 μm^2), medium clusters (2,001 to 32,000 μm^2), and large clusters (32,001 to 512,000 μm^2). Insulin-positive cell clusters do not correspond to islets of Langerhans because not every cell in the islet is insulin positive.

3.3.8. Statistical Analysis

Data were analyzed using the general linear model procedures of SAS 9.2 (Statistical Analysis Software, SAS Institute Inc., Cary, NC, USA) as a completely randomized design with a factorial arrangement including diet, treatment, and diet × treatment. Data were initially analyzed within year with stage of the estrous cycle initially included in the model for year 1. Stage of the estrous cycle did not influence ($P > 0.10$) any of the variables tested and therefore was removed from the model and the data were combined over all stages and analyzed including year in the model as a blocking factor and initial BW as a covariate. Means were separated using the least significant difference approach and were considered significant when $P \leq 0.05$.

3.4. Results

Average daily gain and final BW (kg) were greatest ($P < 0.001$) in OF ewes followed by CON (**Table 3-2**) while a diet × treatment interaction was noted with OF ewes treated with Arg having the greatest values for both measurements ($P \leq 0.004$; **Figure 3-1**).

The changes in BCS and final BCS were greatest ($P < 0.001$) in OF followed by UF ewes while no differences were noted with Arg treatment. Pancreas weight (g) was greater ($P < 0.001$) in OF and CON than UF ewes. Sheep treated with Arg tended to have a greater ($P = 0.09$) pancreas weight (g) than those treated with Sal. Pancreas weight (% of BW) was greater ($P < 0.01$) in UF than OF while CON ewes were intermediate. Arg treated ewes tended to have a greater ($P = 0.09$) pancreas weight (% of BW) than ewes treated with Sal.

Table 3-2. Influence of nutrient restriction and arginine treatment on BW, BCS, pancreatic mass and digestive enzymes in non-pregnant ewes

Item	Dietary Group			SEM	Treatment		SEM	*P*-Values		
	UF	CON	OF		Saline	Arg		Diet	Treatment	Diet × Treatment
[1]Change in BW (kg)	-13.6	-1.13	7.92	0.811	-2.44	-2.09	0.686	< 0.0001	0.71	0.003
Final BW (kg)	46.6^{c}	59.1^{b}	68.1^{a}	0.811	57.8	58.1	0.69	<0.0001	0.71	0.003
[1]Change in BCS (Score 1-5)	-0.488^{c}	0.482^{b}	1.21^{a}	0.0713	0.410	0.390	0.0603	<0.0001	0.81	0.67
Final BCS	2.24^{c}	3.17^{b}	3.91^{a}	0.0800	3.10	3.12	0.068	<0.0001	0.84	0.39
Pancreas Wt										
g	58.0^{b}	68.5^{a}	72.7^{a}	2.12	64.3	68.5	1.79	<0.0001	0.09	0.28
% of BW	1.25^{a}	1.17^{ab}	1.07^{b}	0.039	1.12	1.20	0.033	0.004	0.09	0.88
Protein										
mg/g	120	124	120	2.7	123	120	2.3	0.46	0.33	0.49
g/pancreas	7.02^{b}	8.46^{a}	8.74^{a}	0.339	7.92	8.23	0.293	0.0006	0.44	0.19
mg/kg BW	151^{a}	144^{ab}	128^{b}	6.5	138	144	5.6	0.03	0.47	0.85
α-Amylase										
U/g	91.5^{b}	136^{a}	168^{a}	11.73	136	128	9.9	<0.0001	0.57	0.86
kU/pancreas	5.15^{c}	9.21^{b}	12.7^{a}	0.946	9.14	8.88	0.799	<0.0001	0.82	0.92
U/kg of BW	113^{b}	156^{a}	184^{a}	15.1	153	150	12.7	0.003	0.87	0.96
U/g protein	749^{c}	1099^{b}	1359^{a}	87.0	1090	1049	74.3	<0.0001	0.69	0.75
Trypsin										
U/g	9.74	9.95	10.9	0.657	10.2	10.2	0.55	0.41	0.94	0.54
U/pancreas	567^{b}	672^{ab}	791^{a}	47.7	648	702	40.2	0.003	0.31	0.89
U/kg BW	12.1	11.5	11.8	0.85	11.3	12.3	0.72	0.89	0.30	0.72
U/g protein	80.9	93.4	90.7	5.80	84.7	85.3	4.89	0.43	0.94	0.35

*Data are presented as least square means per treatment ± SEM. Different letters signify means are different ($P < 0.05$). Underfed, UF (n = 42); Control, CON (n = 39); Overfed, OF (n = 39); Arginine, Arg (n = 59); Saline (n = 61). [1]Over the experimental period (5-point scale).

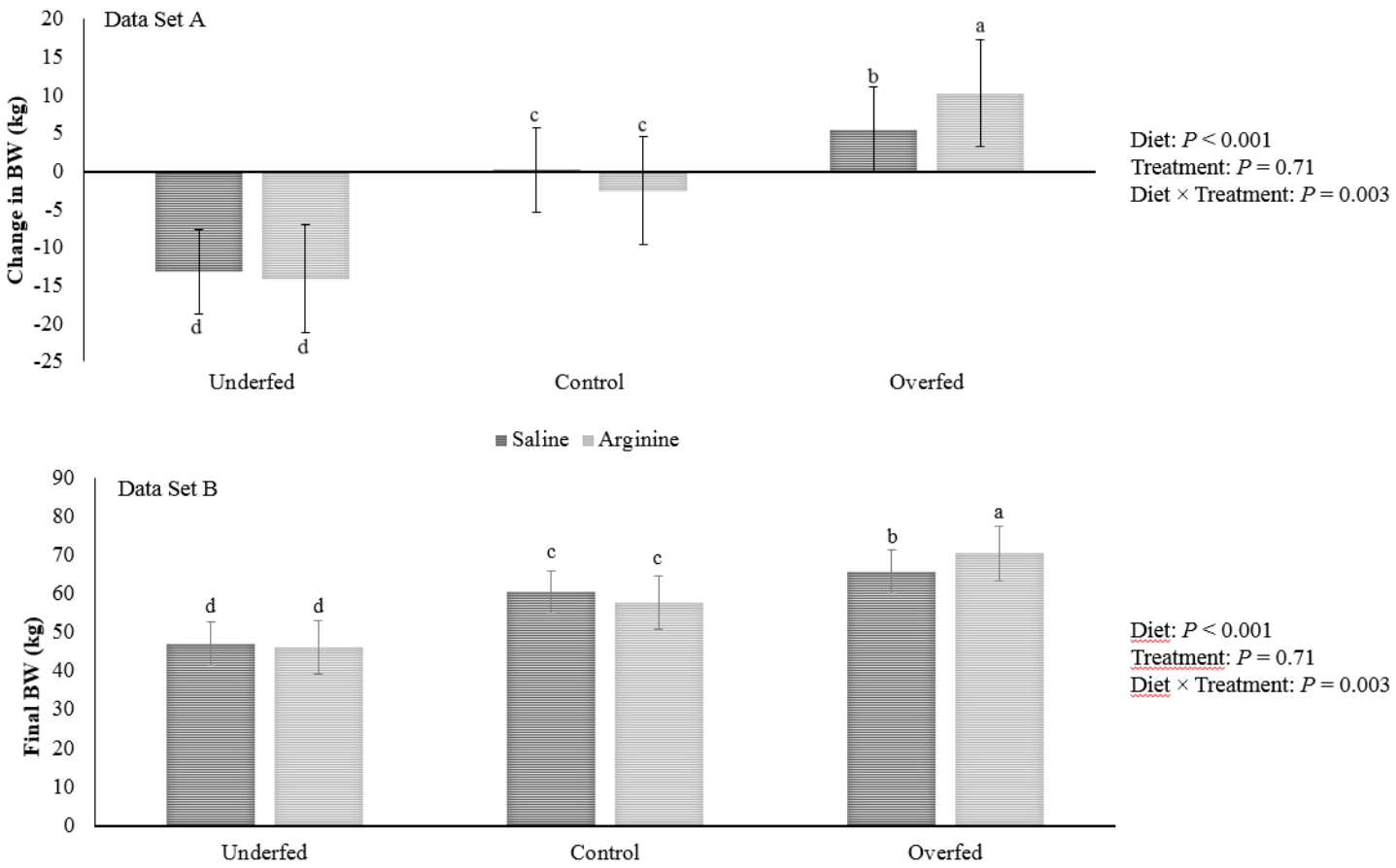

Figure 3-1. Diet × Treatment interaction on change in BW and final BW in non-pregnant ewes.
*Data are presented as least square means per treatment ± SEM. Different letters signify means are different ($P < 0.05$). Underfed (n = 42); Control (n = 39); Overfed (n = 39); Arginine, Arg (n = 59); Saline (n = 61).

No differences ($P > 0.05$) in pancreatic protein concentration (mg/g) were noted between dietary groups or Arg vs Sal treatments. Protein content (g/pancreas) was greater ($P = 0.001$) in CON and OF ewes than UF while protein (% of BW) was greater ($P = 0.03$) in UF than OF with CON intermediate.

The concentration (U/g) of α-amylase activity was greater ($P < 0.001$) in OF and CON than UF ewes. Overfed ewes had the greatest ($P < 0.001$) content of α-amylase activity (kU/pancreas) followed by CON. Both OF and CON had a greater ($P = 0.003$) content of α-amylase activity relative to BW (U/kg BW) than UF ewes. When expressed as U/g protein, the α-amylase activity was greatest ($P < 0.001$) in OF followed by CON and then UF. Arginine treatment had no impact on α-amylase activity.

Trypsin activity (U/g) was not different among treatment groups and when expressed as U/pancreas was greater ($P = 0.003$) in OF than UF with CON not being different from either dietary group. Trypsin activity (U/kg BW and U/g protein) was not different ($P > 0.05$) between diet or Arg treatment.

Serum insulin concentration was greatest ($P < 0.001$) in OF followed by CON and then UF ewes (**Table 3-3**). No effects of diet or Arg treatment ($P > 0.05$) were noted in the pancreatic insulin-positive area (relative to section of tissue) or size of insulin-containing clusters (μm^2) (**Figure 3-2**).

3.5. Discussion

Changes in BW and BCS due to variable amount of feed consumption is well documented for sheep and other species (Church, 1987; Grazul-Bilska et al., 2012; Kaminski et al., 2015). Increased change in BW, final BW, and BCS in ewes provided dietary intake similar

to that in the current study have been previously reported (Grazul-Bilska et al., 2012; Keomanivong et al., 2015a) as overfed ewes tended to gain weight while those restricted in nutrient intake lost weight. The diet × treatment interactions observed for final BW indicated that OF ewes infused with Arg had the greatest final BW compared to other treatments. This may be because of the ability of Arg to increase NO production which is the major vasodilatory factor (Kaminski et al., 2015). This could then allow for increased delivery of nutrients ingested by the overfed ewes. However, although serum Arg concentration increased with Arg treatment, serum nitric oxide metabolite concentration did not differ between dietary treatments or with Arg treatment (Kaminski et al., 2015) suggesting that these responses may not have been mediated by changes in NO synthesis. Surprisingly, a study involving dairy heifers infused with Arg in the peritoneum demonstrated that the proportion of essential amino acids in plasma was greater in heifers exposed to a control rather than an Arg treatment (Yunta et al., 2015). Infusing the ewes directly into the jugular vein in the current study, however, may play a role in the differences observed, as may the difference in the amount of arginine used.

The UF ewes in this study had lower pancreatic weight (g). This is likely the result of reduced metabolizable energy and nutrient intake thus resulting in decreased tissue growth (Ferrell et al., 1986; Swanson et al., 2008a; Keomanivong et al., 2015a). Furthermore, because BW was subjected to a greater decrease than pancreatic weight it is not surprising that UF ewes had a greater pancreatic weight (% of BW) than OF ewes (Salim et al., 2016).

Increasing dietary intake has been associated with greater pancreatic protein concentration (g/pancreas) and the secretion of exocrine digestive enzymes in this and other studies (Swanson et al., 2008b). It has been reported that enhanced pancreatic protein

Table 3-3. Influence of nutrient restriction and arginine treatment on serum insulin and measurements of insulin containing clusters in pancreas in non-pregnant sheep.

Item	Dietary Group				Treatment			*P*-Value		
	UF	CON	OF	SEM	Saline	Arg	SEM	Diet	Treatment	Diet × Treatment
Serum Insulin, µIU/mL	6.97[b]	9.88[b]	13.2[a]	1.142	10.8	9.19	0.977	0.001	0.24	0.88
Pancreatic insulin-positive area (relative to section of tissue), %	1.42	1.30	1.31	0.1486	1.39	1.29	0.124	0.82	0.58	0.66
Insulin-containing clusters within the following ranges (µm²), %										
1 to 2,000	64.5	67.7	64.7	1.97	64.8	66.5	1.64	0.43	0.48	0.67
2,001 to 32,000	34.9	31.5	34.5	1.88	34.4	32.8	1.55	0.37	0.47	0.63
32,001 to 512,000	0.607	0.762	0.760	0.2071	0.734	0.685	0.1748	0.83	0.84	0.60
Average size of insulin-containing clusters (µm²)	2988	3021	3158	253.0	3111	3000	210.3	0.86	0.69	0.73
Smallest insulin-containing cluster (µm²)	251	251	250	0.5	250	250	0.4	0.37	0.93	0.24
Largest insulin-containing cluster (µm²)	48936	55686	46492	6790.8	52364	48379	5643.6	0.61	0.62	0.16

*Data are presented as least square means per treatment ± SEM. Different letters signify means are different ($P < 0.05$). Underfed, UF (n = 42); Control, CON (n = 39); Overfed, OF (n = 39); Arginine, Arg (n = 59); Saline (n = 61).

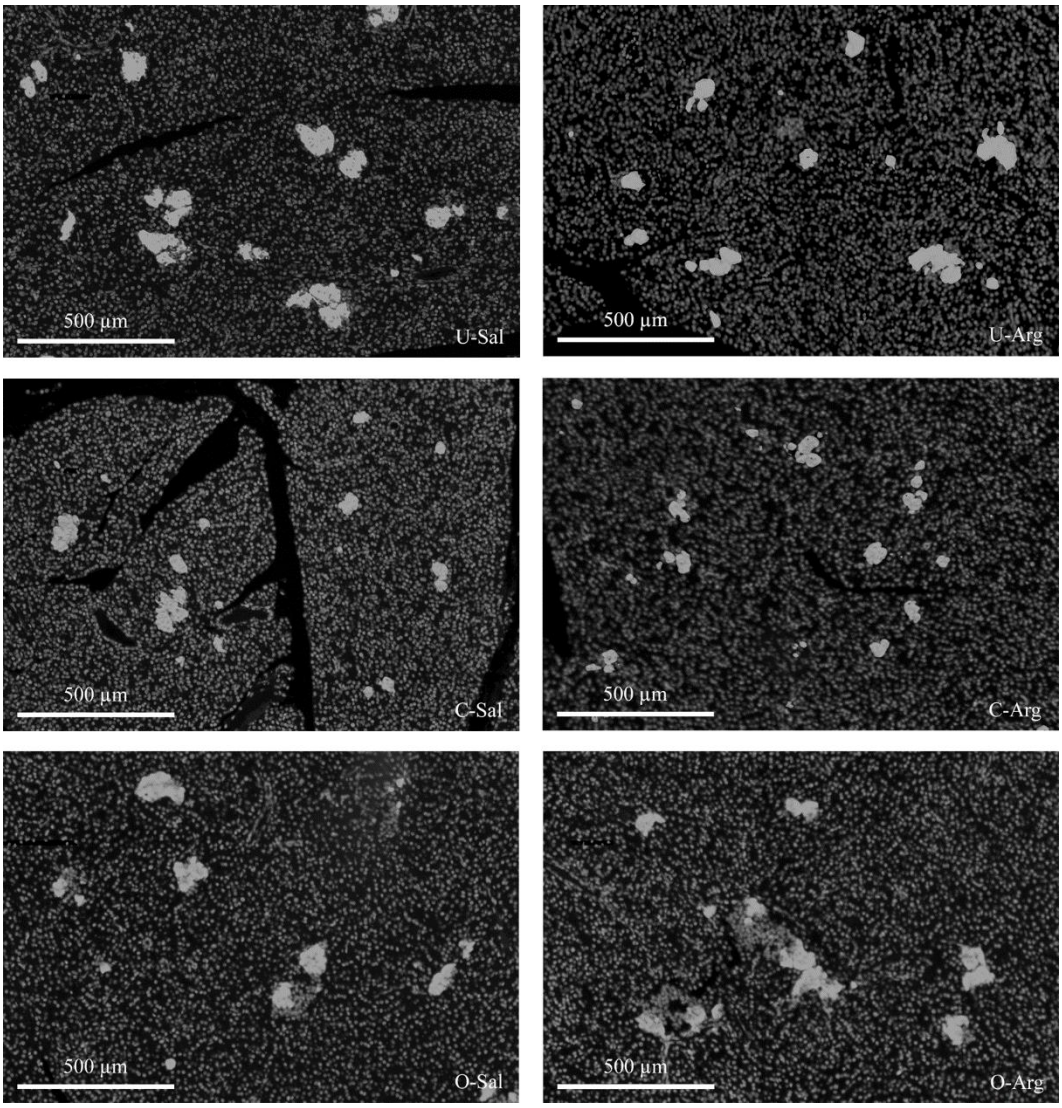

Figure 3-2. Influence of diet and treatment on insulin-positive staining. Sections represent pancreatic tissues of ewes receiving each diet and treatment. Magnification 10x. UF-Sal (Underfed Saline), UF-Arg (Underfed Arginine), CON-Sal (Control Saline), CON-Arg (Control Arginine), OF-Sal (Overfed Saline), OF-Arg (Overfed Arginine).

concentration may increase the hydrolysis of nutrients in the small intestine (Corring, 1980; Harmon, 1992; Swanson et al., 2000; Swanson and Harmon, 2002).

An increase in the content (U/pancreas) of α-amylase and trypsin activity in OF ewes was observed in the current study which is likely due to increased feed intake (Wang et al., 2000; Swanson et al., 2008b) resulting in increases in enzyme concentration (U/g pancreas) or increased tissue mass (g) of the pancreas and therefore tissue enzyme content. Because no difference in the concentration of trypsin (U/g) was noted in this experiment, the increase in content of trypsin activity was likely due to increased tissue mass whereas the increase in α-amylase content was due to increases in both enzyme concentration and tissue mass.

Insulin concentration in serum was greater in OF than CON and UF ewes in the current study. Similarly, greater insulin concentration in overfed and lower insulin concentration in underfed animals were reported by others (Church, 1987; Diskin et al., 2003; Adamiak et al., 2005; Tsiplakou et al., 2012; Kiani, 2013; Kaminski et al., 2015). In this study serum insulin concentration was not affected by treatment with Arg. In addition, Arg treatment during late pregnancy did not affect plasma insulin concentration in ovine fetuses (Oliver et al., 2001). In contrast, high doses of Arg increased insulin concentration in ovine fetuses at late pregnancy, and Arg supplementation increased insulin concentration in calves (Gresores et al., 1997; Hüsier and Blum, 2002). Furthermore, Arg infusion caused an acute increase in insulin concentration in ovine fetuses and dairy cows (Vicini et al., 1988). These data indicate that Arg effects on insulin production depends on dose, reproductive status and species. On the other hand, direct associations between feed intake and serum insulin concentration is well documented for several species (Keomanivong et al., 2015a).

The morphology of insulin containing cell cluster sizes was not affected by diet or Arg-treatment. Although NO has been previously shown to cause changes in exocrine and endocrine function of the monogastric pancreas, it's function on ovine pancreatic function seems to be less clear (Spinas, 1999; Arciszewski, 2007; Kaminski et al., 2015). It is also possible that pancreatic morphology of the ewes was unaltered due to the fact that the animals were mature and pancreatic development had already been achieved. While differences have been observed in our previous studies involving pregnant ewes (Keomanivong et al., 2015a), the amount of time spent under nutrient restriction was significantly longer and there may be different responses within pregnant and non-pregnant ewes.

In the present and our previous study (Kaminski et al., 2015), pancreatic measurements and/or insulin concentration were not affected by stages of the estrous cycle that is characterized by changes of progesterone secretion. These results may be due to the fact that the days examined are close enough that pancreatic enzyme activity and insulin-cluster morphology were not different.

In summary, results obtained from the current experiment have demonstrated that diet but not Arg treatment or stage of the estrous cycle affected pancreatic function including mass, digestive enzyme activity, and serum insulin concentration. These results are supported by the findings in the companion study of Kaminski et al. (2015) who noted a greater impact of diet than Arg or stage of the estrous cycle on measurements of serum metabolites (glucose) and selected hormones (insulin-like growth factor 1, leptin, and progesterone). Thus, contrary to our hypothesis, dose and duration of Arg-treatment used was ineffective as a therapeutic to overcome compromised nutrition in ewes, but plane of nutrition seems to be a major regulator of pancreatic exocrine function.

3.6. Literature Cited

Adamiak, S. J., K. Mackie, R. G. Watt, R. Webb, and K. D. Sinclair. 2005. Impact of nutrition on oocyte quality: cumulative effects of body composition and diet leading to hyperinsulinemia in cattle. Biol. Reprod. 73:918-926.

Arciszewski, M. B. 2007. Expression of neuronal nitric oxide synthase in the pancreas of sheep. Anat. Histol. Embryol. 36:375-381.

Armstrong, D. G., J. G. Gondg, and R. Webb. 2003. Interactions between nutrition and ovarian activity in cattle: physiological, cellular and molecular mechanisms. Repro. Supp. 61:403-414.

Boland, M. P., P. Lonergan, and D. O'Callaghan. 2001. Effect of nutrition on endocrine parameters, ovarian physiology, and oocyte and embryo development. Theriogenology. 55:1323-1340.

Church, D. C. 1987. The ruminant animal: digestive physiology and nutrition. Prentice Hall, Englewood Cliffs, N.J.

Corring, T. 1980. The adaptation of digestive enzymes to the diet: its physiological significance. Reprod. Nutr. Dev. 20:1217-1235.

Diskin, M. G., D. R. Mackey, J. F. Roche, and J. M. Sreenan. 2003. Effects of nutrition and metabolic status on circulating hormones and ovarian follicle development in cattle. Anim. Reprod. Sci. 78:345-370.

Ferrell, C. L., L. J. Koong, and J. A. L. Nienaber. 1986. Effects of previous nutrition on body composition and maintenance energy costs of growing lambs. Br. J. Nut. 56:595-605.

Geiger, R. and H. Fritz. 1986. Trypsin. Methods of Enzymatic Anal. H. Bergmeyer, ed Acad. Press, New York, NY. 119-128.

Grazul-Bilska, A. T., E. Borowczyk, J. J. Bilska, L. P. Reynolds, D. A. Redmer, J. S. Caton, and K. A. Vonnahme. 2012. Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep. Domestic Anim. Endocrin. 43:289-298.

Gresores, A., S. Anderson, D. Hood, G. O. Zerbe, and W. W. Hay -Jr. 1997. Separate and joint effects of arginine and glucose on ovine fetal insulin secretion. Am. J. Physiol. 272:E68-73.

Harmon, D. L. 1992. Impact of nutrition on pancreatic exocrine and endocrine secretion in ruminants: a review. J. Anim. Sci. 70:1290-1301.

Hüsier, B. R. and J. W. Blum. 2002. Metabolic and endocrine changes in response to endotoxin administration with or without oral arginine supplementation. J. Dairy Sci. 85:1927-1935.

Kiani, A. 2013. Temporal changes in plasma concentrations of leptin, IGF-1, insulin and metabolites under extended fasting and re-feeding conditions in growing lambs. Int. J. Endocrinol. Metab. 11:34-40.

Kaminski, S. L., D. A. Redmer, C. S. Bass, D. H. Keisler, L. S. Carlson, K. A. Vonnahme, S. T. Dorsam, and A. T. Grazul-Bilska. 2015. The effects of diet and arginine treatment on serum metabolites and selected hormones during the estrous cycle in sheep. Theriogenology. 83:808-816.

Karsenti, D., Y. Bacq, J. F. Bréchot, N. Mariotte, S. Vol, and J. Tichet. 2001. Serum amylase and lipase activities in normal pregnancy: a prospective case-control study. Am. J. Gastroenterol. 96:697-699.

Keomanivong, F. E., L. E. Camacho, C. O. Lemley, E. A. Kuemper, R. D. Yunusova, P. P. Borowicz, J. D. Kirsch, K. A. Vonnahme, J. S. Caton, and K. C. Swanson. 2015a. Effects of realimentation after nutrient restriction during mid- to late-gestation on pancreatic digestive enzymes, serum insulin and glucose levels, and insulin-containing cell cluster morphology. J. Anim. Physiol. Anim. Nutr. (In press; doi:10.1111/jpn.12480).

Keomanivong, F. E., C. O. Lemley, L. E. Camacho, R. Yunusova, P. P. Borowicz, J. S. Caton, A. M. Meyer, K. A. Vonnahme, and K. C. Swanson. 2015b. Influence of nutrient restriction and melatonin supplementation of pregnant ewes on maternal and fetal pancreatic digestive enzymes and insulin-containing clusters. Animal 9:1-9.

Khayambashi, H., M. Boroumand, A. Boroumand, F. Hekmatyar, and R. C. Barnett. 1971. In vitro inhibition of bovine serum and pancreatic amylase by progesterone. Nature 230:529-531.

Lassala, A., F. W. Bazer, T. A. Cudd, S. Datta, D. H. Keisler, M. C. Satterfield, T. E. Spencer, and G. Wu. 2010. Parenteral administration of l-Arginine prevents fetal growth restriction in undernourished ewes. J. Nutr. 140:1242-1248.

Lassala, A., F. W. Bazer, T. A. Cudd, S. Datta, D. H. Keisler, M. C. Satterfield, T. E. Spencer, and G. Wu. 2011. Parenteral administration of L-arginine enhances fetal survival and growth in sheep carrying multiple fetuses. J. Nutr. 141:849-855.

Morimoto, S., M. A. Cerbón, A. Alvarez-Alvarez, G. Romero-Navarro, and V. Díaz-Sánchez. 2001. Insulin gene expression pattern in rat pancreas during the estrous cycle. Life Sci. 68:2979-2985.

National Research Council. 1996. Nutrient requirements of beef cattle. 7th ed. National Academy Press, Washington, DC.

Oliver, M. H., P. Hawkins, B. H. Breier, P. L. Van Zijl, S. A. Sargison, and J. E. Harding. 2001. Maternal undernutrition during the periconceptual period increases plasma taurine levels and insulin response to glucose but not arginine in the late gestational fetal sheep. Endocrinology. 142:4576-4579.

Salim, H., K. M. Wood, J. P. Cant, and K. C. Swanson. 2016. Influence of feeding increasing levels of dry or modified wet corn distillers grains plus solubles in whole corn grain-based finishing cattle diets on pancreatic α-amylase and trypsin activity. Can. J. Anim. Sci. 96:294-298.

Sartori, R., M. M. Guardieiro, R. S. Surjus, L. F. Melo, A. B. Prata, M. Ishiguro, M. R. Bastos, and A. B. Nascimento. 2013. Metabolic hormones and reproductive function in cattle. Anim. Reprod. 10:199-205.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.

Spinas, G. A. 1999. The dual role of nitric oxide in islet ß-cells. News Physiol. Sci. 14:49-54.

Swanson, K. C. and D. L. Harmon. 2002. Dietary influences on pancreatic α-amylase expression and secretion in ruminants. In Biology of the Intestine in Growing Animals (ed R Zabielski, V Lesniewska, PC Gregory, and B. Westrom), pp. 515-537. Elsevier, Boston, USA.

Swanson, K. C., N. Kelly, H. Salim, Y. J. Wang, S. Holligan, M. Z. Fan, and B. W. McBride. 2008a. Pancreatic mass, cellularity and α-amylase and trypsin activity in feedlot steers fed diets differing in crude protein concentration. J. Anim. Sci. 86:909-915.

Swanson, K. C., N. Kelly, Y. J. Wang, M. Ko, S. Holligan, M. Z. Fan, and B. W. McBride. 2008b. Pancreatic mass, cellularity, and a-amylase and trypsin activity in feedlot steers fed increasing amounts of a high-moisture-corn based diet. Can. J. Anim. Sci. 88: 303-308.

Swanson, K. C., J. C. Matthews, A. D. Matthews, J. A. Howell, C. J. Richards, and D. L. Harmon. 2000. Dietary carbohydrate source and energy intake influence the expression of pancreatic α-amylase in lambs. J. Nutr. 9:2157-2165.

Tsiplakou, E., S. Chadio, and G. Zervas. 2012. The effect of long term under- and over-feeding of sheep on milk and plasma fatty acid profiles and on insulin and leptin concentrations. J. Dairy Res. 79:192-200.

Vonnahme, K. A, J. S. Luther, L. P. Reynolds, C. J. Hammer, D. B. Carlson, D. A. Redmer, and J. S. Caton. 2010. Impacts of maternal selenium and nutritional level on growth, adiposity, and glucose tolerance in female offspring in sheep. Domest Anim Endocrin. 39:240-248.

Wallenfels, K., P. Fold, H. Niermann, H. Bender, and D. Linder. 1978. The enzymic synthesis, by transglucosylation of a homologous series of glycosidically substituted malto-oligosaccharides, and their use as amylase substrates. Carb. Res. 61:359-368.

Wang, X. B., K. Taniguchi, and T. Obitsu. 2000. Response of pancreatic exocrine secretion in sheep fed different type and amount of hay. Asian Aus. J. Anim. Sci. 13:1004-1049.

Wu, G., F. W. Bazer, J. M. Wallace, and T. E. Spencer. 2006. Board-Invited Review: Intrauterine growth retardation: implications for the animal sciences. J. Anim. Sci. 84:2316-2337.

Wu, G. and S. M. Morris Jr. 1998. Arginine metabolism: nitric oxide and beyond. Biochem J. 336:1-17.

Vicini. J. L., J. H. Clark, W. L. Hurley, and J. M. Bahr. 1988. Effects of abomasal or intravenous administration of arginine on milk production, milk composition, and concentrations of somatotropin and insulin in plasma of dairy cows. J. Dairy Sci. 71:658-665.

Yunta, C., K. A. Vonnahme, B. R. Mordhorst, D. M. Hallford, C. O. Lemley, C. Parys, and A. Bach. 2015. Arginine supplementation between 41 and 146 days of pregnancy reduces uterine blood flow in dairy heifers. Theriogenology, 84:43-50.

CHAPTER 4. EFFECTS OF REALIMENTATION AFTER NUTRIENT RESTRICTION DURING MID- TO LATE GESTATION ON PANCREATIC DIGESTIVE ENZYMES, SERUM INSULIN AND GLUCOSE LEVELS, AND INSULIN-CONTAINING CELL CLUSTER MORPHOLOGY[3]

4.1. Abstract

This study examined effects of stage of gestation and nutrient restriction with subsequent realimentation on maternal and fetal bovine pancreatic function. Dietary treatments were assigned on d 30 of pregnancy and included: control (CON; 100% requirements; n = 18) and restricted (R; 60% requirements; n = 30). On d 85, cows were slaughtered (CON, n = 6; R, n = 6), remained on control (CC; n = 12) and restricted (RR; n = 12), or realimented to control (RC; n = 11). On d 140, cows were slaughtered (CC, n = 6; RR, n = 6; RC, n = 5), remained on control (CCC, n = 6; RCC, n = 5), or realimented to control (RRC, n = 6). On d 254, remaining cows were slaughtered and serum samples were collected from the maternal jugular vein and umbilical cord to determine insulin and glucose concentrations. Pancreases from cows and fetuses were removed, weighed, and subsampled for enzyme and histological analysis. As gestation progressed maternal pancreatic α-amylase activity decreased and serum insulin concentrations increased ($P \leq 0.03$). Fetal pancreatic trypsin activity increased ($P < 0.001$) with advancing

[3]The material in this chapter was co-authored by F. E. Keomanivong, L. E. Camacho, C. O. Lemley, E. A. Kuemper, R. D. Yunusova, P. P. Borowicz, J. D. Kirsch, K. A. Vonnahme, J. S. Caton, and K. C. Swanson. It has been accepted for publication by Journal of Animal Physiology and Animal Nutrition, doi: 10.1111/jpn.12480. F. E. Keomanivong had primary responsibility for collecting and analyzing samples in the field. F. E. Keomanivong was the primary developer of the conclusions that are advanced here. F. E. Keomanivong also drafted and revised all versions of this chapter. K. C. Swanson served as proofreader and checked the math in the statistical analysis conducted by F. E. Keomanivong.

gestation. Fetal pancreases subjected to realimentation (CCC vs RCC and RRC) had increased protein and α-amylase activity at d 254 ($P \leq 0.02$) while trypsin (U/g protein; $P = 0.02$) demonstrated the opposite effect. No treatment effects were observed for maternal or fetal pancreatic insulin-containing cell clusters. Fetal serum insulin and glucose levels were reduced with advancing gestation ($P \leq 0.03$). The largest maternal insulin-containing cell cluster was not influenced by advancing gestation while fetal clusters grew throughout ($P = 0.01$). These effects indicate that maternal digestive enzymes are influenced by nutrient restriction and there is a potential for programming of increased fetal digestive enzyme production resulting from previous maternal nutrient restriction.

4.2. Introduction

The nutrient requirements of gestating cattle, along with nutrient availability in feedstuffs, fluctuate throughout the year and meeting dietary needs can be challenging (Freetly et al., 2005; Freetly, et al., 2008). Dietary variations, such as changes in feed intake and composition, influence the production and secretion of pancreatic digestive enzymes important for the break down and absorption of feed in the small intestine (Swanson and Harmon, 2002a; Mader et al., 2009). Studies have also shown significant nutritional impacts on pancreatic endocrine function (Fowden et al., 1989; Sano et al., 1999). The majority of experiments evaluating nutritional effects on pancreatic function in cattle have been conducted in growing animals and less is known about the impacts of nutrition and gestational stage on pancreatic function and development in mature cows and their fetuses, respectively.

Maternal nutrition is responsible for both direct and indirect effects on fetal growth. Fowden and Hill (2001) have shown in ovine species that the pancreatic content of insulin

increases in the fetus during gestation as long as the dam is provided with adequate nutrition. Restricted diets, however, may lead to reduced pancreatic endocrine cell number (Bertram and Hanson, 2001) as well as permanent deficiencies of insulin secretion and β-cell proliferation (Winick and Noble, 1966; Weinkove et al., 1974; Snoeck et al., 1990, Dahri et al., 1995). The proportion of insulin containing tissue within the pancreas of sheep may also be decreased in nutrient restricted fetuses (Limesand et al., 2005). This effect is thought to be caused by diminished mitotic cell division, however, the impact on the pancreas of bovine tissue is unknown.

Responses of the gastrointestinal tract and associated tissues to pregnancy and time of gestation have been evaluated and some authors suggest that, in times of nutritional stress, the maternal body compensates for the loss of nutrients to the developing fetus by sacrificing maternal metabolic needs (Molle et al., 2004; Reed et al., 2007). The fetus is also able to adapt to periods of restriction by decreasing the rate of cell division which could induce lifelong changes in the organ and whole animal functionality (Barker and Clark, 1997). Such an effect is termed developmental programming (Reynolds et al., 2010; Zhang, 2010).

In many species, realimentation has been able to increase the body weight of restricted animals to a level that matches non-restricted contemporaries (Funston et al., 2010; Meyer et al., 2010). Realimentation of nutrient restricted animals was also successful in returning the weights of internal organs to those of their control counterparts (Zubair and Leeson, 1994). Although the growth of the conceptus is minimal during early gestation, changes in cell proliferation can still occur (Schoonmaker, 2013). Carlsson et al. (2010) reported noticeable pancreatic glucagon staining at d 25 with insulin apparent at d 26. Also, between d 89 to 105 small sections of insulin clusters were beginning to assemble into larger groups. Changes in these developmental patterns

have the potential to alter pancreatic function after conception. Although current knowledge of bovine pancreatic growth is minimal, numerous studies conducted in ewes undergoing nutrient restriction from early to mid-gestation followed by realimentation until parturition indicate significant compensatory growth of other areas of the body such as in placentomes (Foote et al., 1958; Robinson et al., 1995; McMullen et al., 2005) and fetal musculature (Gonzalez, et al., 2013). While the majority of fetal growth has been shown to occur during late gestation it is important to determine the most effective timing of realimentation during gestation to avoid adverse physiological effects such as impaired growth or organ function postnatally.

The pancreas has important exocrine and endocrine roles and there is limited information on the functional and morphological changes that take place during gestation, especially in cattle. Determining the physiological effects associated with dietary variation is also critical for obtaining a comprehensive understanding of pancreatic development and maintenance. The objective of this study was to determine the impact of different lengths of nutrient restriction during early- and mid-gestation and subsequent realimentation at different stages of gestation on pancreatic function and development in fetuses. We hypothesize that fetal pancreatic development and maintenance will be altered by nutritional treatment of the mother during advancing gestational age. Moreover, we believe nutrient restriction will be detrimental to pancreatic function while increasing the duration of realimentation may help rescue pancreatic development returning it to a level matching that of control animals. Therefore, the effects of advancing gestation were evaluated (d 85, 140 and 254) using cows receiving adequate nutrition (NRC, 2000). The impacts of dietary intake were assessed by comparing control to restricted treatments and by comparing different lengths of realimentation were accessed by comparing cows realimented for differing periods as compared to controls on d 85, 140 and 254.

4.3. Materials and Methods

All animal care, handling and sample collection was approved by the NDSU Institutional Animal Care and Use Committee.

4.3.1. Animals and Dietary Treatments

Animal use and treatment design have been described previously (Camacho et al., 2014). Briefly, 34 non-lactating, cross-bred, multiparous cows were artificially inseminated. Pregnancy was verified on d 25 or 26 post-insemination using transrectal Doppler ultrasonography. Cows were trained to use the Calan gate feeding system (American Calan, Northwood, NH, USA) with exclusive access to feeders through electronic transponders in order to measure individual feed intake. Diets were composed of grass hay (86% DM, 8% CP, 68.5% NDF, 41.5 % ADF, 58% TDN and 1.3% EE). Intake was adjusted every two weeks relative to body weight (BW) and dietary dry matter (DM) intake was adjusted to meet NRC recommendations according to the stage of gestation (average requirements for periods from d 30 to 85, d 86 to 140, d 141 to 197, and d 198 to 254). Intake for the control cows was 1.33, 1.34, 1.41, and 1.57% of BW (DM basis) for periods of d 30 to 85, d 86 to 140, d 141 to 197, and d 198 to 254 with restricted groups receiving 60% of the control cows relative to BW. There were no feed refusals over the experimental period. Mineral and vitamin supplement (12% Ca; 5% Mg; 5% K; 180 mg/kg Co; 5,100 mg/kg Cu; 375 mg/kg I; 1.2% Fe; 2.7% Mn; 132 mg/kg Se; 2.7% Zn; 570,000 IU/kg Vit A; 160,000 IU/kg Vit D-3; 2,700 IU/kg Vit E; Camacho, et al., 2014) was top-dressed three times a week at a rate of 0.18% of hay DMI to meet requirements relative to BW change of the cows (i.e. fed at a constant percentage of the total diet). On d 30 of pregnancy, cows were randomly assigned to dietary treatments (n = 4 to 5/pen with greater than 1 dietary treatment per pen): 1) control (CON; 100% requirements; n = 18) and 2) restricted (R; 60% requirements; n =

30). On d 85, cows were slaughtered (CON, n = 6; R, n = 6), remained on control (CC; n = 12) and restricted (RR; n = 12), or 3) were realimented to control (RC; n = 11). On d 140, cows were slaughtered (CC, n = 6; RR, n = 6; RC, n = 5), remained on control (CCC, n = 6; RCC, n = 5), or were realimented to control (RRC, n = 6). At d 254, all remaining cows were slaughtered (CON, n = 6; RCC, n = 5; RRC, n = 6; **Figure 4-1**; Camacho, et al., 2014).

4.3.2. Sample Collection

On slaughter d 85 (early-gestation), 140 (mid-gestation) and 254 (late-gestation) of pregnancy, cows were weighed and slaughtered via captive bolt and exsanguination. Blood samples were collected from the maternal jugular vein and umbilical cord, allowed to clot, and centrifuged at 2000 × g for 20 min to separate serum which was stored at -80°C for later analysis of insulin and glucose concentration. Pancreases were removed from both the cow and fetus and trimmed of mesentery and fat. Pancreases were weighed and a subsample of tissue was taken from the body of the pancreas (Swanson et al., 2004) and flash-frozen in isopentane that was super-cooled in liquid nitrogen and then stored at -80˚C until analyses for protein and α-amylase and trypsin activities. A section of pancreatic tissue with an area of approximately 1.5 cm^2 was fixed in 10% formalin and later embedded in paraffin blocks to be used in immunohistochemistry analyses.

4.3.3. Analysis of Pancreatic Protein Concentration, and α-Amylase and Trypsin Activity

Pancreatic tissue (0.25 g) was homogenized in a 0.9% NaCl solution (2.25 mL) using a polytron (Brinkmann Instruments Inc., Westbury, NY, USA). Protein concentration was measured using the Pierce Bicinchoninic acid (BCA) Protein Assay Kit (Fisher Scientific, Pittsburgh, PA, USA) with bovine serum albumin (BSA) used as a standard diluted in saline

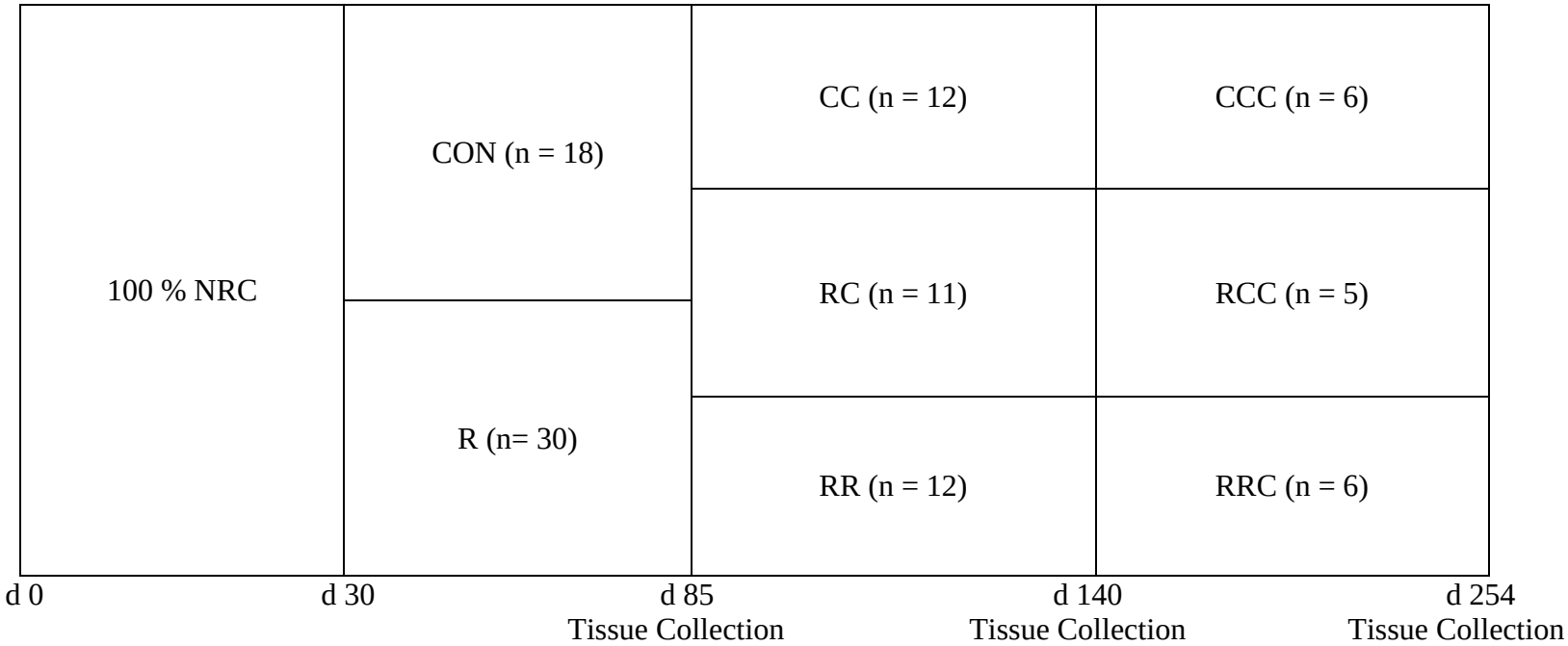

Figure 4-1. Diagram of experimental design adapted from Camacho et al., 2014. Multiparous, non-lactating beef cows were fed grass hay to meet or exceed NRC recommendations (NRC, 2000) until day 30 of gestation. Dietary treatments were assigned on d 30 of pregnancy and included: control (CON; 100% requirements; n = 18) and restricted (R; 60% requirements; n = 30). On d 85, cows were slaughtered (CON, n = 6; R, n = 6), remained on control (CC; n = 12) and restricted (RR; n = 12), or realimented to control (RC; n = 11). On d 140, cows were slaughtered (CC, n = 6; RR, n = 6; RC, n = 5), remained on control (CCC, n = 6; RCC, n = 5), or were realimented to control (RRC, n = 6). On d 254, all remaining cows were slaughtered.

(0.9% NaCl). Activity of α-amylase was determined using the procedure of Wallenfels et al. (1978) utilizing a kit from Teco Diagnostics (Anahein, CA, USA). Trypsin activity was assayed using the methods described by Geiger and Fritz (1986) after activation with 100 U/L enterokinase (Glazer and Steer, 1977; Swanson et al., 2002b). Analyses were adapted for use on a microplate spectrophotometer (SpectraMax 340, Molecular Devices, Sunnyvale, CA, USA). One unit (U) of enzyme activity equals 1 µmole product produced per min. Enzyme activity data are expressed as U/g wet tissue, kU/pancreas or U/pancreas, U/kg BW and U/g protein.

4.3.4. Serum Insulin and Glucose Analysis

Serum samples were analyzed for insulin by using an immunoassay system (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA; Grazul-Bilska, et al., 2012). The interassay coefficient of variation (CV) for the low pool was 4.3% and 2.0% for the high pool and the intra-assay CV was 8.76%. Serum was analyzed for glucose using the hexokinase/glucose-6-phosphate dehydrogenase method (Farrance, 1987) using a kit from Thermo Scientific (Pittsburgh, PA, USA) and a microplate reader (Synergy, H1 Microplate reader, BioTek Instruments, Winooski, VT, USA).

4.3.5. Immunohistochemistry

Maternal and fetal pancreatic tissue was immersion-fixed in formalin and embedded in paraffin blocks (Grazul-Bilska et al., 2009). From each sample, 5-µm sections were obtained from the blocks maintaining at least 100 µm between sections. Tissue sections on the slides were then deparaffinized and rehydrated. After rehydration, slides were incubated in a staining enhancer citrate buffer heated to 95˚C for 5 minutes then cooled to room temperature. Slides were then covered in a solution of 10% donkey serum and 1% bovine serum albumin diluted in tris-buffered saline for a period of 20 minutes. Slides were incubated with 1:100 mouse anti-pig

insulin (Santa Cruz Biotech, Dallas, TX, USA) in 1% BSA and tris-buffered saline overnight at 4˚C on a plate rocker. The following day, the slides were washed with tris-buffered saline with added tween 20 (TBST) and incubated with 1:100 CF™633 Goat Anti-Mouse IgG secondary antibody for one hr in complete darkness. A final wash with TBST was performed and coverslips were applied using mounting medium containing 4', 6- diamidino-2-phenylindole in order to visualize all pancreatic cell nuclei.

4.3.6. Histological analysis

Photomicrographs were taken with a Zeiss Imager.M2 epifluorescence microscope (Carl Zeiss Microscopy, Jena, Germany) using a 10x objective and AxioCam HR camera with a Zeiss piezo automated stage. To describe morphology of the pancreas, the mosaic image of a large tissue area of approximately 144 pictures (12 × 12 pictures) on the slide was taken using the MosaiX module of Zeiss AxioVision software. This method allowed creation of a single image covering all the pancreatic tissue present on the histological slide. The MosaiX images were then analyzed using the ImagePro Premier software (ImagePro Premier 9.0, Media Cybernetics, Silver Spring, MD, USA) for the insulin-positive tissue area (per section of tissue) and size of insulin-containing cell clusters (**Figure 4-2**).

The images were analyzed by creating regions of interest corresponding to the whole area of pancreatic tissue visible on the picture. Within the regions of interest, images were segmented based on positive insulin staining and measurements of total positive insulin staining within pancreatic tissue expressed as percent as well as of individual insulin-containing cell clusters.

Insulin-containing cell clusters were further classified based on size in order to perform population density measurements of small clusters (1 to 2,000 μm^2), medium clusters (2,001 to 32,000 μm^2), large clusters (32,001 to 512,000 μm^2), and giant clusters ($\geq$ 512,000 μm^2). Insulin-

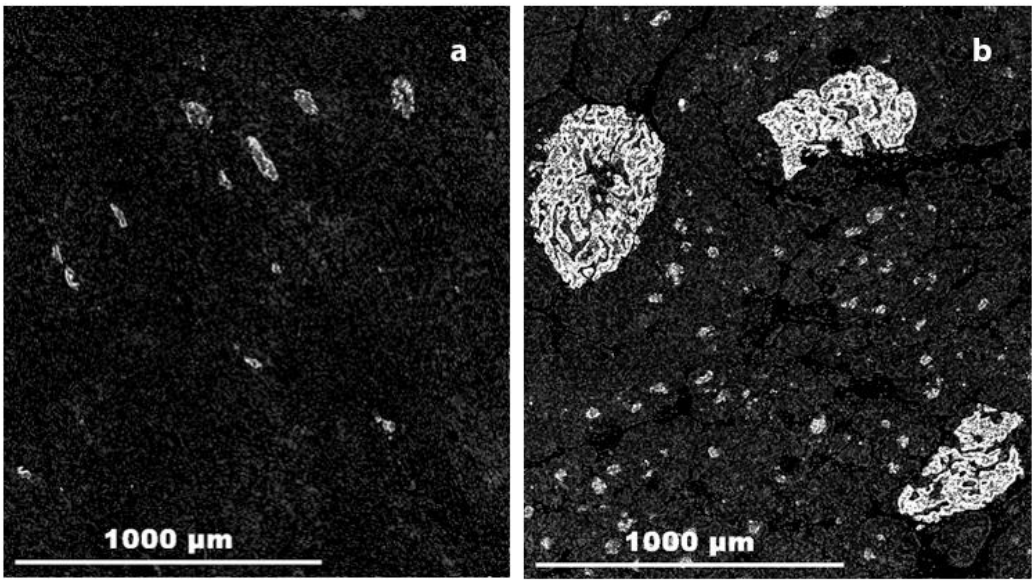

Figure 4-2. Representative images of insulin-containing cell clusters represented by sections of maternal (left image; a) and corresponding fetal (right image; b) pancreatic tissue of cows receiving 100% NRC recommendations. (Tissues have been stained for insulin as shown in white). Magnification 10x.

positive cell clusters do not correspond to islets of Langerhans because not every cell in the islet is insulin positive.

4.3.7. Statistical Analysis

Data were analyzed as a completely randomized design. Fetal sex was removed from the model when significance was found to have a *P*-Value greater than 0.2. Therefore, treatment was included in the model statement for the dams while treatment and sex was included for the fetuses. Differences between means were determined using contrast statements analyzing linear and quadratic effects in the control group (CON, CC, and CCC) over time to determine effects of advancing gestation. Contrast statement coefficients for linear and quadratic effects with unequal spacing of day of gestation were determined using the interactive matrix language (IML) procedure of SAS (statistical analysis software version 9.2, SAS Institute Inc., Cary, NC, USA).

Effects of dietary treatments were determined using contrast statements within slaughter day (d 85, d 140, or d 254 of gestation) to determine the effect of nutrient restriction on d 85 (CON vs R), nutrient restriction on d 140 (CC vs RR and RC), nutrient restriction vs realimentation on d 140 (RC vs RR), nutrient realimentation on d 254 (CCC vs RCC and RRC), and the effect of length of realimentation (RCC vs RRC). Linear and quadratic measurements and comparisons of CON vs R treatments could not be obtained for fetal enzyme activity at d 85 as the pancreas was too small for analysis. Comparison of fetal enzymes from d 140 to d 254 was achieved using the MIXED procedure of SAS and values using this method are indicated with the symbol † in Tables 3 and 4. To determine if there were differences in enzyme activity and endocrine measurements between dams and fetuses, data were analyzed using the MIXED procedure of SAS. Physiological stage (maternal or fetal), treatment, and the interactions between these factors were included in the model. Significance was declared at $P \leq 0.05$ and a tendency was reported if $0.05 > P \leq 0.10$.

4.4. Results

4.4.1. Maternal Enzyme Data

Body weight was not influenced by advancing gestation or dietary treatment except for a tendency ($P = 0.10$) for CC cows to be heavier than RC and RR cows **(Table 4-1)**.

Also, Camacho et al. (2014) found when comparing CC to RR cows, RR cows had decreased BW ($P = 0.05$) compared with CC cows (data not shown). Maternal pancreas absolute weight (g) was not influenced by advancing gestation. Maternal pancreas absolute weight (g) was not influenced by nutrient restriction on d 85, tended to decrease ($P = 0.08$) in RC and RR when compared to CC cows at d 140, was not influenced by realimentation on d 254 (CCC vs.

Table 4-1. Influence of realimentation after nutrient restriction during mid- to late gestation on maternal pancreatic digestive enzymes

	Stage of Gestation											p-value			
	Early (Day 85)		Mid (Day 140)			Late (Day 254)									
Item	CON	R	CC	RC	RR	CCC	RCC	RRC	SEM	L	Q	CON vs R	CC vs RC and RR	RC vs RR	CCC vs RCC and RRC	RCC vs RRC
BW, kg	558	540	606	571	531	610	619	606	28.7	0.22	0.35	0.62	0.10	0.30	0.94	0.73
Pancreas weight, g	393	441	474	380	400	470	518	389	40.1	0.22	0.23	0.36	0.08	0.72	0.73	0.02
Pancreas g/kg of BW	0.706	0.817	0.783	0.660	0.747	0.771	0.835	0.652	0.0594	0.49	0.41	0.16	0.25	0.29	0.69	0.03
Protein																
mg/g tissue	119	132	136	123	130	114	116	119	9.4	0.45	0.08	0.28	0.38	0.58	0.75	0.79
Total g/pancreas	46.1	57.8	64.4	47.1	52.5	54.0	60.3	46.9	6.48	0.60	0.04	0.17	0.05	0.54	0.96	0.13
mg/kg of BW	0.083	0.107	0.106	0.082	0.098	0.088	0.097	0.078	0.0094	0.96	0.06	0.06	0.14	0.23	0.95	0.15
α- Amylase																
U/g	203	138	161	102	155	97.8	101	96.3	35.41	0.03	0.86	0.16	0.43	0.27	0.98	0.91
kU/pancreas	78.0	61.6	73.4	39.9	62.3	46.2	53.1	39.2	15.61	0.10	0.75	0.42	0.22	0.30	1.00	0.52
U/kg of BW	139	110	122	68.9	121	76.1	84.8	65.2	26.81	0.07	0.92	0.41	0.39	0.16	0.97	0.59
U/g protein	1676	1036	1142	789	1159	869	925	791	232.0	0.02	0.31	0.04	0.53	0.25	0.97	0.67
Trypsin																
U/g	1.19	0.767	0.984	1.35	0.958	0.917	0.723	0.441	0.2061	0.36	0.63	0.12	0.47	0.16	0.16	0.32
U/pancreas	433	333	463	507	378	435	371	177	82.4	0.96	0.76	0.35	0.83	0.26	0.09	0.09
U/kg of BW	0.797	0.615	0.753	0.885	0.715	0.729	0.588	0.289	0.1430	0.73	0.89	0.33	0.77	0.38	0.08	0.13
U/g protein	10.0	5.87	7.00	11.1	7.43	8.36	7.10	3.64	1.745	0.62	0.22	0.07	0.26	0.13	0.14	0.15

*CON or C = control, fed at 100% of NRC (2000) recommendations, R = restricted, fed at 60% of NRC (2000) recommendations starting at d 30 and continuing until d 85 (R) or 140 (RR). n = 6, 6 6, 5, 6, 6, 5, 6 for CON, R, CC, RC, RR, CCC, RCC, and RRC treatments, respectively. L = linear effects of stage of gestation with CON, CC, and CCC treatments; Q = quadratic effects of stage of gestation with CON, CC, and CCC treatments; CON vs R; CC vs CR and RR; RC vs RR; CCC vs RCC and RRC; RCC vs RRC = contrast statements within slaughter days (d 85, d 140, or d 254 of gestation).

RCC and RRC), and was greater ($P = 0.02$) in cows undergoing a longer realimentation period (RCC vs. RRC). Pancreas weight relative to BW (g/kg BW) was not influenced by advancing gestation, was not influenced by nutrient restriction on d 85 or 140 (CON vs R, CC vs RC and RR, and RC vs RR), was not influenced by realimentation on d 254 (CCC vs. RCC and RRC), and was greater ($P = 0.03$) in cows undergoing a longer realimentation period (RCC vs. RRC) at d 254. No linear effects were observed for protein concentration (mg/g tissue), a tendency ($P = 0.08$) was observed for quadratic effects over gestation with an increase noted for control treatments from d 85 to d 140 and a decrease until d 254. Dietary treatment did not influence protein concentration (mg/g tissue). Linear effects of advancing gestation were not present for protein content (total g/pancreas), quadratic effects ($P = 0.04$) were observed for control treatments with advancing gestation with an increase from d 85 to d 140 followed by a decrease until d 254 and d 140 protein content (g/pancreas) was greater in CC cows ($P = 0.05$) than in restricted cows (RC and RR). No other treatment effects were observed for protein content (g/pancreas). Advancing gestation did not impact protein content relative to BW (mg/kg of BW); however, there was a tendency for a quadratic effect ($P = 0.06$) with an increase observed from d 85 to d 140 and a decrease until d 254. At d 85, pancreatic protein content relative to BW (mg/kg BW) tended to be greater ($P = 0.06$) in cows subjected to restriction (R vs. CON). Dietary treatment had no other effects on maternal protein content relative to BW (mg/kg BW).

The progression of gestation resulted in a linear decrease ($P \leq 0.03$) in α-amylase (U/g) and no quadratic or dietary treatment effects. Content of α-amylase (kU/pancreas) was not affected by advancing gestation or dietary treatment. A trend ($P = 0.07$) for a linear decrease in total α-amylase activity relative to BW (U/kg BW) was observed and no other effects of α-amylase activity relative to BW (U/kg BW) were observed. A linear decrease ($P = 0.02$) in

specific α-amylase activity (U/g protein) was observed with advancing gestation, no quadratic effects were observed, CON cows had greater (P = 0.04) specific α-amylase activity (U/g protein) than R cows at d 85, and dietary treatment had no other effects.

Concentration of trypsin activity (U/g) was not influenced by advancing gestation or by dietary treatment. Advancing gestation or dietary treatment did not influence trypsin content (U/pancreas) until d 254 when RCC and RRC cows tended (P = 0.09) to have decreased total trypsin activity (U/pancreas) compared with CCC cows and RRC cows tended to have decreased (P = 0.09) total trypsin activity (U/g pancreas) compared with RCC cows. Total trypsin activity relative to BW (U/kg of BW) was not influenced by advancing gestation or dietary treatment until d 254 where there was a trend (P = 0.08) in trypsin activity relative to BW to be greater in CCC cows than those subjected to prior restriction (RCC and RRC). Specific maternal trypsin activity (U/g protein) was not influenced by advancing gestation; however, at d 85 CON cows had greater specific trypsin activity than R cows. No other dietary treatment effects on specific trypsin activity (U/g protein) were observed.

4.4.2. Maternal Endocrine and Histological Data

A linear increase (P = 0.02) in insulin concentration (μIU/mL) in jugular serum occurred as gestation progressed (**Table 4-2**) and no dietary treatment effects were observed.

Glucose (mg/dL) was not influenced by advancing gestation or dietary treatment on d 85. Cows on the RR treatment tended to have greater (P = 0.09) concentrations of jugular serum glucose (mg/dL) than RC cows on d 140 and jugular serum glucose was not influenced by treatment on d 254.

Pancreatic insulin positive area (relative to section of tissue) and percent of small insulin-containing cell clusters were not influenced by gestational stage or dietary treatment. Advancing

Table 4-2. Influence of realimentation after nutrient restriction during mid- to late gestation on maternal serum and pancreatic insulin concentration and measurements of insulin-containing cell clusters

	Stage of Gestation									*P*-value						
	Early (Day 85)		Mid (Day 140)			Late (Day 254)										
Item	CON	R	CC	RC	RR	CCC	RCC	RRC	SEM	L	Q	CON vs R	CC vs RC and RR	RC vs RR	CCC vs RCC and RRC	RCC vs RRC
Insulin, µIU/mL	5.17	8.87	9.27	5.07	10.2	13.5	13.2	14.8	2.514	0.02	0.63	0.26	0.57	0.14	0.86	0.63
Glucose, mg/dL	93.4	99.5	96.1	83.8	94.9	90.6	88.6	85.4	4.738	0.57	0.51	0.32	0.21	0.09	0.49	0.62
Pancreatic insulin-positive tissue area (relative to section of tissue), %	1.08	1.31	1.26	1.16	1.17	0.971	1.18	0.943	0.5041	0.76	0.58	0.71	0.80	0.97	0.83	0.59
Insulin-containing cell clusters within the following ranges (μm^2), %																
1 to 2,000	56.9	45.2	56.7	57.1	55.5	49.1	48.9	63.7	10.82	0.44	0.78	0.39	0.95	0.87	0.43	0.12
2,002 to 32,000	36.8	43.9	39.7	39.1	40.8	48.4	47.6	33.9	9.08	0.19	0.90	0.53	0.96	0.84	0.31	0.09
32,001 to 512,000	6.36	10.9	3.60	2.80	3.64	1.79	3.00	1.48	3.185	0.18	0.61	0.25	0.87	0.77	0.87	0.58
Average size of insulin-containing cell clusters, (μm^2)	7032	10946	6001	5160	5992	5409	6583	4109	2493.5	0.54	0.80	0.21	0.82	0.72	0.98	0.26
Smallest insulin- containing cell clusters, (μm^2)	251	273	248	251	248	254	254	252	8.2	0.60	0.52	0.04	0.86	0.67	0.83	0.70
Largest insulin-containing cell clusters, (μm^2)	57820	87886	111610	50122	58091	39301	54520	58200	43889	0.46	0.09	0.58	0.09	0.84	0.64	0.92

gestation as well as dietary treatment on d 85 or d 140 did not influence the proportion of medium insulin-containing cell clusters. There was a tendency for medium insulin-containing cell clusters during late-gestation (d 254; $P = 0.09$) to be greater in cows undergoing realimentation at d 85 vs d 140 (RCC vs RRC). Proportion of large insulin-containing cell clusters and the average size of insulin-containing cell clusters were not influenced by advancing gestation or dietary treatment. Advancing gestation did not influence the size of the smallest insulin-containing cell cluster. Cows offered an adequate diet had the smallest insulin-containing cell clusters ($P = 0.04$) when compared to restricted animals on d 85 with no influences on d 140 and d 254. There was a trend for a quadratic effect ($P = 0.09$) for the size of the largest insulin-containing cell cluster with the largest cluster observed on d 140, no treatment effects were observed on d 85, there was a trend ($P = 0.09$) for the CC treatment to have the largest clusters compared with RC and RR cows on d 140 and no effects were observed on d 254.

4.4.3. Fetal Enzyme Data

There was a quadratic effect ($P < 0.001$) with advancing gestation for fetal BW (kg) and pancreas weight (g) as weights increased to a greater extent from mid- to late- than from early- to mid-gestation. Fetal BW (kg) and pancreas weight (g) were not influenced by dietary treatment (**Table 4-3**).

Relative pancreas weight (g/kg BW) was not influenced by dietary treatment. Although the pancreas at d 85 was too small for enzyme analysis, a comparison of controls at d 140 and 254 was performed to evaluate differences in relation to gestational stage. Pancreatic protein concentration (mg/g tissue) was not influenced by advancing gestation or by dietary treatment on d 140, but realimented cows (RCC and RRC) had greater ($P = 0.001$) protein concentration than

Table 4-3. Influence of realimentation after nutrient restriction during mid- to late gestation on fetal pancreatic digestive enzymes

	Stage of Gestation									*P*-value						
	Early (Day 85)		Mid (Day 140)			Late (Day 254)										
Item	CON	R	CC	RC	RR	CCC	RCC	RRC	SEM	L or Mid- vs Late[†]	Q	CON vs R	CC vs RC and RR	RC vs RR	CCC vs RCC and RRC	RCC vs RRC
BW, kg	0.117	0.204	2.00	2.16	2.09	34.5	35.1	37.1	1.121	<0.001	<0.001	0.95	0.92	0.96	0.22	0.19
Pancreas weight, g	0.105	0.524	1.38	1.90	1.26	24.1	23.6	25.4	0.9499	<0.001	<0.001	0.73	0.85	0.62	0.71	0.18
Pancreas g/kg of BW	0.901	1.07	0.756	0.837	0.737	0.706	0.659	0.690	0.0825	0.52	0.98	0.12	0.74	0.38	0.73	0.78
Protein																
mg/g tissue	-	-	81.6	82.8	81.8	84.3	129	127	10.10	0.84	-	-	0.95	0.94	0.001	0.93
Total g/pancreas	-	-	0.096	0.169	0.071	2.04	3.05	3.28	0.3195	<0.001	-	-	0.95	0.82	0.005	0.61
mg/kg of BW	-	-	0.062	0.070	0.061	0.059	0.087	0.089	0.0104	0.81	-	-	0.76	0.53	0.02	0.89
α- Amylase																
U/g	-	-	0.676	0.730	0.923	1.16	5.01	6.65	1.1981	0.75	-	-	0.91	0.91	0.002	0.31
kU/pancreas	-	-	0.003	0.000	0.005	0.029	0.120	0.168	0.0301	0.51	-	-	1.00	0.90	0.002	0.24
U/kg of BW	-	-	0.436	0.708	0.647	0.825	3.21	4.45	0.7113	0.67	-	-	0.76	0.95	0.001	0.20
U/g protein	-	-	7.99	9.03	10.4	14.5	37.6	53.9	9.104	0.58	-	-	0.86	0.91	0.005	0.19
Trypsin																
U/g	-	-	0.120	0.170	0.143	0.564	0.669	0.625	0.0729	<0.001	-	-	0.65	0.79	0.32	0.65
U/pancreas	-	-	-0.220	0.508	-0.567	14.2	15.8	15.8	2.0009	<0.001	-	-	0.93	0.70	0.49	0.99
U/kg of BW	-	-	0.085	0.127	0.100	0.401	0.440	0.424	0.0478	<0.001	-	-	0.60	0.68	0.80	0.80
U/g protein	-	-	1.32	2.25	1.49	8.23	4.83	4.97	1.121	<0.001	-	-	0.67	0.63	0.02	0.93

[1]CON or C = control, fed at 100% of NRC (2000) recommendations, R = restricted, fed at 60% of NRC (2000) recommendations starting at d 30 and continuing until d 85 (R) or 140 (RR). n = 6, 6 6, 5, 6, 6, 5, 6 for CON, R, CC, RC, RR, CCC, RCC, and RRC treatments, respectively. L = linear effects of stage of gestation with CON, CC, and CCC treatments; Q = quadratic effects of stage of gestation with CON, CC, and CCC treatments; CON vs R; CC vs CR and RR; RC vs RR; CCC vs RCC and RRC; RCC vs RRC = contrast statements within slaughter days (d 85, d 140, or d 254 of gestation).

[*]The fetal pancreas at d 85 was too small for enzyme analysis and therefore was excluded from the data set as indicated by a dashed line (-). Linear contrast for BW, pancreas weight (g) and pancreas weight (g/kg BW). CC vs CCC for all other variables.

CCC cows at d 254. Total protein content (g/pancreas) increased ($P < 0.001$) from d 140 to d 254, was not influenced by treatment on d 140, and was greater ($P = 0.005$) in realimented groups (RCC and RRC) compared to CCC at d 254.

Gestational stage did not influence concentration and content of α-amylase activity (U/g; kU/pancreas, U/kg BW, and U/g protein), nor did dietary treatment on d 140. However, realimentation increased ($P = 0.02$) α-amylase activity as compared to controls (RCC and RRC vs. CCC).

As gestation advanced from d 140 to d 254, trypsin activity (U/g, U/pancreas, U/kg of BW, and U/g protein) increased. Trypsin specific activity (U/g protein) was not affected by dietary treatment on d 140 but on d 254 trypsin activity (U/g, U/pancreas, U/kg of BW, and U/g protein) was greater ($P = 0.02$) in CCC vs RCC and RRC cows.

4.4.4. Fetal Endocrine and Histological Analysis

Serum insulin (μIU/mL) concentration in the uterine umbilical cord decreased ($P < 0.001$) from d 140 to 254 but was not influenced by dietary treatment. Serum glucose (mg/dL) decreased from d 140 to 254 ($P = 0.03$) but was not influenced by dietary treatment. Pancreatic insulin-positive tissue area (relative to section of tissue) linearly increased ($P = 0.03$) as gestation progressed (**Table 4-4**), was not affected by treatment on d 85, demonstrated a trend ($P = 0.07$) for fetuses from RR cows to have reduced insulin-positive tissue area compared to those realimented at day 85 (RC) on d 140 and was not influenced by treatment on d 254.

The percent of small insulin-containing cell clusters was not influenced by gestational stage or dietary treatment. The number of medium clusters (2,001 to 32,000 μm^2) responded quadratically ($P = 0.02$) with percentage of cell clusters increasing to a greater extent between d 85 to 140 than d 140 to 254. Dietary treatment did not influence number of medium clusters. The

Table 4-4. Influence of realimentation after nutrient restriction during mid- to late gestation on fetal serum and pancreatic insulin concentration and measurements of insulin-containing cell clusters

	Stage of Gestation									*P*-value						
	Early (Day 85)		Mid (Day 140)			Late (Day 254)										
Item	CON	R	CC	RC	RR	CCC	RCC	RRC	SEM	L	Q	CON vs R	CC vs RC and RR	RC vs RR	CCC vs RCC and RRC	RCC vs RRC
Insulin, µIU/mL	-	-	77.5	72.9	78.0	19.4	19.0	18.1	6.01	<0.001	-	-	0.78	0.54	0.91	0.91
Glucose, mg/dL	-	-	91.0	101	105	26.2	28.4	43.3	21.48	0.03	-	-	0.63	0.88	0.70	0.62
Pancreatic insulin-positive tissue area (relative to section of tissue), %	3.19	5.05	10.1	17.1	10.8	12.1	10.7	8.15	3.629	0.03	0.16	0.68	0.16	0.07	0.38	0.40
Insulin-containing cell clusters within the following ranges (µm^2), %																
1 to 2,000	73.8	88.5	69.6	62.3	71.4	59.9	61.1	60.4	9.91	0.16	0.97	0.25	0.71	0.33	0.92	0.94
2,001 to 32,000	10.2	9.84	30.7	36.1	28.9	38.3	38.7	38.3	5.828	<0.001	0.02	0.96	0.67	0.19	0.97	0.94
32,001 to 512,000	0.456	0.315	0.618	0.945	0.539	1.17	0.997	1.21	0.378	0.06	0.81	0.77	0.65	0.26	0.85	0.50
> 512,001	0.031	-0.062	0.052	-0.031	0.059	0.266	0.210	0.180	0.1217	0.05	0.55	0.55	0.67	0.44	0.48	0.77
Average size of insulin-containing cell clusters, (µm^2)	1859	215	3440	2857	3205	8867	6786	6817	1716	0.01	0.71	0.60	0.82	0.88	0.32	0.99
Smallest insulin-containing cell clusters, (µm^2)	251	255	246	245	249	245	250	246	4.7	0.30	0.43	0.53	0.73	0.36	0.48	0.24
Largest insulin-containing cell clusters, (µm^2)	100022	902280	522837	282972	526970	2260855	1752921	1427699	77904.5	0.01	0.64	0.90	0.84	0.74	0.31	0.62

[a]CON or C = control, fed at 100% of NRC (2000) recommendations, R = restricted, fed at 60% of NRC (2000) recommendations starting at d 30 and continuing until d 85 (R) or 140 (RR). n = 6, 6 6, 5, 6, 6, 5, 6 for CON, R, CC, RC, RR, CCC, RCC, and RRC treatments, respectively. L = linear effects of stage of gestation with CON, CC, and CCC treatments; Q = quadratic effects of stage of gestation with CON, CC, and CCC treatments; CON vs R; CC vs CR and RR; RC vs RR; CCC vs RCC and RRC; RCC vs RRC = contrast statements within slaughter days (d 85, d 140, or d 254 of gestation).

percent of large clusters (32,001 to 512,000 μm^2) tended to increase (P = 0.06) as gestation progressed and was not influenced by dietary treatment. The percentage of giant clusters (> 512,001 μm^2) increased (P = 0.05) with advancing gestation but was not influenced by dietary treatment. The average size of insulin-containing cell clusters increased (P = 0.01) with advancing gestation and was not influenced by dietary treatment. The size of the smallest cluster did not differ with advancing gestation and between dietary treatments. The size of the largest cluster increased (P = 0.01) as gestation progressed and was not influenced by dietary treatment.

4.4.5. Comparison of Maternal and Fetal Enzyme Data

The relative pancreatic weight (g/kg of BW) in the cows and fetuses was not influenced by treatment (**Table 4-5**). The fetal pancreas had a greater (P < 0.001) percent of pancreatic insulin-positive tissue area (relative to section of tissue; **Table 4-6**) than maternal pancreas.

Fetal pancreatic tissue had a larger percent (P < 0.001) of small insulin-containing cell clusters than maternal tissue. A greater percent of medium insulin-containing cell clusters (P < 0.001) and large insulin-containing cell clusters (P < 0.001) were observed in maternal tissues compared with fetal tissues. A greater percent of giant insulin-containing cell clusters were observed in fetal pancreatic tissue (P = 0.002). The average size of the insulin-containing cell clusters was greater (P = 0.05) in the maternal compared with the fetal pancreas while the smallest clusters were not different among maternal and fetal tissues. The largest clusters (P < 0.001) were observed in fetal tissue as compared to maternal tissue.

Interactions for physiological stage and treatment effects were observed for trypsin activity (U/g, U/pancreas, U/kg of BW, U/g protein), insulin concentration (μIU/mL) and glucose concentration (mg/dL). Interactions were also observed for the pancreatic insulin-

Table 4-5. Comparison of maternal vs fetal pancreatic digestive enzymes during mid- to late gestation

	Physiological Stage			
Item	Maternal	Fetal	SEM	*P*-Value
BW, kg[a]	590	18.8	7.64	<0.001
Pancreas weight, g[a]	438	13.0	10.85	<0.001
Pancreas g/kg of BW	0.742	0.738	0.0240	0.92
Protein				
mg/g tissue[b]	123	97.7	3.84	<0.001
Total g/pancreas	54.2	1.47	1.942	<0.001
mg/kg of BW	0.092	0.072	0.0039	<0.001
α- Amylase				
U/g	119	2.44	10.034	<0.001
kU/pancreas	52.4	0.053	4.4205	<0.001
U/kg of BW	89.6	1.67	7.774	<0.001
U/g protein	946	21.4	65.81	<0.001
Trypsin				
U/g[b]	0.896	0.386	0.0567	<0.001
U/pancreas[b]	388	7.80	22.939	<0.001
U/kg of BW[b]	0.660	0.269	0.0411	<0.001
U/g protein[b]	7.44	3.98	0.553	<0.001

[*]Data are presented as least square means ± SEM, n = 48.
[a]Treatment ($p \leq 0.05$).
[b]Physiological stage × Treatment ($p \leq 0.05$).

positive area (relative to section of tissue) percentage, the average size of insulin-containing cell clusters (μm^2), and the largest insulin-containing cell cluster (μm^2). The observed interactions however, were primarily because of differences in magnitude of response to treatment between maternal and fetal tissues and therefore will not be discussed further.

4.5. Discussion

4.5.1. Maternal Enzyme Data

The increase in pancreatic weight (g and g/kg BW) in cows realimented for a longer period (RCC vs RRC) may be due to the increased amount of time that the realimented cows were exposed to greater intake resulting in compensatory growth. Prolonged restriction in DM intake and subsequent metabolizable energy, in addition to the timing of gestation, can have

Table 4-6. Comparison of fetal vs maternal serum and pancreatic insulin concentration and measurements of insulin-containing cell clusters

	Physiological Stage			*P* - Value
Item	Maternal	Fetal	SEM	
Insulin, μIU/mL[a,b]	11.01	48.3	1.854	<0.001
Glucose, mg/dL[a,b]	89.9	63.9	5.92	0.003
Pancreatic insulin-positive tissue area (relative to section of tissue), %[a,b]	1.06	9.52	0.574	<0.001
Insulin-containing cell clusters within the following ranges (μm^2), %				
1 to 2,000[a]	55.5	68.5	2.38	<0.001
2,001 to 32,000[a]	40.8	28.7	1.75	<0.001
32,001 to 512,000	3.27	0.76	0.577	<0.001
> 512,001	0.000	0.08	0.020	0.002
Average size of insulin-containing cell clusters, (μm^2) [b]	5770	4162	593.3	0.05
Smallest insulin-containing cell clusters, (μm^2)	252	248	1.6	0.12
Largest insulin-containing cell clusters, (μm^2) [a,b]	60513	848290	119740	<0.001

[*]Data are presented as least square means ± SEM, n = 48.
[a]Treatment ($p \leq 0.05$).
[b]Physiological stage × Treatment ($p \leq 0.05$).

significant effects on tissue growth and development impacting the organ's overall mass (Ferrell et al., 1986).

While little is known about the effects of pregnancy on pancreatic digestive enzymes in mature cattle, a linear decrease was observed in α-amylase activity with advancing gestation. Mizoguchi and Imamichi (1986) found that during the progression of pregnancy, pancreatic α-amylase in mice decreased to levels lower than that of the non-pregnant controls. They believed the change in activity to be related to hormones such as adrenaline, glucagon, thyroxine and cortisol. The cows in the current study may also have been exposed to elevated levels of adrenaline and cortisol as it is possible that they experienced greater stress through feed restriction and the additional strain of pregnancy. Croom et al. (1992) also reported that the

nervous system plays a major role in the control of pancreatic exocrine secretion and states that stimulation of the vagus nerve causes enhanced secretion of α-amylase. Because the vagus nerve interfaces with the parasympathetic nervous system it seems logical that, as adrenaline or cortisol block this interface, α-amylase activity would be reduced.

Although no differences in trypsin activity were observed due to nutrient restriction in the current study, several trends during late gestation indicate that previously nutrient restricted cows had a reduced capacity for trypsin production compared to that of their control treatment counterparts. Examination of realimentation strategies also indicate an impact on trypsin at d 254 with animals enduring prolonged restriction exhibiting an even greater reduction than those realimented early in gestation. Animals experiencing prolonged nutrient restriction are often more likely to exhibit signs of reduced capacity of digestive enzymes despite realimentation (Huquet et al., 2006).

4.5.2. Maternal Endocrine and Histological Data

A study conducted by Reynolds et al. (1990) tested the effects of day of gestation on maternal plasma insulin and glucose concentration and found no effect of day of gestation. Their findings do not agree with the results of this trial, as cows on the present study exhibited an increase in serum insulin concentration (μIU/mL) as gestation progressed. Several reasons may exist for the results observed here. The first possible cause might be related to the insulin resistance that frequently accommodates advancing gestation (Salin, et al., 2012). As a result of insulin resistance, the body's normal cellular response to the hormone is reduced and the pancreas of these animals often begins producing more insulin as a way of compensation. Another potential cause may be the increased amount of metabolizable protein provided to the cows through realimentation because of increased dietary feed intake. A study done by

Sletmoen-Olson et al. (2000b) evaluated the impact of three different levels of undegradable intake protein (low, medium and high) resulting in increased supply of metabolizable protein provided during late gestation (months 7, 8 and 9). The researchers found that the plasma insulin levels increased when levels of UIP offered increased. They also found that insulin levels escalated during months 8 and 9 corresponding to late gestation. Along with the cows being realimented in the current trial, the amount of feed offered also increased to ensure the animals were provided with enough feed to meet NRC recommendations. These increases in insulin concentration may have been caused by the greater levels of amino acids found in the supplements (Sletmoen-Olson et al., 2000a) and the ability of these amino acids to stimulate insulin production.

A quadratic effect in pancreatic insulin concentration with advancing gestation was noted with CC cows having the greatest values for the largest insulin-containing cell clusters at d 140 which is similar to results from multiple studies that report a substantial increase in serum and plasma insulin levels during mid-gestation of ewes which then seem to decrease as pregnancy progresses to final term (Blom et al., 1976; Vernon et al., 1981). While the differing realimentation strategies did not influence endocrine measurements in the maternal pancreas, trends were observed on d 254 with cows exposed to early realimentation having a greater percentage of medium insulin-containing cell clusters when compared against those remaining restricted until d 140. Perhaps this observation can be explained by compensatory gain as the pancreas of animals subjected to earlier realimentation would likely be working to secrete sufficient levels of insulin into the body's blood stream to offset the loss caused by previous nutrient restriction. Therefore, the pancreas could have developed a greater number of medium

insulin-containing cell clusters capable of secreting larger quantities of insulin and compensating for prior dietary insults.

4.5.3. Fetal Enzyme Data

As expected, a gain in fetal weight was observed as pregnancy progressed with the majority of fetal growth and development occurring during the last third of gestation (Robinson et al., 1977) as indicated by the quadratic effect with advancing gestation. The lack of an effect of dietary treatments on fetal BW (g) or pancreas weight (g or g/kg BW) agree with the findings of Long et al. (2010) who found no differences in BW or pancreas weight of fetuses from nutrient restricted dams regardless of realimentation strategy. Pancreas weight (g), protein concentration (total g/pancreas) and trypsin activity (U/g, U/pancreas, U/kg of BW, and U/g protein) increased from d 140 to d 254. In agreement with our findings, increases in bovine fetal pancreatic digestive enzymes were also observed as gestation progressed in a study conducted by Track et al. (1972) who noted the rising levels of trypsin activity continued until the calves had reached 12 weeks post-partum. Unfortunately, no postpartum measurements could be collected during this study.

At d 254, a clear impact of realimentation can be observed when evaluating pancreatic enzyme activity. The RCC and RRC treatments had greater protein concentration and content (mg/g, g/pancreas and g/kg BW), and α-amylase activity (U/g, kU/pancreas, U/kg of BW, U/g protein) indicating that the pancreas may have been subjected to compensatory development with an overshot in compensation. Trypsin activity (U/g protein) however, was greater in the CON treatment. This suggests a potential programming effect of early- to mid-gestation nutrient restriction on fetal pancreatic development of the exocrine pancreas.

4.5.4. Fetal Endocrine and Histological Data

In the current study, both insulin (μIU/mL) and glucose (mg/dL) concentration decreased from d 140 to d 254. While the majority of fetal growth occurs in the last trimester, Aldoretta et al. (1998) reported that the fetal pancreas of ovine species develops in the late first to early second trimester. This allows for the production of measurable insulin concentrations by mid-gestation. Additionally, studies in cattle have shown that serum insulin levels increase from 90 to 150 days of gestation and then begin to decline as pregnancy continues (Padodar et al., 2014). D'Agostino et al. (1985) also noted a decrease in pancreatic insulin concentrations of the fetal bovine between the mid-second and third trimester. This reduction may be the result of the pancreas compensating for its rapidly developing exocrine function (Frazier et al., 1981).

Progression of gestation resulted in an increase in fetal pancreatic insulin-positive tissue area (relative to section of tissue), along with percent of medium, large, and giant insulin-containing cell clusters. An increase was also found in the average size of clusters and in the size of the largest clusters found within each area of pancreatic tissue analyzed. Although we observed linear increases there was a numerically greater increase from early- to mid-gestation than from mid- to late-gestation which agrees with the plasma insulin data.

At d 140 the percentage of insulin-positive tissue area (relative to section of tissue) tended to decrease in fetuses from cows subjected to prolonged nutrient restriction. Dietary treatment had no impact on the size or distribution of insulin-containing cell clusters. Results from studies conducted in sheep by Limesand et al. (2006) and Fowden and Hill (2001) reported pancreatic islets from nutritionally deprived fetuses had reduced mass and insulin secretion. A study by Gonzalez et al. (2013) investigating fetal muscle fiber reported that realimentation after nutrient restriction until d 140 was able to support compensatory growth and return fiber size and

muscle progenitor numbers to those of control fetuses in bovine animals by d 254. The conclusions drawn by the authors may explain the lack of differences in fetal tissue at d 254. While the theory behind compensatory growth is well documented, the results found in this study appear to be more likely due to the maternal ability to maintain fetal pancreatic endocrine development as well as maintain their own metabolic needs in the face of nutrient restriction.

4.5.5. Comparison of Maternal and Fetal Enzyme Data

Cows in this study had greater digestive enzyme activity than fetuses. The immaturity of the fetal exocrine pancreatic function is expected as fetuses receive their nutrition from the dams and, therefore, are not required to directly digest any feed components (Snoeck et al., 1990).

4.5.6. Comparison of Maternal and Fetal Endocrine and Histological Data

The concentration of insulin in umbilical serum was greater than that in the maternal jugular vein. The level of glucose in the umbilical cord, however, was lower than that in maternal circulation. This is not surprising as glucose is transported across the placenta resulting in a constant supply from the dam, and, further, fetal gluconeogenesis is limited (Porter, 2012).

Similar to a recent study in sheep, the insulin-positive tissue area (relative to section of tissue) was greater in fetuses than dams (Keomanivong et al., 2015). A greater percentage of small and giant insulin-containing cell clusters along with the largest clusters measured were found in fetal pancreatic tissue. Maternal tissue had a greater percentage of medium and large insulin-containing cell clusters. The differences found between fetal and maternal pancreatic insulin-positive tissue area (relative to section of tissue) and the size distribution of insulin-containing cell clusters indicates a dramatic shift in endocrine functionality as the fetus develops. Merkwitz et al. (2012) also examined the morphology of the fetal and cow pancreas and found islets of two differing sizes. The percent of smaller islets (interlobular islets) was greater in fetal

tissue. Larger islets (perilobular giant islets) were also present although they appeared less frequently. Islets of intermediate size were shown to persist into adulthood while perilobular giant islets underwent regression. Results from the current study support these findings as demonstrated in **Figure 4-2**. It is also not surprising that the average size of maternal insulin containing cell clusters would be greater than that found in fetal tissue. The increased number of small clusters along with the limited giant clusters found in fetal tissue in contrast to the greater percentages of medium and large clusters found in maternal tissue could therefore reasonably result in a larger average size of insulin-containing cell clusters in maternal tissue. More work is needed in this area, however, to determine the timing of regression and the impact on pancreatic function as the calf matures.

4.6. Conclusion

Our results suggest that maternal digestive enzymes are influenced by nutrient restriction and that the fetal pancreas undergoes significant shifts of escalation and regression in endocrine and exocrine function due to stage of gestation and maternal nutrient restriction. Realimentation was able to reverse the impact of restriction and, in some cases, increase the concentration of fetal pancreatic protein and enzyme activity as compared to the controls. It is not known if these differences would persist after parturition and into adulthood. Feeding strategies during mid- to late gestation can alter pancreatic physiology of the cow and fetal pancreatic development.

4.7. Literature Cited

Aldoretta, P. W., T. D. Carver, and W. W. Hay -Jr. 1998. Maturation of glucose-stimulated insulin secretion in fetal sheep. Biol. Neonate. 73:375-386.

Barker, D. J. and P. M. Clark. 1997. Fetal undernutrition and disease in later life. Rev. Reprod. 2:105-112.

Bertram, C. E., and M. A. Hanson. 2001. Animal models and programming of the metabolic syndrome. Brit. Med. Bull. 60:103-121.

Blom, A. K., K. Hove, and J. J. Nedkvitne. 1976. Plasma insulin and growth hormone concentrations in pregnant sheep I: diurnal variations in mid- and late pregnancy. Acta Endocrinologica. 82:553-560.

Camacho, L. E., C. O. Lemley, M. L. Van Emon, J. S. Caton, K. C. Swanson, and K. A. Vonnahme. 2014. Effects of maternal nutrient restriction followed by realimentation during early and mid-gestation on beef cows. I. Maternal performance and organ weights at different stages of gestation. J. Anim. Sci. 92:520-529.

Carlsson, G. L., R. S. Heller, P. Serup, and P. Hyttel. 2010. Immunohistochemistry of pancreatic development in cattle and pig. Anatomia. Histologia. Embryologia. 39:107-119.

Croom, W. J. -Jr., L. S. Bull, and I. L. Taylor. 1992. Regulation of pancreatic exocrine secretion in ruminants: a review. J. Nutr. 122.1:191-202.

D'Agostino, J., J. B. Field, and M. L. Frazier. 1985. Ontogeny of immunoreactive insulin in the fetal bovine pancreas. Endocrinol. 116:1108-1116.

Dahri, S., B. Reusen, C. Remacle, and J. J. Hoet. 1995. Nutritional influences on pancreatic development and potential links with non-insulin-dependent diabetes. Proc. Nutr. Soc. 54:345-356.

Farrance, I. 1987. Plasma glucose methods, a review. Clin. Biochem. Rev. 8:55–68.

Ferrell, C. L., L. J. Koong, and J. A. L. Nienaber. 1986. Effects of previous nutrition on body composition and maintenance energy costs of growing lambs. Brit. J. Nutr. 56:595-605.

Foote, W. C., A. L. Pope, C. R. Earl, A. B. Chapman, and L. E. Casida. 1958. Reproduction in the yearling ewe as affected by breed and sequence of feeding levels: II. Effects on fetal development. J. Anim. Sci. 18:463–474.

Fowden, A. L., and D.J. Hill. 2001. Intra-uterine programming of the endocrine pancreas. Brit. Med. Bull. 60:123-142.

Fowden, A. L., P. Hughes, and R. S. Comline. 1989. The effects of insulin on the growth rate of the sheep fetus during late gestation. Q. J. Exp. Physiol. 74:703-714.

Frazier, M. L., R. A. Montagna, and G. F. Saunders. 1981. Insulin gene expression during development of the fetal bovine pancreas. Biochem. 20:367-371.

Freetly, H. C., C. L. Ferrell, and T. G. Jenkins. 2005. Nutritionally altering weight gain patterns of pregnant heifers and young cows changes the time that feed resources are offered without any differences in production. J. Anim. Sci. 83:916-1026.

Freetly, H. C., J. A. Nienaber, and T. Brown-Brandl. 2008. Partitioning of energy in pregnant beef cows during nutritionally induced body weight fluctuation. J. Anim. Sci. 86:370-377.

Funston, R. N., D. M. Larson, and K. A. Vonnahme. 2010. Effects of maternal nutrition on conceptus growth and offspring performance: implications for beef cattle production. J. Anim. Sci. 88:E205-E215.

Glazer, G., and M. L. Steer. 1977. Requirements for activation of trypsinogen and chymotrypsinogen in rabbit pancreatic juice. Anal. Biochem. 77:130–140.

Gonzalez, J. M., L. E. Camacho, S. M. Ebarb, K. C. Swanson, K. A. Vonnahme, A. M. Stelzleni, and S. E. Johnson. 2013. Realimentation of nutrient restricted pregnant beef cows supports compensatory fetal muscle growth. J. Anim. Sci. 91:4797-4806.

Grazul-Bilska, A. T., J. S. Caton, W. Arndt, K. Burchill, C. Thorson, E. Borowczyk, J. J. Bilski, D. A. Redmer, L. P. Reynolds, and K. A. Vonnahme. 2009. Cellular proliferation in ovine fetal ovaries: Effects of energy restrictions and selenium in maternal diet. Reproduction. 137:699–707.

Grazul-Bilska, A. T., E. Borowczyk, J. J. Bilski, L. P. Reynolds, D. A. Redmer, J. S. Caton, and K. A. Vonnahme. 2012. Overfeeding and underfeeding have detrimental effects on oocyte quality measured by in vitro fertilization and early embryonic development in sheep. Dom. Anim. Endocrinol. 43:289–298.

Huguet, A., G. Savary, E. Bobillier, Y. Lebreton, and I. Le Huërou-Luron. 2006. Effects of level of feed intake on pancreatic exocrine secretions during the early post weaning period in piglets. J. Anim. Sci. 84:2965-2972.

Keomanivong, F. E., C. O. Lemley, L. E. Camacho, R. Yunusova, P. P. Borowicz, J. S. Caton, A. M. Meyer, K. A. Vonnahme, and K. C. Swanson. 2015. Influence of nutrient restriction and melatonin supplementation of pregnant ewes on maternal and fetal pancreatic digestive enzymes, islet morphology and plasma insulin concentration. Animal. 9:1-9.

Limesand, S. W., J. Jensen, J. C. Hutton, and W. W. Hay. 2005. Diminished β-cell replication contributes to reduced β-cell mass in fetal sheep with intrauterine growth restriction. Am. J. Physiol. 288:R1297-R1305.

Limesand, S. W., P. J. Rozance, G. O. Zerbe, J. C. Hutton, and W. W. Hay. 2006. Attenuated insulin release and storage in fetal sheep pancreatic islets with intrauterine growth restriction. Endocrinology. 147:1488-1497.

Long, N. M., M. J. Prado-Cooper, C. R. Krehbiel, U. DeSilva, R. P. Wetteman. 2010. Effects of nutrient restriction of bovine dams during early gestation on postnatal growth, carcass and organ characteristics, and gene expression in adipose tissue and muscle. J. Anim. Sci. 88:3251-3261.

Mader, C. J., Y. R. Montanholi, Y. L. Wang, S. P. Miller, I. B. Mandell, B. W. McBride, and K. C. Swanson. 2009. Relationships among measures of growth performance and efficiency with carcass traits, visceral organ mass, and pancreatic digestive enzymes in feedlot cattle. J. Anim. Sci. 87:1548-1557.

McMullen, S., J. C. Osgerby, J. S. Milne, J. M. Wallace, and D. C. Wathes. 2005. The effects of acute nutrient restriction in the mid-gestational ewe on maternal and fetal nutrient status, the expression of placental growth factors and fetal growth. Placenta. 26:25–33.

Merkwitz, C., P. Lochhead, J. Böttger, M. Matz-Soja, M. Sakurai, R. Gebhardt, and A. M. Ricken. 2012. Dual origin, development, and fate of bovine pancreatic islets. J. Anat. 222:358-71.

Mizoguchi, J., and T. Imamichi. 1986. Decrease in amylase (EC 3.4.21.4) synthesis in lactating rats. Brit. J. Nutr. 57:291-299.

Molle, J. D. C., E. J. Scholljegerdes, S. L. Lake, V. Nayigihugu, R. L. Atkinson, L. R. Miller, S. P. Ford, W. J. Means, J. S. Caton, and B. W. Hess. 2004. Effects of maternal nutrient restriction during early to mid-gestation on cow and fetal visceral organ measurements. Proc., WSASAS. 55:405–409.

Meyer, A. L., J. J. Reed, K. A. Vonnahme, S. A. Soto-Navarro, L. P. Reynolds, S. P. Ford, B. W. Hess, and J. S. Caton. 2010. Effects of stage of gestation and nutrient restriction during

early to mid-gestation on maternal and fetal visceral organ mass and indices of jejunal growth and vascularity in beef cows. J. Anim. Sci. 88:2410-2424.

National Research Council. 2000: Nutritional Requirements of Beef Cattle. 7th rev. ed. Natl. Acad. Press, Washington, D.C.

Padodar, R. J., J. S. Arya, and J. Ninan. 2014. Assessment of blood glucose and serum insulin profiles at different stages of gestation in triple cross-bred cattle. Indian J. Anim. Res. 48:94-96.

Porter, T. E. 2012. Development and function of the fetal endocrine system. Endocrinology of Pregnancy. 13:387-406.

Reed, J. J., M. A. Ward, K. A. Vonnahme, T. L. Neville, S. L. Julius, P. P. Borowicz, J. B. Taylor, D. A. Redmer, A. T. Grazul-Bilska, L.P. Reynolds, and J. S. Caton. 2007. Effects of selenium supply and dietary restriction on maternal and fetal body weight, visceral organ mass and cellularity estimates, and jejunal vascularity in pregnant ewe lambs. J. Anim. Sci. 85: 721-2733.

Reynolds, L. P., P. P. Borowicz, J. S. Caton, K. A. Vonnahme, J. S. Luther, C. J. Hammer, K. R. Maddock Carlin, A. T. Grazul-Bilska, and D. A. Redmer. 2010. Developmental programming: The concept, large animal models, and the key role of uteroplacental vascular development. J. Anim. Sci. 88:E61-72

Reynolds, L. P., C. L. Ferrell, D. A. Robertson, and J. Klindt. 1990. Growth hormone, insulin and glucose concentrations in bovine fetal and maternal plasma at several stages of gestation. J. Anim. Sci. 68:725-733.

Robinson, J. S., C. K. Kind, F. Lok, P. Owens, and J. Owen. 1995. Placental control of fetal growth. Reprod. Fert. Dev. 7:333–344.

Robinson, J. J., I. McDonald, C. Fraser, and I. McHattie. 1977. Studies on reproduction in prolific ewes. I. Growth of the products of conception. J. Agric. Sci. Cambridge. 88:539-552.

Salin, S., J. Taponen, K. Elo, I. Simpura, A. Vanhatalo, R. Boston, and T. Kokkonen. 2012. Effects of abomasal infusion of tallow or camelina oil on responses to glucose and insulin in dairy cows during late pregnancy. J. Dairy Sci. 95:3812-5206.

Sano, H., A. Takebeyashi, Y. Kodama, H. Nakamura, H, Ito, Y, Arino, T. Fujita, H. Takahashi, and K. Ambo. 1999. Effect of feed restriction and cold exposure on glucose metabolism in response to feeding and insulin in sheep. J. Anim. Sci. 77:2564-2573.

Schoonmaker, J. 2013. Effect of maternal nutrition on calf health and growth. Tri-State Dairy Nutr. Conf. 63-80

Sletmoen-Olson, K. A., J. S. Caton, K. C. Olson, D. A. Redmer, J. D. Kirsch, and L. P. Reynolds. 2000a. Undergraded intake protein supplementation: II. Effects on plasma hormone and metabolite concentrations in periparturient beef cows fed low-quality hay during gestation and lactation. J. Anim. Sci. 78:456-463.

Sletmoen-Olson, K. A., J. S. Caton, K. C. Olson, and L. P. Reynolds. 2000b. Undergraded intake protein supplementation. I. Effects on forage utilization and performance of periparturient beef cows fed low quality hay. J. Anim. Sci. 78:449-455.

Snoeck, A., C. Remacle, B. Reusens, and J. J. Hoet. 1990. Effects of low protein diet during pregnancy on the fetal rat endocrine pancreas. Biol. Neonate. 57:107–118.

Swanson, K. C., J. A. Benson, J. C. Matthews, and D. L. Harmon. 2004. Pancreatic exocrine secretion and plasma concentration of some gastrointestinal hormones in response to abomasal infusion of starch hydrolyzate and/or casein. J. Anim. Sci. 82:1781-1787.

Swanson, K. C., and D. L. Harmon. 2002a. Dietary influences on pancreatic α-amylase expression and secretion in ruminants. Biology of the Intestine in Growing Animals. R. Zabielski, V. Lesniewska, P.C. Gregory, and B. Westrom, ed. 515–537, Elsevier, Boston, MA.

Swanson, K. C., J. C. Matthews, C. A. Woods, and D. L. Harmon. 2002b. Postruminal administration of partially hydrolyzed starch and casein influences pancreatic alpha-amylase expression in calves. J. Nutr. 132:376-381.

Swanson, K. C., N. Kelly, H. Salim, Y. J. Wang, S. Holligan, M. Z. Fan, and B. W. McBride. 2014. Pancreatic mass, cellularity, and α-amylase and trypsin activity in feedlot steers fed diets differing in crude protein concentration. J. Anim. Sci. 86:909–915.

Track, N. S., M. Bokermann, C. Creutzfeldt, H, Schmidt, and W. Creutzfeldt. 1972. Enzymatic and ultrastructural development of the bovine exocrine pancreas. Comp. Biochem. Physiol. 43B:313-332.

Vernon, R. G., R. A. Clegg, and D. J. Flint. 1981: Metabolism of sheep adipose tissue during pregnancy and lactation adaptation and regulation. Biochem. J. 200:307-314.

Weinkove, C., E. A. Weinkove, and B. L. Pimstone. 1974. Microassays for glucose and insulin. South African Med. J. 48:365–368.

Winick, M., and A. Noble. 1996. Cellular response in rats during malnutrition at various ages. J. Nutr. 89:300–306.

Zhang, L., 2010. Fetal pancreatic beta-cell development and insulin secretion in ovine maternal over-nutrition and under-nutrition models. Univ. of Wyoming. Laramie, WY. Doctoral Thesis.

Zubair, A. K., and S. Leeson. 1994.. Effect of early feed restriction and realimentation on heat production and changes in sizes of digestive organs of male broilers. Poult, Sci. 73:529-538.

CHAPTER 5. INFLUENCE OF DRY-ROLLED CORN PROCESSING AND DISTILLER'S GRAIN INCLUSION RATE ON RUMINAL PH, AMMONIA AND VOLATILE FATTY ACID CONCENTRATION, IN VITRO METHANE CONCENTRATION AND ENZYME ACTIVITY

5.1. Abstract

The objective was to determine the effects of degree of dry-rolled corn processing and dried distiller's grains with solubles (DDGS) inclusion rate on ruminal pH, NH_3 and volatile fatty acid (VFA) concentration, total in vitro gas and methane production, and enzyme activity in cattle fed high-concentrate diets. Eight ruminally cannulated Holstein steers (526 ± 3.6 kg) were assigned randomly to four dietary treatments in a 2 × 2 factorial arrangement consisting of 1) 65% coarse-rolled corn (2.5 mm) with 20% DDGS, 2) 45% coarse-rolled corn with 40% DDGS, 3) 65% fine-rolled corn (1.7 mm) with 20% DDGS and 4) 45% fine-rolled corn with 40% DDGS. Diets met NRC recommendations and were offered for ad libitum intake. The experimental design was a 4 × 4 Latin square with 17 d periods allowing for 7 d of diet adaptation, 7 d of sample collection, and 3 d of rest in which all steers were offered an intermediate ration. Results showed no differences in ruminal pH among treatments. However, steers consuming 40% DDGS tended ($P = 0.07$) to have a pH less than 5.5 for a longer period of time (h/d) than those consuming 20% DDGS. Ruminal NH_3 was greater in steers receiving 20% DDGS ($P = 0.02$). Steers fed fine-rolled corn had greater ($P = 0.02$) concentrations of butyric acid while those consuming coarse-rolled corn tended ($P = 0.06$) to have greater levels of isovaleric acid. No difference in DM degradation rate was noted between treatments. In vitro methane concentration of the headspace was unaffected by treatment. Steers fed diets containing

20% DDGS had greater ($P < 0.001$) ruminal α-amylase activity (U/L ruminal fluid; U/L/kg starch disappearance) while those fed 40% DDGS had greater ($P = 0.01$) trypsin activity (U/L/kg CP disappearance). An interaction between degree of corn processing × DDGS was noted for measurements of trypsin activity (U/L/kg CP disappearance; $P = 0.02$) and maltase (U/L of ruminal fluid; $P = 0.004$). In conclusion, these differences are likely the result of the final nutrient profile of each treatment. Overall, there were minimal effects on ruminal fermentation and in vitro methane concentration with increasing degree of dry-rolling or DDGS inclusion indicating limited effects on ruminal health and environmental sustainability.

5.2. Introduction

Ethanol is a commonly produced alternative fuel which is largely manufactured in the USA using corn grown in the midwestern part of the country. The production of ethanol also supplies a by-product known as dried corn distiller's grains with solubles (DDGS) which provides a valuable feed source for ruminants (Dicostanzo and Write, 2012). In addition to being an economical supplement, DDGS provides approximately 10% more energy than corn (Klopfenstein et al., 2008) as well as nearly 30% protein, 5 to 10% fat and 1% phosphorus (Jacob et al., 2010). Feeding DDGS often results in improved feed efficiency in finishing cattle (Swanson et al., 2014).

Although the available information on particle size reduction of rolled corn when fed with DDGS is limited, Loe et al. (2006) reported an increase in intake when offering finely vs. coarsely rolled corn in diets containing corn gluten feed. The rate of digestibility has also been shown to increase in cattle consuming mechanically processed corn grain (Galyean, et al., 1979;

Galyean, 1996). However, attention must be given to diet formulation when implementing various processed corn to avoid issues with ruminal acidosis.

While it is crucial to prevent acidosis, studies have shown that diets containing more corn relative to forage will lower the pH of the rumen which may result in a reduction in the NH_3 concentration (Zhang et al., 2015). These results are likely due to the ability of the starch-fermenting bacteria to assimilate NH_3 and increase propionate production (Lana et al., 1998; Russell, 1998). This change in the acetate:propionate ratio is associated with a reduction in CH_4 emission from the rumen (Luepp et al., 2009). The quantity of CH_4 being produced during the enteric fermentation of diets high in starch may also be reduced as cattle fed these diets produce less hydrogen in the rumen. In addition, an improved feed efficiency provides a shorter time to market allowing less opportunity of CH_4 emission to occur (Swanson et al., 2014).

The effects of feeding distiller's grains on CH_4 production have been variable. Distiller's grains contain greater concentrations of fat and fiber. The fat found in these by-products may reduce or eliminate protozoa as well as methanogenic bacteria in the rumen helping to mitigate CH_4 emissions by altering the hydrogen sink through bio-hydrogenation via propionate production (Massé et al., 2014). Fiber, however, is concentrated nearly 3-fold during ethanol production and possesses greater methanogenic potential than that of starch (Behlke et al., 2008).

The microbes in the rumen are responsible for the enzymatic activity and consequential nutrient breakdown and the production of VFA and CH_4. When cattle consume diets high in starch, the amylolytic organisms are likely to become more prevalent (Hobson and Stewart, 2012), whereas diets containing greater amounts of DDGS may cause a rise in CP and, thereby, increase the activity of trypsin (Gao et al., 2015).

Corn distiller's grains commonly are mixed with cattle rations in ranges from 10 to 50% (DM basis), depending on the goal of supplementation. With this in mind, developing feeding strategies is important to determine the optimum corn processing method and DDGS inclusion rates to obtain the greatest benefit for the ruminal environment and subsequent nutrient breakdown. Due to the greater concentrations of starch found in the diets containing 65% corn and 20% DDGS, we hypothesized that ruminal pH would be lower in the steers fed the 20% DDGS diets. We also hypothesized that the CH_4 emission would decrease as more reducing equivalent would be shifted toward propionate production while acetate production decreased. In terms of rumen enzyme activity, we hypothesized that ruminal digestive enzymes would change with diet because of changes in substrate available for fermentation. Therefore, the objective of this study was to determine the influence of the degree of dry-rolled corn processing and dried distiller's grains with solubles (DDGS) inclusion rate on ruminal pH, NH_3 and volatile fatty acid (VFA) concentration, total in vitro gas and CH_4 production, and enzyme activity in cattle fed high-concentrate diets.

5.3. Materials and Methods

5.3.1. Animals, Experimental Design and Dietary Treatments

All animal care, handling, and surgical procedures were approved by the North Dakota State University Animal Care and Use Committee.

Eight Holstein steers weighing 526 ± 3.6 kg were surgically fitted with a ruminal cannula (Bar Diamond, Inc., Parma, ID) and were used in a 4 × 4 Latin square design consisting of 4 periods with 4 dietary treatments. Two animals were assigned to each treatment per period to determine the impact of dry-rolled corn processing and DDGS inclusion rate on ruminal pH,

ammonia and VFA concentration, gas production and enteric CH_4 concentration, and ruminal digestive enzyme activity. Steers were housed in individual tie stalls (1.0 × 2.2 m) in a temperature controlled environment at the North Dakota State University Animal Nutrition and Physiology Center. Dietary treatments (**Table 5-1**) were offered to ensure ad libitum intake and approximately 6% feed refusal daily.

Table 5-1. Dietary composition and analyzed nutrient concentration of diets (DM basis).

	Coarse-rolled corn		Fine-rolled corn	
Dietary component, % of DM	20% DDGS	40% DDGS	20% DDGS	40% DDGS
Coarse-rolled corn	65.0	45.0	-	-
Fine-rolled corn	-	-	65.0	45.0
DDGS[a]	20.0	40.0	20.0	40.0
Grass-legume hay	5.0	5.0	5.0	5.0
Corn silage	5.0	5.0	5.0	5.0
Limestone	1.56	1.90	1.56	1.90
Urea	0.85	-	0.85	-
Salt	0.20	0.20	0.20	0.20
Vitamin premix	0.01	0.01	0.01	0.01
Trace mineral premix	0.05	0.05	0.05	0.05
Monensin/tylosin premix	0.03	0.03	0.03	0.03
Fine-ground corn	2.05	2.56	2.05	2.56
Chromium oxide	0.25	0.25	0.25	0.25
Feed Analysis				
DM, % of as fed	82.2	82.9	82.4	83.6
OM, % of DM	94.9	93.7	95.1	93.8
CP, % of DM	16.3	17.9	15.9	17.4
aNDF, % of DM	27.1	30.2	24.5	30.5
ADF, % of DM	9.02	11.1	8.47	11.0
Ether extract, % of DM	4.45	4.92	3.77	4.86
Calcium, % of DM	0.79	0.93	0.76	1.00
Phosphorus, % of DM	0.41	0.54	0.41	0.53
Starch, % of DM	48.0	39.4	48.2	40.8
DIP % of DM[*,b]	5.80	7.20	5.80	7.20

[*]Calculated NRC, 1996.
[a]Dried corn distiller's grains with solubles (DDGS)
[b]Degradable intake protein (DIP)

Treatments consisted of 1) 65% coarse-rolled corn (2.5 mm) and 20% DDGS, and 2) 45% coarse-rolled corn and 40% DDGS, 3) 65% fine-rolled corn (1.7 mm) and 20% DDGS and

4) 45% fine-rolled corn and 40% DDGS. Diets were formulated to meet or exceed requirements for degradable intake protein (DIP), metabolizable protein (MP), vitamins, and minerals (NRC, 1996). Before the initiation of the experiment, steers were adapted to high grain diets over a period of 21 days. A preliminary 7 days on the animal's respective treatment preceded 7 days of sample collection for each period. This was then followed by a 3-day rest period where steers were offered an intermediate diet to allow for adaptation to the next dietary treatment.

5.3.2. Laboratory Analysis

Coarse- and fine-rolled corn samples were analyzed for particle size following the procedure of ASAE (2003) using a sieve shaker (Tyler Ro-Tap;W. S. Tyler, Mentor, OH). Thirteen sieves including a bottom pan were used. The sieves ranged in size from 3,360 to 53 μm. Particle size was calculated using the equations of Baker and Herrman (2002) and the geometric mean diameter was reported (Swanson et al., 2014). Complete rations were mixed prior to each period and stored in a cooler at 4° C.

Orts were collected at 07:00 daily. Each animal's consumption was calculated and animals were immediately offered fresh feed by 08:00. Feed samples were collected immediately following ration mixing and were composited over each collection period. All samples were stored at -20˚C until analyses.

Feed samples were dried for 48 hrs at 60˚C in a forced air Grieve SB-350 oven (The Grieve Corporation, Round Lake, IL, USA) and ground to pass a 2-mm screen using a Wiley mill (Model #3; Arthur H. Thomas, Philadelphia, PA, USA). Feed samples were analyzed for DM, and ash (Procedure numbers: 934.01, 2001.11, and 942.05 respectively; AOAC, 1990), CP (Kjeldahl method) as well as aNDF and ADF (Goering and Van Soest 1970). Starch was

analyzed using the methods of Herrera-Saldana and Huber (1989) on a microplate spectrophotometer (Synergy, H1 Microplate reader, BioTek Instruments, Winooski, VT, USA).

5.3.3. Ruminal pH Determination

Ruminal pH was determined using a wireless pH sensor (Kahne Ltd., Auckland, NZ) with measurements taken every 5 min from d 3 to 5 of the collection period. Sensors were calibrated with 7.0 and 4.0 pH solutions before each period and were manually inserted into the rumen and placed in the liquid phase of the ventral sac.

5.3.4. NH3 and VFA Analysis

Approximately 200 mL of ruminal fluid was collected into Whirl-pak bags (Nasco, Fort Atkinson, WI and Modesto, CA, USA; 532-mL) from d 3 to 5 in a manner that allowed a sample to be collected every other hr in a 24-hr cycle. Samples were taken at 02:00, 08:00, 14:00 and 20:00 hr on d 3; 04:00, 10:00, 16:00, and 22:00 hr on d 4 and 06:00, 12:00, 18:00, and 00:00 hr on d 5 and stored frozen (−20°C) until the end of the collection period at which point they were thawed, equally composited and centrifuged at 2000 × g for 20 min. The liquid portion was filtered through a 0.45-µm filter and the supernatant separated and analyzed for NH_3 (Broderick and Kang, 1980). Ruminal VFA concentrations were determined by GLC (Hewlett Packard 5890A Series II GC, Wilmington, DE) and separated on a capillary column (Nukol, Supelco, Bellefonte, PA) using 2-ethyl butyric acid as the internal standard (Goetsch and Gaylean, 1983).

5.3.5. Gas Production Rate

Gas production was measured on d 1 and d 7 of each collection period with 2 replicates per animal and, therefore, 8 replicates per treatment. Dietary samples were dried for 48 hr at 60˚C in a forced air Grieve SB-350 oven (The Grieve Corporation, Round Lake, IL) and ground to pass a 2-mm screen using a Wiley mill (Model #3; Arthur H. Thomas, Philadelphia, PA). The

in vitro procedure consisted of adding 0.375 g of the treatment diet to a fermentation vessel (Gas Pressure Monitor, Ankom Technology Corp., Macedon, NY) along with 212.5 mL of a McDougall's buffer (McDougall, 1948) and ruminal fluid in a 4:1 ratio. McDougall's buffer was prepared by 07:00 the morning of each analysis with the collection of ruminal fluid immediately following. Each module was purged with CO_2, sealed, and then placed in an oscillating water bath at 39°C for 24 h, with the oscillation set at 125 rpm. A wireless gas pressure monitoring system was used to measure the changes in pressure inside the flask relative to atmospheric pressure as a consequence of the gas produced during fermentation. Data obtained from this system were converted from pressure to volume using the formula reported by López et al. (2007).

To measure the concentration of CH_4 in the headspace of the Ankom flask, 15 mL of gas was collected at 24 hr of incubation from the septa port using a gas tight syringe and analyzed using subsequent gas chromatograph (GC) (Model No. 8610C, SRI Instruments, Torrance, CA 90502; Borhan et al., 2012).

5.3.6. Ruminal α-Amylase, Trypsin Activity, and Maltase Activity

Ruminal fluid (100 µL) was added to a 0.9% NaCl solution (700 µL). Activity of α-amylase was determined using the procedure of Wallenfels et al. (1978) utilizing a kit from Teco Diagnostics (Anahein, CA). Trypsin activity was assayed using the methods described by Geiger and Fritz (1986; Swanson et al., 2008). Maltase activity was determined using the methods described by Bauer (1996) with a modification of the Turner and Moran (1982) technique. Analyses were adapted for use on a microplate spectrophotometer (SpectraMax 340, Molecular Devices). One unit (U) of enzyme activity equals 1 µmole product produced per min. Enzyme activity data are expressed as α-amylase U/L ruminal fluid, α-amylase U/L/kg starch intake, α-

amylase/kg starch disappearance, trypsin U/L ruminal fluid, trypsin U/L/kg CP intake, trypsin U/L/kg CP disappearance, and maltase U/L ruminal fluid.

5.3.7. Calculations and Statistical Analysis

Data were analyzed as a 2 × 2 factorial using the Mixed procedure of SAS (SAS Inst. Inc., Cary, NC). The model included the effects of animal, period, degree of dry-roll processing (coarse vs fine), DDGS inclusion rate (20 vs. 40% DDGS), and the interaction between degree of dry-roll processing × DDGS inclusion rate. Ruminal pH and the concentration of VFA over time were measured using the Mixed procedure of SAS with the model including animal, period, corn, DDGS, hr, corn × DDGS, corn × hr, and DDGS × hr.

Individual measurements of gas production were calculated using a nonlinear model in SAS and the equation gas=A*(1-exp(-(b*(time-c)+d*(sqrt(time)-sqrt(c))))) where A is equal to the asymptotic gas volume formation (mL), b is equal to the fractional rate (hr^{-1}), c is the lag time before measurable gas production (hr), and d is a constant ($hr^{-1/2}$; France et al., 2005). Degradation rate at 1, 12, and 24 hr were calculated using the predicted components of the gas production model. Gas production model components and degradation rates were analyzed using repeated measures (run; d 1 and d 7) in the MIXED procedure of SAS with the effects of animal, period, degree of dry-roll processing (coarse vs fine), DDGS inclusion rate (20 vs. 40% DDGS), degree of dry-roll processing × DDGS inclusion rate, run, degree of dry-roll corn processing × run, DDGS inclusion rate × run, and degree of dry-roll processing × DDGS inclusion rate × run. Appropriate (minimize information criterion) covariance structures were utilized (Wang and Goonewarden, 2004). There were no interactions between run and dietary treatments so only main effect means of degree dry-roll processing and DDGS inclusion rate are presented. Statistical significance was declared at $P \leq 0.05$.

5.4. Results

Feed intake was not influenced by corn particle size or DDGS inclusion rate (12.4 ± 1.40 kg DM). No differences were observed in ruminal pH between treatments ($P > 0.05$) however, the amount of time the rumen spent below pH 5.5 tended ($P = 0.07$) to be longer in cattle fed diets supplemented with 40% DDGS (**Figure 5-1**).

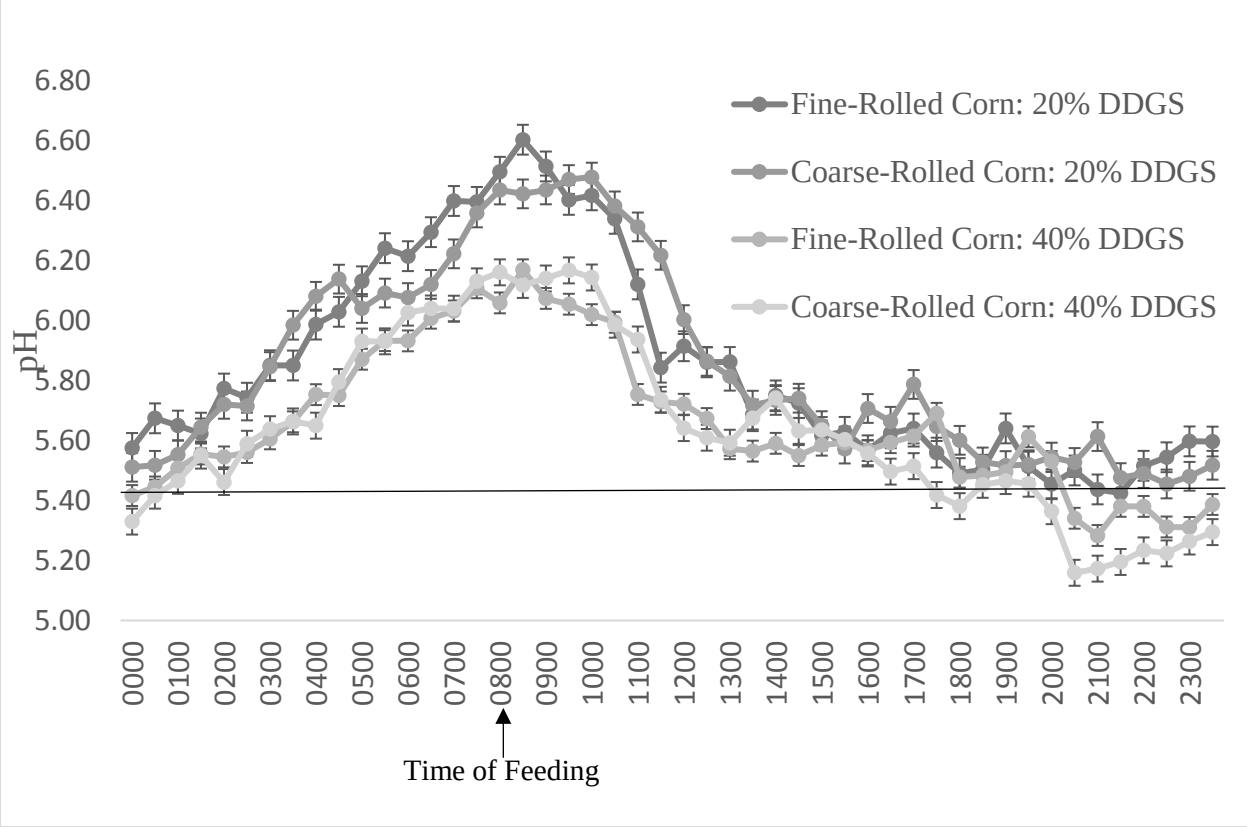

Figure 5-1. Data are represented as ruminal pH over a 24-hr period. Effects of dry-rolled corn processing method ($P = 0.85$), dried distiller's grains with solubles (DDGS) inclusion level ($P = 0.12$), Dry-rolled corn processing method*DDGS inclusion level ($P = 0.72$), and hr of the day ($P < 0.001$). The number of hr below a pH reading of 5.5 tended to be greater ($P = 0.07$) in cattle consuming 40 vs 20% DDGS.

Ruminal NH_3 concentration was greater ($P = 0.02$) in diets containing 20% DDGS (**Table 5-2)**.

Table 5-2. Ruminal pH and VFA profiles of steers fed coarse- vs fine-rolled corn with 20% vs 40% dried distiller's grains with solubles (DDGS)

	Coarse-rolled corn		Fine-rolled corn			P-Values			
	20% DDGS	40% DDGS	20% DDGS	40% DDGS	SEM[a]	Corn	DDGS	Corn × DDGS	Hr
Ruminal pH	5.96	5.68	5.88	5.70	0.134	0.85	0.12	0.72	<0.001
Minimum	5.31	5.04	5.22	5.10	0.197	0.94	0.32	0.68	-
Maximum	6.69	6.77	7.04	6.78	0.214	0.44	0.69	0.49	-
Time < 5.5, h/d	3.02	11.1	4.66	5.82	2.125	0.40	0.07	0.14	-
Ruminal NH_3, mM	13.3	10.4	13.4	9.8	13.31	0.87	0.02	0.80	<0.001
Total VFA, mM	184	183	197	198	9.7	0.14	0.99	0.91	<0.001
				VFA, mol/100 mol					
Acetic	33.1	34.1	32.0	32.7	1.44	0.41	0.56	0.92	0.18
Propionic	22.0	24.7	25.2	22.2	2.19	0.87	0.96	0.20	<0.001
Isobutyric	2.79	2.77	2.48	2.58	0.159	0.12	0.82	0.73	<0.001
Butyric	17.4	15.3	19.6	21.4	1.68	0.02	0.93	0.24	0.45
Isovaleric	16.5	14.7	11.9	10.9	2.07	0.06	0.50	0.84	<0.001
Valeric	8.24	8.51	8.83	10.2	0.642	0.10	0.23	0.42	0.05
Acetate:Propionate	1.65	1.52	1.39	1.70	3.4	0.84	0.66	0.28	0.001

[*]Data are presented as least square means per treatment ± SEM, n = 8

The concentration of butyric acid was greater ($P = 0.02$) in cattle consuming fine-rolled corn while isovaleric acid tended ($P = 0.06$) to be greater in cattle consuming coarse-rolled corn.

The in vitro gas production measurements showed no significant differences between treatments (**Table 5-3**).

Steers fed diets containing 20% DDGS had greater ($P < 0.001$) ruminal α-amylase activity (U/L ruminal fluid; U/L/kg starch disappearance) while steers consuming rations with 40% DDGS had greater ($P = 0.01$) trypsin activity (U/L/kg CP disappearance; **Table 5-4**).

Trypsin activity was also shown to have an interaction between degree of corn processing × DDGS when calculated as U/L/kg CP disappearance ($P = 0.02$). The diet composed of coarse-rolled corn with 40% DDGS had the lowest ($P = 0.004$) maltase activity (U/L of ruminal fluid).

5.5. Discussion

A previous study by Gonzalez et al. (2012) found that increased distribution of feeding throughout the day can moderate ruminal pH by decreasing the amount of acid production per unit of time and increasing the salvation rate and frequency. Despite being offered feed for ad libitum intake it is interesting to note the pH was the highest at 08:00 each day. These results are likely due to the addition of salivary buffers such as bicarbonate and phosphate ions as the animal awoke and the rumen prepared for the ingestion of fresh feed (Erdman, 1988).

It is also interesting to note that diets high in starch are more likely to cause a lower ruminal pH. Despite the lack of starch found in distiller's grains, cattle consuming 40% DDGS tended to have a ruminal pH lower than 5.5 for a longer period of time than those consuming 20% DDGS. While this may seem surprising it has also been shown that decreasing the particle size of corn grain decreases ruminal pH (Krause and Combs, 2003). The decrease in

Table 5-3. Gas production and in vitro methane concentration of steers fed coarse- vs fine-rolled corn with 20% vs 40% dried distiller's grains with solubles (DDGS)

	Coarse-rolled corn		Fine-rolled corn			*P* - Values		
	20% DDGS	40% DDGS	20% DDGS	40% DDGS	SEM	Corn	DDGS	Corn × DDGS
A, mL	187	161	163	171	26.7	0.79	0.76	0.53
b, hr^{-1}	0.147	0.110	0.112	0.084	0.0203	0.15	0.12	0.83
c, hr	0.202	0.036	0.128	-0.011	0.1070	0.58	0.17	0.90
d, $hr^{-1/2}$	1.63	1.61	2.14	1.62	0.545	0.64	0.62	0.65
Ruminal degradation rate (hr^{-1}) at:								
1 hr	0.263	0.213	0.263	0.293	0.0964	0.58	0.89	0.59
12 hr	0.185	0.157	0.174	0.177	0.0411	0.90	0.74	0.68
24 hr	0.176	0.150	0.164	0.164	0.0365	0.98	0.70	0.70
Methane, % of gas	108	121	115	126	12.6	0.68	0.44	0.94

Data are presented as least square means per treatment ± SEM, n = 4 per treatment. A = asymptote, b = fractional rate (hr^{-1}), c = lag time (hr), d = rate constant ($hr^{-1/2}$). gas=A(1-exp(-(b*(time-c)+d*(sqrt(time)-sqrt(c)))))

Table 5-4. Ruminal enzyme activity of steers fed coarse- vs fine-rolled corn with 20% vs 40% dried distiller's grains with solubles (DDGS)

	Coarse-Rolled		Fine-Rolled			P-Values			
	20% DDGS	40% DDGS	20% DDGS	40% DDGS	SEM	Corn	DDGS	Corn × DDGS	Hr
α-Amylase									
U/L Ruminal fluid	1491	1297	1500	1097	104.8	0.13	<0.001	0.11	0.02
U/L/kg Starch intake	264	267	254	228	22.9	0.07	0.41	0.28	0.04
U/L/kg Starch disappearance	8610	6244	8850	5533	641.4	0.58	<0.001	0.27	0.05
Trypsin									
U/L Ruminal fluid	2359	2185	2156	2394	192.7	0.98	0.81	0.13	0.35
U/L/kg CP intake	1214	1020	1102	1111	115.5	0.88	0.20	0.15	0.52
U/L/kg CP disappearance	3055[a]	3083[a]	2597[a]	3623[b]	275.8	0.84	0.01	0.02	0.46
Maltase									
U/L Ruminal fluid	323[a]	276[b]	300[ab]	324[a]	19.1	0.30	0.33	0.004	0.003

[*]Data are presented as least square means per treatment ± SEM, n = 8.

ruminal pH with increasing DDGS inclusion also could be related the pH of DDGS as research has suggested that the low pH of DDGS can directly decrease ruminal pH (Felix et al., 2012).

The greater ruminal NH_3 concentration in diets containing 20% DDGS was somewhat unexpected as research has shown that reduced CP typically results in reduced ruminal ammonia concentration (Frank et al., 2002; Todd et al., 2006). However, diets containing 20% DDGS also included urea to meet the DIP requirement (NRC, 1996). Consequently, the increase in NH_3 may have been the result of the urea being rapidly hydrolyzed to ammonia by bacterial urease (Habib et al., 2009).

In regards to the VFA concentration, studies conducted by Secrist et al. (1995) and Plascenia et al. (2009) reported that the concentration of butyrate was higher in cattle consuming coarse-rolled corn. Therefore, initially we would have expected propionate to be higher in diets containing fine-rolled corn. This increase is generally associated with an increase in butyrate. While only the concentration of butyrate was shown to increase in the current study, this may indicate the rate of fermentation of soluble fiber in the DDGS is more rapid than first hypothesized.

No differences were seen in the overall in vitro gas production measurements and the lack of response of CH_4 concentration in the headspace of the in vitro flasks to dietary treatment is similar to the results observed for the ruminal acetate:propionate ratios. Acetate production vs propionate production has been shown to be associated with increased CH_4 concentration (Moss et al., 2000 and Whitelaw et al., 1984). As no changes to the acetate:propionate ratio were found in the current study, it was not surprising that CH_4 concentrations did not differ between steers from the different dietary treatments.

The degree of dry-rolled processing in this study generally did not influence ruminal pH and in vitro gas or CH_4 production potentially because of adequate buffering occurring in both of the diets. Perhaps if a larger difference in rolled-corn particle size or DDGS inclusion rate was used in our study, differences in ruminal fermentation parameters and in vitro gas production would be more prevalent.

The greater ruminal α-amylase activity (U/L ruminal fluid; U/L/kg starch disappearance) found in diets containing 20% DDGS was likely because of the greater amount of corn (and thus starch) in the 20% DDGS diets with the ratio being 65:20% corn:DDGS while diets containing 40% DDGS had 45:40% corn:DDGS. These results indicate that the greater levels of starch found within the grain stimulated the ruminal microbes to increase α-amylase production for starch breakdown.

The increased trypsin activity (U/L/kg CP disappearance) in steers consuming rations with 40% DDGS is likely due to the increased true protein concentrations found in the DDGS which would require more trypsin for protein breakdown. The increased level of CP found within diets containing 40% DDGS, in addition to the increased exposure of the fine-rolled corn grain was likely the cause of the greater production of microbial trypsin (U/L/kg CP disappearance).

Diets high in starch require the ruminal microbes to hydrolyze starch into maltose and glucose (Coleman, 1969). Due to the starch being removed during the processing of DDGS and the lower inclusion rate of the coarse-rolled-corn (Secrist et al., 1995), it is not surprising that maltase (U/L of ruminal fluid) in treatments containing coarse-rolled corn with 40% DDGS is lowest.

5.6. Conclusion

In conclusion, it seems that the ratio of corn grain to level of DDGS in a cattle ration has a greater impact on in vitro gas production than the corn processing method. It may be that differences in available dietary starch were not significant enough to cause a change in the amount of gas produced and, despite a rise in ruminal butyrate, acetate and propionate were not affected which may have reduced the likelihood of hydrogen capture and subsequent CH_4 mitigation. The increased concentrations of starch in diets containing 20% DDGS along with the higher CP levels in DDGS resulted in tendencies for increased α-amylase and trypsin activity, respectively, suggesting that the ruminal microbes are adapting to changes in substrate. Overall, using fine- vs. coarse-rolled corn and 20 vs. 40% inclusion rate of distiller's grains did not result in any large differences in the ruminal environment.

5.7. Literature Cited

AOAC. 1990. Official Methods of Analysis. 15th Ed. ed. Arlington, VA.

ASAE. 2003. Method of determining and expressing fineness of feed materials by sieving. ASAE Standard ASAE S319.2

Baker, S., and T. Herrman. 2002. Evaluating particle size. MF-2051 Feed Manufacturing. Dept. Grain Sci. Ind., Kansas State Univ., Manhattan.

Bauer, M. L. 1996. Nutritional regulation of small intestinal glucose absorption in ruminants. Ph.D. dissertation., University of Kentucky, Lexington.

Behlke, E. J., T.G. Sanderson, T. J. Klopfenstein, and J. L Miner. 2008. Ruminal methane production following the replacement of dietary corn with dried distiller's grains. Nebraska Beef Rep. 50.

Borhan, M. S., S. Rahman, and H. K. Ahn. 2012. Dry anaerobic digestion of fresh feedyard manure: a case study in a laboratory setting. Int. J. Emerg. Sci. 2:509-525.

Broderick, G. A., and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63:64-75.

Coleman, G. S. 1969. The metabolism of starch, maltose, glucose, and some other sugars by the rumen ciliate Entodinium caudatum. J. Gen. Microbiol. 87:303-332.

Dicostanzo, A., and C. L. Wright. 2012. Feeding ethanol coproducts to beef cattle. In: Liu K. S., Rosentrater K. A., editors, Distillers grains: Production, properties, and utilization. CRC Press, Boca Raton, FL. 237-264.

Erdman, R. A. 1988. Dietary buffering requirements of the lactating dairy cow: A review. J. Dairy Sci. 71:3246–3266.

Felix, T. L., T. A. Murphy, and S. C. Loerch. 2012. Effects of dietary inclusion and NaOH treatment of dried distiller's grains with solubles on ruminal metabolism of feedlot cattle. J. Anim. Sci. 90: 4951-4961.

France, J., S. Lopez, E. Kebread, A. Bannink, M. S. Dhanoa, and J. Dijkstra. 2005. A general compartmental model for interpreting gas production profiles. Anim. Feed Sci. Technol. 123-124.

Frank, B., M. Persson, and G. Gustafsson. 2002. Feeding dairy cows for decreased ammonia emission. Livestock Prod Sci. 76:171–179.

Galyean, M. L. 1996. Protein levels in beef cattle finishing diets: Industry application, university research, and systems results. J. Anim. Sci. 74:2860-2870.

Galyean, M. L., D. G. Wagner, and F. N. Owens. 1979. Corn particle size and site and extent of digestion by steers. J. Anim. Sci. 49:204-210.

Gao, W., A. Chen, B. Zhang, P. Kong, C. Liu, and J. Zhao. 2015. Rumen degradability and post-ruminal digestion of dry matter, nitrogen and amino acids of three protein supplements. Asian-Australas. J. Anim. Sci. 28:485-493.

Geiger, R. and H. Fritz. 1986. Trypsin. In: Bergmeyer H, editor. Methods of Enzymatic Analysis. New York: Elsevier: 119-128.

Goering, H. K., and P. J. Van Soest. 1970. Forage analyses. Agriculture Handbook 379. Agric. Res. Serv. U.S. Dept. of Agriculture.

Gonzalez, L. A., X. Manteca, S. Calsamiglia, K. S. Schwartzkopf-Genswein, and A. Ferret. 2012. Ruminal acidosis in feedlot cattle: Interplay between feed ingredients, rumen function and feeding behavior (a review). Anim. Feed Sci. Technol. 172:66-79.

Goetsch, A. L., and M. L. Gaylean. 1983. Influence of feeding frequency on passage of fluid particulate markers in steers fed a concentrate diet. Can. J. Anim. Sci. 63:727-730.

Habib, M. A., M. S. Hossain, and A. K. F. H. Bhuiyan. 2009. Impact of urea-based diets on production of Red Chittagong cattle. Bangladesh Veterinarian. 26:74-79

Herrera-Saldana, R., and J. T. Huber. 1989. Influence of varying protein and starch degradabilities on performance of lactating cows. J. Dairy Sci. 72:1477-1483.

Hobson, P. N., and C. S. Stewart. 2012. The rumen microbial ecosystem. Springer Science and Business Media.

Jacob, M. E., Z. D. Paddock, D. G. Renter, K. F. Lechtenberg, and T. G. Nagaraja. 2010. Inclusion of Dried or Wet Distillers' Grains at Different Levels in Diets of Feedlot Cattle Affects Fecal Shedding of Escherichia coli O157:H7. Appl. Environ. Microbiol. 76:7238-7242.

Klopfenstein, T. J., G. E. Erickson, and V. R. Bremer. 2008. Board-invited review: Use of distillers by-products in the beef cattle feeding industry. J. Anim. Sci. 86:1223-1231.

Krause, K. M. and D. K. Combs. 2003. Effects of forage particle size, forage source, and grain fermentability on performance and ruminal pH in midlactation cows. J. Dairy Sci. 86:1382-1397.

Lana, R. P., J. B. Russell, and M. E. Van Amburgh. 1998. The role of pH in regulating ruminal methane and ammonia production. J. Anim. Sci. 76:2190-2196

Leupp, J. L., G. P. Lardy, K. K. Karges, M. L. Gibson, and J. S. Caton. 2009. Effects of increasing level of corn distillers dried grains with solubles on intake, digestion, and ruminal fermentation in steers fed seventy percent concentrate diets. J. Anim. Sci. 87:2906-2912.

Loe, E. R., M. L. Bauer, and G. P. Lardy. 2006. Grain source and processing in diets containing varying concentrations of wet corn gluten feed for finishing cattle. J. Anim Sci. 84:986-996.

López, S., M. S. Dhano, J. Dijkstra, A. Bannink, E. Kebreab, and J. France. 2007. Some methodological and analytical considerations regarding application of the gas production technique. Anim. Feed Sci. Technol. 135:139-156.

Massé, D. I., G. Jarret, C. Benchaar, and N. M. Cata Saady. 2014. Effect of corn Dried Distiller Grains with Solubles (DDGS) in dairy cow diets on manure bioenergy production potential. Animal. 4:82-92.

Moss, A. R., J. P. Jouanyb, and J. Newboldc. 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49:231-253.

National Research Council. 1996. Nutrient Requirements of Beef Cattle. 7th Ed. National Academy Press, Washington, DC.

Plascencia, A., V. M. González-Vizcarra, M. A. López-Soto, D. May, L. C. Pujol, G. F. Ruiz, and S. H. Vega. 2009. Influence of cracked, coarse ground or fine ground corn on digestion, dry matter intake and milk yield in Holstein cows. J. Appl. Anim. Res. 35:149-154.

Russell, J. B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. J. Dairy Sci. 81:3222-3230.

Secrist, D. S., W. J. Hill, F. N. Owens, and S. D. Welty. 1995. Effects of corn particle size on feedlot steer performance and carcass characteristics. Anim. Sci. Res. Rep. 99-104, (http://www.ansi.okstate.edu/research/ researchreport).

Swanson, K. C., N. Kelly, H, Salim, Y. J. Wang, S. Holligan, M. Z. Fan, and B. W. McBride. 2008. Pancreatic mass, cellularity and α-amylase and trypsin activity in feedlot steers fed diets differing in crude protein concentration. J. Anim. Sci. 86:909-915.

Swanson, K. C., A. Islas, Z. E. Carlson, R. S. Goulart, T. C. Gilbery, and M. L. Bauer 2014. Influence of dry-rolled corn processing and increasing dried corn distiller's grains plus solubles inclusion for finishing cattle on growth performance and feeding behavior. J. Anim. Sci. 92:2531-2357.

Todd, R. W., N. A. Cole, and N. R. Clark. 2006. Reducing Crude Protein in beef cattle diets reduces ammonia emissions from artificial feedyard surfaces. J. Environ. Qual. 35:404-411.

Turner, R. J. and A. Moran. 1982. Heterogeneity of sodium-dependent D-glucose transport sites along the proximal tubule: Evidence from vesicle studies. Am. J. Physiol. 242:F406-F414.

Wallenfels, K., P. Fold, H. Niermann, H. Bender, and D. Linder. 1978. The enzymatic synthesis, by transglycosylation of a homologous series of glycosidically substituted malto-oligosaccharides, and their use as amylase substrates. Carbohydrate Research. 61:359-68.

Wang, Z., and L. A. Goonewardene. 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. Can. J. Anim. Sci. 84:1-11.

Whitelaw, F. G., M. J. Eadie, L. A. Bruce, and W. J. Shand. 1984. Methane formation in faunated and ciliate-free cattle and its relationship with rumen volatile fatty acid proportions. Brit. J. Nut. 52:261-275.

Zhang, X., H. Zhang, Z. Wang, X. Zhang, H. Zou, C. Tan, and Q. Peng. 2015. Effects of dietary carbohydrate composition on rumen fermentation characteristics and microbial population in vitro. Italian J. Anim. Sci. 14:3366

CHAPTER 6. IMPACT OF GRAIN SOURCE AND DRIED DISTILLER'S GRAIN WITH SOLUBLES WITH LOW OR MODERATE OIL CONCENTRATIONS ON RUMINAL PH, AMMONIA AND VOLATILE FATTY ACID CONCENTRATION, IN VITRO METHANE AND CARON DIOXIDE CONCENTRATION AND ENZYME ACTIVITY

6.1. Abstract

This study was conducted to examine the effect of grain source (rolled-corn vs rolled-barley) and dried distiller's grains with solubles (DDGS) oil concentration (low = 4.5% vs moderate = 7.9%) on ruminal pH, ammonia (NH_3) and volatile fatty acid (VFA) concentrations, in vitro enteric methane (CH_4) and carbon dioxide (CO_2) production, and digestive enzyme activity. Eight ruminally cannulated Holstein steers (715 ± 61.4 kg) were randomly assigned to four dietary treatments in a 2 × 2 factorial arrangement consisting of 1) rolled-corn and low-oil DDGS, 2) rolled-corn and moderate-oil DDGS, 3) rolled-barley and low-oil DDGS and 4) rolled-barley and moderate-oil DDGS. Diets were formulated to meet NRC recommendations and were offered for ad libitum intake. The experiment was designed as a 4 × 4 Latin square with 24-d periods allowing for 7 d of diet adaptation, 7 d of sample collection, and a 10-d transition period in which all steers were offered an intermediate ration. Feed intake was greater ($P = 0.01$) in steers consuming low-oil DDGS diets. No differences were noted for rumen NH_3 or total VFA concentration. There was a trend ($P = 0.05$) for isovaleric acid to be greater in diets containing moderate-oil DDGS while valeric acid was greater ($P = 0.03$) in rolled-barley rations. The lag time in gas production was greater ($P = 0.03$) in corn-based rations while diets containing barley had a faster ($P = 0.03$) degradation rate. Total CH_4 and CO_2 concentration showed no difference between treatments. Grain × DDGS interactions ($P < 0.03$) were observed when evaluating α-

amylase (U/L ruminal fluid and U/kg starch intake) with steers consuming rolled-barley and moderate-oil DDGS diets having the lowest activity. Steers provided rolled-corn diets had the greatest ($P = 0.01$) α-amylase activity (U/kg starch disappearance). Grain × DDGS interactions ($P < 0.03$) were observed when evaluating trypsin as steers consuming rolled-corn diets and low-oil DDGS had the greatest enzyme activity (U/L ruminal fluid, U/kg CP intake, U/kg CP disappearance). Maltase activity (U/L ruminal fluid) was greater ($P = 0.01$) in steers provided rolled-corn rations. Steers consuming diets with low-oil DDGS also exhibited greater ($P < 0.001$) maltase activity. Despite variation in rumen enzyme activity, ruminal pH was not influenced by dietary treatment. These results indicate that feeding different grain sources (corn or barley) or DDGS with differing oil concentration (moderate or low) were not detrimental to the ruminal microbes or environment.

6.2. Introduction

Corn and barley are commonly used in cattle rations throughout North America. This is largely due to the grain accessibility and the need to provide sufficient amounts of dietary energy for effective growth and development. Due to the high starch concentration and thus dietary NE found in corn, it is one of the most prevalent choices. Although barley has been shown to contain 5 to 10 percent less energy than corn (Milner et. al., 1995), it also is a common feed source because its starch is more available for rapid ruminal digestion. This rapid digestion has been shown to result in a greater production of VFA within the ruminal fluid (Aschenbach et al., 2011), which can lead to acidotic conditions and, consequently, reduced rumen function of amylolytic bacteria and protozoa impeding the breakdown of starch (Stone et al., 2004). Despite

this issue, barley is known to contain greater concentrations of protein and a more balanced supply of amino acids allowing for the possibility of reduced supplementation (Salo et al., 2016).

Distiller's grains are also typically incorporated into rations to reduce feed costs while maintaining an energy value similar to, or greater than grain (Klopfenstein et al., 2008; Dicostanzo, 2012). In recent years, ethanol plants have begun extracting greater levels of oil to be used in biodiesel production and providing additional commodities for a value-added market (Saunders et al., 2009; Bremer et al., 2014).

Different grain sources and changes in distiller's by-products have the potential to impact cattle production in both negative and positive ways through altered nutrient composition, thereby affecting the rumen environment including impacting the microbial and enzymatic action on feed (Hobson and Stewart, 2012). Therefore, the objective of this study was to determine the impact of corn- vs. barley-based grains in combination with low- vs. moderate-oil concentration distiller's grain on ruminal pH, ammonia (NH_3) and volatile fatty acid (VFA) concentration, in vitro gas production, and enteric methane (CH_4) and carbon dioxide (CO_2) concentration, and ruminal digestive enzyme activity.

6.3. Materials and Methods

6.3.1. Animals, Experimental Design and Dietary Treatments

All animal care, handling, and surgical procedures were approved by the North Dakota State University Animal Care and Use Committee.

Eight Holstein steers weighing 715 ± 61.4 kg were surgically fitted with a ruminal cannula (Bar Diamond, Inc., Parma, ID) randomly assigned to four dietary treatments in a 2 × 2 factorial arrangement. Two animals were assigned to each treatment per period to determine the

impact of grain source (rolled-corn vs rolled-barley) and dried distiller's grains with solubles (DDGS) oil concentration (low = 4.5% vs moderate = 7.9%) on ruminal pH, ammonia (NH_3) and volatile fatty acid (VFA) concentration, in vitro enteric methane (CH_4) and carbon dioxide (CO_2) concentration, and digestive enzyme activity. Steers were housed in individual tie stalls (1.0 × 2.2 m) in a temperature-controlled environment. Dietary treatments (**Tables 6-1 and 6-2**) were offered to ensure ad libitum intake and approximately 6% feed refusal daily.

Dietary treatments consisted of 1) rolled-corn and low-oil DDGS, 2) rolled-corn and moderate-oil DDGS, 3) rolled-barley and low-oil DDGS and 4) rolled-barley and moderate-oil DDGS. Diets were formulated to meet or exceed requirements for degradable intake protein, MP, vitamins, and minerals (NRC, 1996). Corn silage was used as the source of forage and was included in the diet at 20% on a DM basis. Before the initiation of the experiment, steers were adapted to high grain diets over 21 days. Each treatment period consisted of 24 days allowing for 7 d of diet adaptation, 7 d of sample collection, and a 10-d rest period in which all steers were offered an intermediate dietary transition before being provided the next dietary treatment.

6.3.2. Laboratory Analysis

Dietary treatments were prepared weekly and stored at 4˚C. Samples were collected immediately following ration mixing and preserved at -20˚C until analyses. Orts were collected at 07:00 daily. Each animal's consumption was calculated and animals were offered fresh feed by 08:00.

Table 6-1. Dietary composition and analyzed nutrient concentration of diets (DM basis)

	Rolled-Corn		Rolled-Barley	
Dietary Component, % of DM	Low-Oil DDGS	Moderate-Oil DDGS	Low-Oil DDGS	Moderate-Oil DDGS
Rolled Corn	50	50	-	-
Barley	-	-	50	50
Low-Oil DDGS	25		25	
Moderate-Oil DDGS		25		25
Corn Silage	20	20	20	20
Limestone	2	2	2	2
Urea	0.15	0.15	-	-
Salt	0.05	0.05	0.05	0.05
Vitamin Premix[b]	0.01	0.01	0.01	0.01
Mineral Premix[c]	0.05	0.05	0.05	0.05
Monensin[d]	0.02	0.02	0.02	0.02
Tylosin[e]	0.01	0.01	0.01	0.01
Fine-ground corn	2.46	2.46	2.61	2.61
Chromium oxide	0.25	0.25	0.25	0.25
Feed Analysis				
DM, % of as fed	89.1	97.2	89.0	97.0
OM, % of DM	83.1	90.3	82.5	90.0
CP, % of DM	13.7	14.0	14.8	14.8
aNDF, % of DM	29.8	31.8	32.6	34.7
ADF, % of DM	11.9	12.5	13.3	14.1
Ether Extract, % of DM	3.49	4.18	2.40	3.11
Calcium, % of DM	1.09	1.16	1.15	1.07
Phosphorus, % of DM	0.46	0.46	0.50	0.48
Starch, % of DM	43.6	42.1	37.1	37.5

[a]Calculated NRC, 1996
[b]Contained 48,510 kIU/kg vitamin A and 4,630.5 kIU/kg vitamin D.
[c]Contained 3.62% Ca, 2.56% Cu,16% Zn, 6.5% Fe, 4.0% Mn, 1.050 mg/kg I, and 250 mg/kg Co.
[d]Contained 176.4 g monensin/kg premix.
[e]Contained 88.2 g tylosin/kg premix.

Table 6-2. Analyzed Nutrient Concentration of DDGS (DM basis)

Item	Low-Oil DDGS	Moderate-Oil DDGS
DM, % of as fed	87.2	90.5
OM, % of DM	80.3	85.1
CP, % of DM	31.6	32.6
aNDF, % of DM	34.8	46.1
ADF, % of DM	10.9	14.2
Ether Extract, % of DM	4.5	7.9
Calcium, % of DM	0.04	0.04
Phosphorus, % of DM	1.04	0.93
Starch, % of DM	8.87	3.57

After each trial period, feed samples were dried for 48 hrs at 60˚C in a forced air Grieve SB-350 oven (The Grieve Corporation, Round Lake, IL, USA) and ground to pass a 2-mm screen using a Wiley mill (Model #3; Arthur H. Thomas, Philadelphia, PA, USA). Feed samples were analyzed for DM and ash (Procedure numbers: 934.01, 2001.11, and 942.05 respectively; AOAC, 1990), CP (Kjeldahl method) and aNDF and ADF (Goering and Van Soest 1970). Starch was analyzed using the methods of Herrera-Saldana and Huber (1989) using a microplate spectrophotometer (Synergy, H1 Microplate reader, BioTek Instruments, Winooski, VT, USA).

6.3.3. Ruminal pH, NH3, and VFA Analysis

Approximately 200 mL of ruminal fluid was collected into whirl-pak bags (Nasco; 532-mL) from d 3 to 5 in a manner that allowed a sample to be collected every other hr in a 24-hr cycle. Samples were taken at 02:00, 08:00, 14:00 and 20:00 hr on d 3, 04:00, 10:00, 16:00, and 22:00 hr on d 4 and 06:00, 12:00, 18:00, and 00:00 hr on d 5. During collections, ruminal pH was immediately measured using an IQ Scientific pH meter (Hach Company, Loveland, CO, USA) before being allowed to cool. Once pH was recorded samples were stored frozen (−20° C) until the end of the collection period at which point they were thawed, equally composited and centrifuged at 2000 × g for 20 min. The liquid portion was filtered through a 0.45-µm filter and

the supernatant separated and analyzed for NH_3 (Broderick and Kang, 1980). Ruminal VFA concentrations were determined by GLC (Hewlett Packard 5890A Series II GC, Wilmington, DE, USA) and separated on a capillary column (Nukol, Supelco, Bellefonte, PA, USA) using 2-ethyl butyric acid as the internal standard (Goetsch and Gaylean, 1983).

6.3.4. Gas Production Rate

Gas production was measured on d 1 and d 7 of each collection period with 2 replicates per animal and therefore 8 replicates per treatment. The in vitro procedure consisted of adding 0.375 g of the treatment diet to an Ankom fermentation vessel (Gas Pressure Monitor, Ankom Technology Corp., Macedon, NY, USA) along with 212.5 mL of a McDougall's buffer (McDougall, 1948) and ruminal fluid solution in a 4:1 ratio. McDougall's buffer was prepared by 07:00 the morning of each analysis with the collection of ruminal fluid immediately following. Each module was purged with CO_2, sealed with the Ankom pressure monitor cap (Ankom Technology Corp.), and then placed in an oscillating water bath (Northwest Scientific Incorporated) at 39°C for 24 h, with the oscillation set at 125 rpm. A wireless gas pressure monitoring system was used to measure the changes in pressure inside the flask relative to atmospheric pressure as a consequence of the gas produced during fermentation. Data were transferred via an Ankom wireless system to a computer equipped with software that allows the data to be stored in a spreadsheet. Data obtained from this system were converted from pressure units to volume units (mL) using the formula reported by López et al. (2007).

To measure the concentration of methane produced in the headspace of the Ankom flask, 15 mL of gas was collected from the septa port using a gas tight syringe and tested using subsequent gas chromatograph (GC) analysis (Model No. 8610C, SRI Instruments, Torrance, CA, USA, 90502; Borhan et al., 2012).

6.3.5. Rumen α-Amylase, Trypsin Activity, and Maltase Activity

Ruminal fluid (100 µL) was added to a 0.9% NaCl solution (700 µL) and homogenized using a Vortex Genie 2 mixer from Sigma-Aldrich (St. Louis, MO, USA). Activity of α-amylase was determined using the procedure of Wallenfels et al. (1978) utilizing a kit from Teco Diagnostics (Anahein, CA, USA). Trypsin activity was assayed using the methods described by Geiger and Fritz (1986). Maltase activity was determined using the methods described by Bauer (1996) with a modification of the Turner and Moran (1982) technique. Analyses were adapted for use on a microplate spectrophotometer (SpectraMax 340, Molecular Devices.) One unit (U) of enzyme activity equals 1 µmole product produced per min. Enzyme activity data are expressed as α-amylase U/L ruminal fluid, α-amylase U/kg starch intake, α-amylase U/kg starch disappearance, trypsin U/L ruminal fluid, trypsin U/kg crude protein intake, trypsin U/kg crude protein disappearance, and maltase U/L ruminal fluid.

6.3.6. Calculations and Statistical Analysis

Data were analyzed as a 2 × 2 factorial using the Mixed procedure of SAS (SAS Inst. Inc., Cary, NC). The model included the effects of animal, period, grain source (rolled-corn vs rolled-barley) and dried distiller's grains with solubles (DDGS) oil concentration (low vs moderate), and the interaction between Grain × Oil. Ruminal pH and the concentration of VFA over time were measured using the Mixed procedure of SAS with the model including animal, period, Grain, Oil, Hr, Grain × Oil, Grain × Hr, and Oil × Hr.

Individual measurements of gas production were calculated using a nonlinear model in SAS and the equation gas=A*(1-exp(-(b*(time-c)+d*(sqrt(time)-sqrt(c))))) where A is equal to the asymptotic gas formation (mL gas), b is equal to the fractional rate (hr^{-1}), c is the lag time before measurable gas production (hr), and d is a rate constant ($hr^{-1/2}$; France et al., 2005).

Degradation rate at 1, 12, and 24 hr were calculated using the predicted components of the gas production model. Gas production data were analyzed using repeated measures (run; d 1 and d 7) in the MIXED procedure of SAS with the effects of animal, period, grain source (rolled-corn vs rolled-barley) and dried distiller's grains with solubles (DDGS) oil concentration (low vs moderate), Grain× Oil, run, Grain × run, Oil × run, and Grain × Oil × run. Appropriate (minimize information criterion) covariance structures were utilized (Wang and Goonewarden, 2004). There were no interactions between run and dietary treatments so only main effect means of Grain and Oil are presented. Statistical significance was declared at $P \leq 0.05$.

6.4. Results

Feed intake was greater ($P = 0.01$) in steers consuming low-oil DDGS. The concentration of ruminal NH_3 in the current trial was not different between treatments despite the use of barley vs corn (**Table 6-3**).

Total VFA concentration also showed no difference ($P > 0.05$) however, a trend ($P = 0.05$) was observed with isovaleric acid concentration to be higher in diets with moderate-oil DDGS while valeric acid concentration was lower ($P = 0.03$) in diets containing rolled-corn.

Table 6-3. Ruminal NH_3 and VFA profiles of steers consuming corn- vs barley-based diets with variable distiller's oil concentration

	Rolled-Corn		Rolled-Barley			*P* - Values			
	Low-Oil DDGS	Moderate-Oil DDGS	Low-Oil DDGS	Moderate-Oil DDGS	SEM	Grain	Oil	Grain × Oil	Hr
Feed Intake, kg/d	21.7	21.2	21.9	21.5	0.84	0.19	0.01	0.73	-
Ruminal ammonia, mM	13.8	11.1	10.9	10.4	1.48	0.26	0.33	0.33	<0.001
Total VFA, mM	197	193	184	186	9.2	0.30	0.91	0.72	<0.001
			VFA, mol/100 mol						
Acetic	31.1	33.0	33.2	30.6	1.74	0.92	0.83	0.21	0.68
Proprionic	22.8	23.7	23.4	23.5	1.60	0.89	0.75	0.80	<0.001
Isobutyric	3.71	3.70	3.86	3.70	0.211	0.73	0.71	0.74	<0.001
Butyric	22.0	19.3	18.6	19.9	1.89	0.46	0.72	0.30	0.35
Isovaleric	11.2	12.3	9.58	11.5	0.728	0.12	0.05	0.57	<0.001
Valeric	9.21	7.97	11.4	10.8	1.057	0.03	0.39	0.76	0.01
Acetate:Propionate	1.49	1.48	1.55	1.40	0.147	0.95	0.60	0.63	<0.001

[*] Data are presented as least square means per treatment ± SEM, n = 8

The lag time in gas production was longer ($P = 0.03$) in corn vs barley-based rations. The rate of ruminal degradation was greater ($P = 0.03$) for barley at 1 hr and tended to be greater at 12 hr ($P = 0.06$) and 24 hr ($P = 0.07$). No differences ($P > 0.05$) in CH_4 and CO_2 concentration were observed among the dietary treatments (**Table 6-4**).

Ruminal α-amylase activity (U/L ruminal fluid, U/kg starch intake; $P = 0.02$) was greater in steers consuming low-oil DDGS while an interaction ($P = 0.03$) between grain source and DDGS oil concentration for ruminal α-amylase activity (U/L ruminal fluid, U/kg starch intake; $P = 0.02$) was observed as barley diets with moderate oil DDGS had the lowest activity. Activity of α-amylase (U/kg starch disappearance) was greater ($P = 0.01$) in corn-based rations. Steers fed the diet containing corn with low-oil DDGS had the greatest (interaction $P \leq 0.03$) trypsin activity (U/kg CP intake and U/kg CP disappearance) while steers fed corn-based diets ($P = 0.01$) and low-oil DDGS ($P < 0.01$) had greater maltase activity (U/L ruminal fluid; **Table 6-5**).

Ruminal pH was not influenced ($P = 0.13$) by dietary treatment but was shown to change depending on the hr of the day ($P < 0.001$; **Figure 6-1**) with time points near 07:00 being the highest.

6.5. Discussion

Ruminal ammonia concentrations are impacted by the amount and degradability of the crude protein in the diet along with the rate of fermentation (Oh et al., 2008). The concentration of ruminal ammonia, however, was not influenced by the dietary treatments in the current trial. This is likely formulated to contain similar levels of CP and because grain type and DDGS oil concentration did not greatly affected protein metabolism in the rumen.

Table 6-4. Gas production and in vitro CH_4 and CO_2concentration of steers consuming corn- vs barley-based diets with variable distiller's oil concentration

	Rolled-Corn		Rolled-Barley			*P* - Values		
	Low-Oil DDGS	Moderate-Oil DDGS	Low-Oil DDGS	Moderate-Oil DDGS	SEM	Grain	Oil	Grain × Oil
A, mL	188	116	191	121	52.4	0.87	0.17	0.97
b, hr^{-1}	0.153	0.126	0.100	0.095	0.0282	0.13	0.56	0.67
c, hr	0.148	0.144	0.057	0.027	0.0484	0.03	0.72	0.78
D, $hr^{-1/2}$	0.482	0.456	0.407	0.297	0.1374	0.39	0.61	0.75
Ruminal degradation rate (hr^{-1}) at:								
1 hr	0.128	0.110	0.227	0.198	0.4253	0.03	0.57	0.89
12 hr	0.108	0.100	0.174	0.147	0.0304	0.06	0.56	0.76
24 hr	0.105	0.098	0.168	0.142	0.0293	0.07	0.56	0.74
Methane, % of gas	22.2	24.8	24.4	23.3	1.58	0.80	0.61	0.22
CO_2, % of gas	59.4	55.8	57.1	54.5	2.16	0.41	0.17	0.84
CH_4:CO_2	0.385	0.462	0.443	0.440	0.0333	0.57	0.25	0.22

Data are presented as least square means per treatment ± SEM, n = 4 per treatment. A = asymptote, b = fractional rate (hr^{-1}), c = lag time (hr), d = rate constant ($hr^{-1/2}$). gas=A(1-exp(-(b*(time-c)+d*(sqrt(time)-sqrt(c)))))

Table 6-5. Rumen enzyme activity of steers consuming corn- vs barley-based diets with variable distiller's oil concentration

	Rolled-Corn		Rolled-Barley					
	Low-Oil DDGS	Moderate-Oil DDGS	Low-Oil DDGS	Moderate-Oil DDGS	SEM	Grain	Oil	Grain × Oil
Feed Intake, kg/d	21.7	21.2	21.9	21.5	0.84	0.19	0.01	0.73
Amylase								
U/L Ruminal fluid	132[a]	131[a]	144[a]	106[b]	13.5	0.45	0.02	0.03
U/L/kg Starch Intake	98.2[ab]	102[ab]	116[a]	86.0[b]	12.16	0.94	0.08	0.02
U/L/kg Starch Disappearance	9969	10030	9094	7258	767.1	0.01	0.20	0.17
Trypsin								
U/L Ruminal fluid	275[a]	218[b]	207[b]	211[b]	14.4	0.002	0.02	0.01
U/L/kg CP Intake	875[a]	705[b]	633[b]	644[b]	52.2	<0.001	0.05	0.03
U/L/kg CP Disappearance	6106[a]	4565[b]	4835[b]	4541[b]	399.2	0.01	0.001	0.02
Maltase								
U/L Ruminal fluid	334	258	280	222	28.0	0.009	<0.001	0.56

[*]Data are presented as least square means per treatment ± SEM, n = 8.

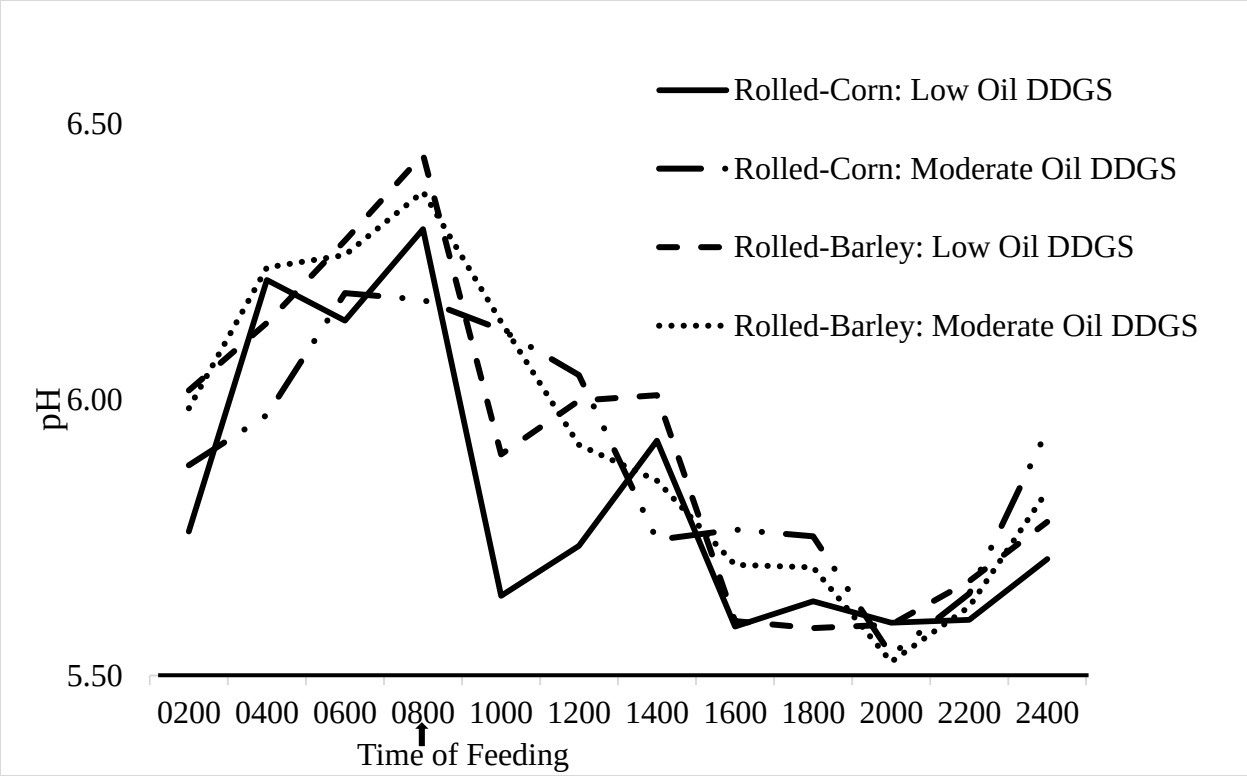

Figure 6-1. Data are represented as ruminal pH over a 24-hr period. Effects of Grain (P = 0.13), Oil (P = 0.24), Grain × Oil (P = 0.24), and Hr of the day (P < 0.001).

After testing the effect of moderate vs high fat diets, Chan et al. (1997) found an increase in the level of butyric acid in the ruminal fluid from cattle fed the high fat rations while acetate and propionate were unaffected. The levels of acetate and propionate were also not influenced in the current study. However, despite the varying levels of DDGS oil, no effects on butyric acid were seen. Although the total VFA concentration, along with acetate butyrate and propionate showed no differences between dietary treatments, there was an impact on isovaleric acid and valeric acid with isovaleric acid tending to be greater in moderate-oil DDGS rations while valeric acid was greater in barley-based rations. These results are surprising as valeric acid is increased during the microbial deamination and decarboxylation of valine, leucine and isoleucine (Raun, 1961) and these amino acids are generally found to be higher in corn. The lower concentrations

of valeric acid found in the rumen fluid of the steers consuming rolled-corn are similar to results seen in a study conducted by Payne and Morris (1970). When examining the effect of adding urea to high grain rations, they found the proportion of n-valeric acid to be lower in the ruminal fluid from steers provided the supplement. In order to provide a sufficient level of protein, rolled-corn diets in the current study were also supplemented with urea. However, no overall effects were seen on ruminal NH_3 concentration

It has been shown that rolled corn is not digested as extensively in the rumen as rolled barley (Galloway et al., 1993; Yang et al., 1997). This may indicate why the gas production lag time was greater in corn-based rations while the rumen degradation rates were lower. The increased lag time until gas production may also have been greater in corn-based rations related to previous rankings based on lower enzymatic and in situ starch and dry matter degradation rates compared to barley (Herrera-Saldana et al., 1990).

Many studies have indicated that providing cattle with rations composed of high grain rather than forage will result in reduced methane concentration. In fact, when evaluating variable grain sources, previous research has estimated CH_4 losses of cattle consuming corn-based rations to be around 3.5% of GE intake (Houghton et al., 1996) while for cattle consuming barley-based diets it is predicted to result in a loss of 6.5 to 12% of GE (Johnson et al., 2000). Other studies have found reductions in methane when cattle are provided diets with increasing levels of fat (Gerber et al., 2013; Hünerberg et al., 2013; Massé et al., 2014). Typically, these results will occur due to the inhibited growth of the rumen protozoa and reduction of methanogens (Beauchemin and McGinn, 2004; Knapp et al., 2014). However, no differences were found in the total in vitro gas production of CH_4 and CO_2 concentration in the current study. This may indicate that the rations provided were not different enough to cause a variation in methane

production. Overall, the finding that the acetate:propionate ratio was unaffected by treatment is consistent with the lack of an effect in methane concentration as the acetate: propionate ratio is often the greatest indicator of methane production (Russell, 1998).

An interaction for lowered ruminal α-amylase activity (U/L ruminal fluid, U/kg starch intake) was observed in barley diets containing moderate oil DDGS. Reducing the fat concentration in DDGS generally results in a change in the ruminal acetate-to-propionate ratios. This has been shown to favor amylolytic bacterial growth (DiCostanzo and Crawford, 2013). As no differences were seen in the acetate to propionate levels in the current trial however, the increased α-amylase may be due to the impact of higher DMI by steers fed the low-oil DDGS diets. Another possibility includes the fact that low-fat DDGS have been shown to enhance ruminal microorganism growth and activity while higher-fat DDGS suppress it (DiCostanzo, 2013). The ruminal degradation rate will also influence enzymatic activity as lower levels were found in steers consuming corn in the current experiment indicating that the rumen bacteria were producing more amylase in order to further break down the corn during its slower degradation.

Steers fed the diet containing corn with low-oil DDGS had the greatest trypsin activity (U/kg CP intake and U/kg CP disappearance). This may be due to the increased levels of fat in moderate oil DDGS reducing the interaction of microorganisms with the dietary protein and reducing its digestibility (Brooks et al., 1954) and need for trypsin. Another cause may be due to the protein in barley undergoing faster ruminal degradability than the protein in corn which therefore would require increased levels of trypsin to meet sufficient breakdown (Nikkhah, 2012).

When starch is digested by α-amylase it is broken down into smaller polysaccharides and disaccharides including maltose (Coleman, 1969). This maltose then requires the action of

maltase to continue nutrient breakdown. The increased activity of α-amylase in the diets containing corn would then require greater amounts of maltase.

While the ruminal pH was not impacted by dietary treatment, it is interesting to note that the highest pH measurements occurred between 0600 and 0700. This may have been because of increased salivation acting as a buffer. Although the animals were provided feed for ad libitum intake, bunks were cleaned and rations replaced every morning during this time. Also during this time, the animals were most active and consumed the greatest portion of feed. Rumen pH then began to drop as the day continued indicating that the rumen microbes were becoming more active and feed was undergoing nutrient breakdown (Gonzalez et al., 2012).

In conclusion, reducing oil concentrations of DDGS may be beneficial in improving rumen enzymatic function which could lead to improved digestion and nutrient utilization. The lack of change in ruminal pH, NH_3, total in vitro gas production and the concentrations of CH_4 and CO_2 indicate that feeding high-concentrate diets containing DDGS with differing oil concentration in both corn- and barley-based diets indicates that there was no negative influence on ruminal fermentation even though ruminal digestive enzymes seem to be moderated by both grain source and DDGS oil concentration.

6.6. Literature Cited

AOAC. 1990. Official Methods of Analysis. 15th Ed. ed. Arlington, VA.

Aschenbach J. R., G. B. Penner, F. Stumpff, and G. Gabel. 2011. Ruminant Nutrition

Bauer, M. L. 1996. Nutritional regulation of small intestinal glucose absorption in ruminants.

Ph.D. dissertation., University of Kentucky, Lexington.

Beauchemin, K. A. and S. M. McGinn. 2004. Methane emissions from feedlot cattle fed barley or corn diets. J. Anim. Sci. 83: 653-661

Bremer, M. L., B. L. Nuttleman, C. J. Bittner, D. B. Burken, G. E. Erickson, J. C. MacDonald, and T. J. Klopfenstein. 2014. Energy value of de-oiled modified distillers grains plus solubles in beef feedlot diets. J. Anim. Sci. 92 (Suppl. 2): 211. (Abstr.)

Broderick, G. A. and J. H. Kang. 1980. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and in vitro media. J. Dairy Sci. 63: 64-75

Brooks, C. C., G. B. Garner, C. W. Gehrke, M. E. Muhrer, and W. H. Pfander. 1954. The Effect of Added Fat on the Digestion of Cellulose and Protein by Ovine Rumen Microorganisms1. J. Anim. Sci. 13:758-764

Chan, S. C., J. T. Huber, C. B. Theurer, Z. Wu, K. H. Chen, and J. M. Simas. 1997. J. Dairy. Sci. 80: 152-159

Coleman, G. S. 1969. The metabolism of starch, maltose, glucose, and some other sugars by the rumen ciliate Entodinium caudatum. J. Gen. Microbiol. 87: 303-332

DiCostanzo, A. and G. I. Crawford. 2013. Low fat distillers grains with solubles (LF-DDGS) in feedlot finishing diets. Ag. Utilization Research Institution, Saint Paul, MN.

Dicostanzo, A. and C. L. Wright. 2012. Feeding ethanol coproducts to beef cattle. In: Liu K. S., Rosentrater K. A., editors, Distillers grains: Production, properties, and utilization. CRC Press, Boca Raton, FL. 237-264

France, J., S. Lopez, E. Kebread, A. Bannink, M. S. Dhanoa, and J. Dijkstra. 2005. A general compartmental model for interpreting gas production profiles. Anim. Feed Sci. Technol. 123-124

Galloway, D. L., A. L. Goetsch, L. A. Jr. Forster, A. C. Brake, and Z. B. Johnson. 1993. Digestion, feed intake, and live weight gain by cattle consuming bermudagrass and supplemented with different grains. J. Anim. Sci. 71:1288–1297

Geiger, R. and H. Fritz. Trypsin. In: Bergmeyer H, editor. 1986. Methods of Enzymatic Analysis. New York: Elsevier: 119-128.

Gerber, P. J., B. Henderson, and H. P. S. Makkar. 2013. Mitigation of greenhouse gas emission in livestock production: A review of technical options for non-CO_2 emissions. Food and Agricultural Organization of the United Nations

Goering, H. K., and P. J. Van Soest. 1970. Forage analyses. Agriculture Handbook 379. Agric. Res. Serv. U.S. Dept. of Agriculture.

Gonzalez, L. A., X. Manteca, S. Calsamiglia, K. S. Schwartzkopf-Genswein, and A. Ferret. 2012. Ruminal acidosis in feedlot cattle: Interplay between feed ingredients, rumen function and feeding behavior (a review). Anim. Feed Sci. Technol. 172: 66-79.

Goetsch, A. L., and M. L. Gaylean. 1983. Influence of feeding frequency on passage of fluid particulate markers in steers fed a concentrate diet. Can. J. Anim. Sci. 63: 727-730.

Herrera-Saldana, R., and J. T. Huber. 1989. Influence of varying protein and starch degradabilities on performance of lactating cows. J. Dairy Sci. 72: 1477–1483.

Herrera-Saldana, R., J. T. Huber, and M. H. Poore. 1990. Dry matter, crude protein, and starch degradability of five cereal grains. J. Dairy Sci. 73:2386–2393.

Hobson, P. N., and C. S. Stewart. 2012. The rumen microbial ecosystem. Springer Science and Business Media

Houghton, J. T., L. G. Meira Foilho, B. Lim, K. Tre ′anton, I. Mamaty, Y. Bonduki, D. J. Griggs, and B. A. Callander. 1996. Greenhouse Gas Inventory Reference Manual. Revised 1996

IPCC Guidelines for National Greenhouse Gas Inventories. Vol. 3. Agriculture, Intergovernmental Panel on Climate Change, Geneva, Switzerland.

Hünerberg M., S. M. McGinn, K. A. Beauchemin, E. K. Okine, O. M. Harstad, and T. A. McAllister. 2013. Effect of dried distillers grains plus solubles on enteric methane emissions and nitrogen excretion from growing beef cattle. J. Anim. Sci. 91: 2846-2857

Johnson, D. E., K. A. Johnson, G. M. Ward, and M. E. Branine. 2000. Ruminants and other animals, Chapter 8. Pages 112–133 in Atmospheric Methane: Its Role in the Global Environment. M. A. K. Khalil, ed. Springer-Verlag, Berlin Heidelberg, Germany.

Klopfenstein, T. J., G. E. Erickson, and V. R. Bremer. 2008. Board-invited review: Use of distillers by-products in the beef cattle feeding industry. J. Anim. Sci. 86: 1223-1231.

Knapp, J. R., G. L. Laur, P. A. Vadas, W. P. Weiss, and J. M. Tricarico. 2014. Invited review: Enteric methane in dairy cattle production: Quantifying the opportunities and impact of reducing emissions. J. Dairy Sci. 97: 3231-3261.

Lana, R. P., J. B. Russell, M. E. Van Amburgh. 1998. The role of pH in regulating ruminal methane and ammonia production. J. Anim. Sci. 76: 2190-2196

López, S., M. S. Dhano, J. Dijkstra, A. Bannink, E. Kebreab, and J. France. 2007. Some methodological and analytical considerations regarding application of the gas production technique. Anim. Feed Sci. Technol. 135: 139–156

Massé, D. I., G. Jarret, C. Benchaar, and N. M. Cata Saady. 2014. Effect of corn dried distiller grains with solubles (DDGS) in dairy cow diets on manure bioenergy production potential. Animal. 4: 82-92

McDougall, E. I. 1948. Studies on ruminant saliva. I. The composition and output of sheeps saliva. Biochem. J. 43: 99-109.

Milner, T. J., J. G. P. Bowman, and B. F. Sowell. 1995. Effects of barley variety or corn on feedlot performance and feeding behavior. Proc. West. Sect., Am. Soc. Anim. Sci. and West Branch Can. Soc. Anim. Sci. 46: 539-542.

Nikkhah, A. 2012. Barley grain for ruminants, a global treasure or tragedy. J. Anim. Sci. Biotech. 3:22

NRC. 1996. Nutrient Requirements of Beef Cattle. 7th Ed. National Academy Press, Washington, DC.

Oh, Y., J. Kim, K. Kim, C. Choi, S. Kang, I. Nam, D. Kim, M. Song, C. Kim, and K. Park. 2008. Effects of level and degradability of dietary protein on ruminal fermentation and concentrations of soluble non-ammonia nitrogen in ruminal and omasal digesta of Hanwoo steers. Asian-Aust. J. Anim. Sci. 21:392-403

Payne, E., and J. G. Morris. 1970. The effect of urea supplementation of high grain rations on the metabolism of fatty acids in the rumen of sheep and cattle. Proceedings of the Australian Society of Animal Production 8: 60.

Raun, N. S. 1961. Dietary factors affecting volatile fatty acid production in the rumen. Retrospective Theses and Dissertation. Paper 1951

Russell, J. B. 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. J. Dairy Sci. 81: 3222-3230.

Salo, S., M. Urge, and G. Animut. 2016. Effects of supplementation with different forms of barley on feed intake, digestibility, live weight change and carcass characteristics of Hararghe Highland sheep fed natural pasture. J. Food Process. Technol. 7: 3

Saunders, J. A. and K. A. Rosentrater. 2009. Properties of solvent extracted low-oil corn distillers dried grains with solubles. Biomass and Bioenergy 33: 1486–1490.

Stone, W. C. 2004. Nutritional approaches to minimize subacute ruminal acidosis and laminitis in dairy cattle. J. Dairy Sci. 87: E12–E26

Turner, R. J., and A. Moran. 1982. Heterogeneity of sodium-dependent D-glucose transport sites along the proximal tubule: Evidence from vesicle studies. Am. J. Physiol. 242: F406-F414

Wallenfels, K., P. Fold, H. Niermann, H. Bender, and D. Linder. 1978. The enzymatic synthesis, by transglycosylation of a homologous series of glycosidically substituted malto-oligosaccharides, and their use as amylase substrates. Carbohydrate Research. 61: 359–68.

Wang, Z. and L. A. Goonewardene. 2004. The use of MIXED models in the analysis of animal experiments with repeated measures data. Can. J. Anim. Sci. 84:1-11

Yang, W. Z., K. A. Beauchemin, K. M. Koenig, and L. M. Rode. 1997. Comparison of hull-less barley, barley, or corn for lactating cows: Effects on extent of digestion and milk production. J. Dairy. Sci. 80:2475-24.

CHAPTER 7. SUMMARY AND CONCLUSIONS

The enzymes found within the ruminant digestive system play an essential role in the absorption of nutrients. They also have an undeniable impact on the production of VFA and ruminant produced greenhouse gases. With the world's population growing as quickly as it is, the need to provide sufficient animal products and improve environmental sustainability will continue to rise. Therefore, gaining a better understanding of how enzymatic activity is influenced is crucial.

The studies conducted and presented in this dissertation have allowed for the opportunity to determine how stage of maturity and level of feed intake impacts the production of enzymes in both the rumen and small intestine. It has been shown that the level of enzymatic activity found in the digestive system of the fetus is highly dependent on its maternal nutrient intake. Unfortunately, undergoing periods of feed restriction is common for livestock throughout the world and being able to determine ways of ensuring appropriate growth and development of the fetus is essential.

The addition of specific supplements to feed has the potential to impact enzymatic activity. In the Experiment 1, we investigated the influence of melatonin supplementation. While nutrient restriction decreased the pancreatic mass of the dam and fetus, melatonin was only able to rescue the maternal tissue. Melatonin also increased the α-amylase activity (U/g, kU/pancreas, U/kg BW) in the dam after it had been reduced during feed restriction. While these results may seem positive, both nutrient restriction and melatonin supplementation were found to result in decreased maternal pancreatic insulin-positive tissue area (relative to section of tissue). When comparing ewes to fetuses, it was found that ewes had a greater percentage of medium

insulin-containing cell clusters (2001 - 32,000 µm2) while fetuses had more ($P < 0.001$) pancreatic insulin-positive area (relative to section of tissue) and a greater percent of small, large and giant insulin-containing cell clusters. While some benefits were seen with supplementation, the results were not enough to recommend melatonin to be used as a therapeutic agent capable of mitigating reduced pancreatic function due to nutrient restriction.

Experiment 2 investigated the impact of providing the essential amino acid arginine to mature animals during different stages of estrus. During this trial, it was noted that nutrient intake influenced body weight of the animal in addition to its pancreatic weight and enzymatic function with overfed animals having higher enzyme activity. The stage of estrus did not affect pancreatic function. The use of arginine did not influence pancreatic function which may indicate that the dosage used was not sufficient. More research on other doses is needed.

The influence of maternal nutrition on fetal development has long been an area of concern. However, it has been shown that the majority of fetal growth is occurring during the final trimester of gestation. Because of this, the advantage of realimentation during the different stages of pregnancy was examined in Experiment 3 in the hopes of allowing the fetuses to return to levels matching their control counterparts. The results also indicated a benefit in regards to showing an increase in the protein content and α-amylase activity in the realimentated fetus. Trypsin however, showed an opposite effect. Regardless, the idea of compensatory development was indicated in this study and, when implemented at the appropriate time, may prove to provide a rescue mechanism during times of reduced maternal feed intake.

In addition to the level of feed intake, the impact of the nutrients provided in the diet is also important for the determination of enzymatic activity. While the feeds offered may be of an identical grain source such as corn and byproduct such as distiller's grain, the initial feed

processing methods and level of DDGS inclusion are capable of altering the rumen environment. In Experiment 4, steers were fed either fine- or course-rolled corn in addition to 20% or 40% DDGS. Steers fed diets containing 20% DDGS were found to have greater ruminal α-amylase activity (U/L ruminal fluid; α-amylase/kg starch disappearance) while those fed 40% DDGS had greater trypsin activity (trypsin/kg CP disappearance). The nutrient profile of the diet had no impact on the level of gas produced or the concentration of methane. While some changes were observed in enzymatic activity, the results indicate that degree of dry-roll processing and DDGS inclusion level used in this experiment does not impact ruminal health and environmental sustainability.

Finally, Experiment 5 examined the impact of providing different grain sources along with DDGS of different oil concentrations. Steers were fed either corn or barley-based rations along with moderate or low-oil concentration DDGS. During this trial, it was shown that feeding corn resulted in greater enzymatic activity when compared to barley and maintained a longer lag time before producing gas in vitro. It was also found that the ruminal degradation rate of barley was greater than corn. However, despite the breakdown of these feeds at different rates, no changes in the overall gas production or concentration of methane and carbon dioxide were observed.

As variable results have been found during these studies there is no question that the nutrition provided to the maternal animal plays a large impact on the development of the fetal pancreas. There is also no denying that nutrition in general will affect the enzymatic activities of the ruminant digestive system. Understand the greatest ways to utilize this knowledge and improve animal performance and environmental sustainability is key. Therefore, further

research is warranted and this will forever be an area to explore as the world's population continues to increase.